Universidade do Minho
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Study the roles of human galectin-3 using the yeast *Saccharomyces cerevisiae* and colorectal cancer cells as eukaryotic models

abril de 2017

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Tese de Doutoramento em Biologia Molecular e Ambiental
Especialidade em Biologia Celular e Saúde

Trabalho efetuado sob a orientação da
Professora Doutora Cândida Manuel Ribeiro Simões Lucas
e da
Professora Doutora Ana Arminda Lopes Preto de Almeida

abril de 2017

STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagirism or any form of falsification of results in the process of the thesis elaboration.

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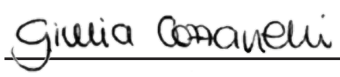
Signature 

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Abstract

Study the roles of human galectin-3 using the yeast* Saccharomyces cerevisiae *and colorectal cancer cells as eukaryotic models.

Galectins are lectins characterized by a conserved CRD with a high affinity for β-galactosides. When localized extracellularly, they mainly interact with glycans on the surface of the cells, promoting cell-cell adhesion, the onset of immune response, and the recognition and clearance of pathogens. When intracellular, they interact with other proteins, modulating cell proliferation, cell cycle progression and apoptosis. The focus of the present work was galectin-3, the only *chimera* type galectin, aiming at turning the yeast *Saccharomyces cerevisiae* into a suitable model to study the roles and mechanisms of action of gal-3. Specifically, the work intended to assess the ability of extracellular gal-3 to recognize and bind yeast cells, as well as the ability of intracellular gal-3 to interfere with yeast cell survival and proliferation, in particular when interacting with the oncoprotein KRAS. The work also aimed at uncover the role of gal-3/KRAS/$p16^{INK4a}$ axis regulation in colorectal cancer (CRC) cells. Thus, both *S. cerevisiae* and human cancer cell lines were used as eukaryotic models.

The effects caused by gal-3 on yeast biological processes were compared with those caused by other galectins with different structure and number of CRDs (the *proto-type* gal-1 and gal-7, and the *tandem repeat* gal-4). *S. cerevisiae* and *Candida albicans* were used, as species with a very different biology, and because *C. albicans* was the only yeast in the literature tested in regard to gal-3. Each galectin caused a different pattern of effects, generally stronger on *S. cerevisiae* than on *C. albicans*. Gal-3 decreased viability and increased cell size, ROS level and DNA alterations, but did not induce membrane rupture, plasmatic nor mitochondrial, indicating that the stress it induces is not associated with cell death (necrotic or programmed). Gal-3 effects were mostly caused by its CRD (as shown using the truncated version of the galectin), and mediated by the Ras/cAMP/PKA pathway (demonstrated using the *S. cerevisiae* mutants). Gal-4 and gal-7 increased the levels of ROS and membrane rupture, without affecting viability. Gal-1 did not induce any significant alteration. A microarray-based analysis of the binding ability of galectins, in accordance with the results above, showed that all galectins except gal-1 bound to whole cells of *S. cerevisiae* and *C. albicans*, more

efficiently to *S. cerevisiae*, in particular the correspondent *Δras2* mutant and cell wall and membrane sub-cellular fractions.

A *S. cerevisiae*-based high throughput platform (HTP) expressing human gal-3 and KRAS was built with the purpose of achieving the expression of both human cDNAs in the same strain. This is meant to enable *in vivo* study of the functional relations between the two proteins, and to serve as a HTP for pharmacological screening of drugs/molecules targeting either gal-3, KRAS or their interaction. Two different genetic backgrounds were used (W303-1A and BY4741), wild type as well as *Δras1* and *Δras2* mutants. Gal-3 expression was fully achieved, altering growth rate and chronological life span. These phenotypes depended on the presence of Ras1 and/or Ras2. Human KRAS expression in wt yeast also caused phenotype variations, decreasing yeast resistance to various stress stimuli, most possibly due to the hyperactivation of the Ras/cAMP/PKA pathway. The double expression of gal-3 and KRAS in the same *S. cerevisiae* strain was attempted using several cloning strategies, and will be pursued in the future. Bearing this goal in mind, as well as the potential use for which the platform was built, human CRC cell lines were used to better understand the gal-3/KRAS interaction and their role in CRC progression. The pivotal interaction of gal-3 and KRAS were confirmed, and a third protein was shown to belong to this regulation axis, $p16^{INK4a}$. The three proteins physically interacted and co-localized in CRC cells, and there seems to be a reciprocal regulatory mechanism that might control their expression levels, adjusting it to the growing needs of the cancer cell.

In conclusion, this work breaks through several boundaries: (i) galectins bind to yeasts, (ii) the binding is specific, (iii) it causes also specific responses from the yeast cell, (iv) these are mediated by Ras pathway, and (v) once intracellular, gal-3 induces different responses still mediated by Ras pathway. Importantly, in opposition to the scarce literature available, the work showed *S. cerevisiae* to be more sensitive than *C. albicans*, stressing the strain-dependent specificity of galectins recognition, at the same time discarding the suggestion that gal-3 might have the role of distinguishing between pathogenic and non- pathogenic yeasts *in vivo*. Moreover, $p16^{INK4a}$ was identified as a new member of KRAS/gal-3 regulation axis in CRC cells. All taken, the work launched the foundations for assuming and using *S. cerevisiae* as a model to study gal-3.

Resumo

Estudo da galectina-3 humana usando a levedura* Saccharomyces cerevisiae *e células de cancro colorretal como modelos eucarióticos.

As galectinas são proteínas caraterizadas por possuírem um domínio de reconhecimento de carbohidratos (CRD) com elevada afinidade para β-galactosídeos. Quando no meio extracelular, as galectinas interagem com glucanos na superfície das células, promovendo reconhecimento e adesão, a resposta imunitária e a eliminação de patogéneos. Intracelularmente, as galectinas interagem com outras proteínas modulando a proliferação e o progresso do ciclo celular e a morte celular programada. O trabalho focou na galectina-3, o único membro da família das galectinas *quiméricas*. O objectivo do trabalho foi transformar a levedura *Saccharomyces cerevisiae* num modelo celular para estudar os papéis da gal-3. Para isso foi verificada a habilidade da gal-3, quando extracelular, de reconhecer à célula de levedura, quando intracelular, de interferir com a sobrevivência e capacidade proliferativa da levedura, muito em particular em relação à oncoproteína KRAS. O trabalho tinha também como objetivo perceber o papel da regulação do eixo of gal-3/KRAS/$p16^{INK4a}$ em células de cancro colorretal (CCR). Neste contexto foram usados a *S. cerevisiae* e células de cancro humano como modelos eucariotas.

Os efeitos da presença extracelular de gal-3 foram comparados com os causados por outras galectinas com diferentes estrutura, as gal-1 e gal-7 do grupo de galectinas *proto-tipo*, e a gal-4 do grupo de galectinas *repetição em tandem*. Os efeitos destas galectinas foram comparados em estirpes de *S. cerevisiae* e *Candida albicans,* que foram escolhidas pela sua distinta biologia, e porque *C. albicans* era a única espécie de levedura referida na literatura sobre efeitos de galectinas em leveduras. Cada galectina provocou uma combinação de efeitos diferente, que foram genericamente mais fortes em *S. cerevisiae* do que em *C. albicans.* Em particular, gal-3 diminuiu a viabilidade da levedura e aumentou o tamanho das células, os níveis de ROS e as alterações no DNA, mas não induziu ruptura de membranas, plasmática ou mitocondrial, indicando que gal-3 induz stress sem acionar nenhum processo de morte celular, necrótica ou programada. A utilização de uma gal-3 truncada no terminal N mostrou que o CRD é suficiente para induzir os efeitos observados. A utilização de leveduras mutadas nos genes *RAS* mostrou que a gal-3 opera através da via Ras/cAMP/PKA. Em comparação,

gal-1 não induziu qualquer efeito significativo, enquanto as gal-4 e gal-7 provocaram respostas que sugerem uma resposta de stress genérica. Através de um ensaio de *binding microarrays* com células de levedura inteiras e fracções sub-celulares, verificou-se que todas as galectinas, excepto gal-1, se ligam à parede e membrana de *S. cerevisiae* e *C. albican*s, mais eficientemente de *S. cerevisiae*, e mais ainda ao mutante *Δras2* desta levedura.

Foi construída uma plataforma de estirpes de levedura exprimindo os cDNA de gal-3 e KRAS humanos, com o objectivo de obter a expressão conjunta destas duas proteínas. A plataforma destina-se ao estudo das suas funções e interação, bem como a servir de ferramenta para ensaios em larga escala de moléculas e drogas farmacológicas dirigidas para a gal-3, o KRAS, ou para a sua interação. Foram usadas leveduras de dois fungos genéticos distintos (W303-1A e BY4741), selvagens e mutantes defectivos nos genes *RAS1* e *RAS2*. A expressão da gal-3 em levedura alterou a taxa de crescimento e o envelhecimento cronológico na dependência da via Ras/cAMP/PKA. A expressão do KRAS em levedura também provocou alterações de comportamento na extirpe selvagem devidas possivelmente à sobre-ativação da mesma via. A obtenção de uma estirpe exprimindo simultaneamente gal-3 e KRAS foi tentada usando várias estratégias de clonagem, e será possivelmente alcançada no futuro. Tendo em conta os objectivos a que se destina a plataforma, foram usadas células de CCR. A interação da gal-3 e KRAS foi confirmada e demonstrou-se interação deste complexo com a $p16^{INK4a}$. Estas três proteínas interagem fisicamente e colocalizam em células de CCR, parecendo haver um mecanismo de regulação reciproca que parece controlar os seus níveis de expressão, ajustando-os mediante as necessidades de crescimento da célula de cancro.

Em conclusão, o trabalho quebra barreiras mostrando: (i) que as galectinas se ligam às células de levedura, (ii) de forma específica, (iii) induzindo respostas também específicas, (iv) mediadas pela via Ras, (v) igualmente quando a gal-3 é expressa intracelularmente em *S. cerevisiae*. Em oposição à escassa literatura disponível, mostrou-se que *S. cerevisiae* é mais sensível que *C. albicans,* sublinhando a influência da estirpe na especificidade da resposta e ligação às galectinas, e afastando a sugestão de que gal-3 possa exercer *in vivo* uma função discriminatória entre leveduras patogénicas e não-patogénicas. Além disso, foi identificada a $p16^{INK4a}$ como um novo membro do eixo de regulação KRAS/gal-3 em CCR. Globalmente o trabalho lançou as fundações para a utilização da levedura *S. cerevisiae* como modelo para estudar gal-3.

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List of common abbreviations

μ_g = growth rate

aa = amino acid

AFet = Asialofetuin

AFg = Asialofibrinogen

AMP = adenosine monophosphate

AP-1 = activator protein 1

APC = adenomatous polyposis coli

APO-1 = apoptosis antigen 1

AV = Annexin V

Bcl-2 = B cell lymphoma 2

cAMP = 3',5'-cyclic adenosine monophosphate

CDK = cyclin dependent kinase

CDKN2A = CDK inhibitor 2A

cDNA = complementary DNA

CFU = colony forming unit

CKI = CDK inhibitor

Cln = cyclin

CLS = chronological life span

ConA = Concanavalin A

CRC = colorectal carcinoma

CRD = carbohydrate recognition domain

CRE = cAMP dependent response element

CREB = cAMP response element-binding protein

cyt *c* = cytochrome *c*

DAMP = damage-associate molecular pattern

DAPI = 4',6-diamidino-2-phenylindole

DHE = dihydroethidium

DIC = differential interference contrast

$DiOC_6$ = 3,3'-dihexyloxacarbocyanine iodide

DNA = deoxyribonucleic acid

ECM = extracellular matrix

EGF = epidermal growth factor

EGFR = epidermal growth factor receptor

ER = endoplasmic reticulum

ERK = extracellular signal–regulated kinase

FBS = foetal bovine serum

Fet = Fetuin

Fg = Fibrinogen

G1 phase = gap 1 phase

G2 phase = gap 2 phase

gal-1 = galectin-1

gal-2 = galectin-2

gal-3 = galectin-3

gal-4 = galectin-4

gal-5 = galectin-5

gal-7 = galectin-7

gal-8 = galectin-8

gal-9 = galectin-9

gal-10 = galectin-10

gal-12 = galectin-12

gal-13 = galectin-13

gal-14 = galectin-14

GAP = GTPase activating protein

GDP = guanosine diphosphate

GEF = guanine-nucleotide exchange factors

GFP = green fluorescent protein

GSK-3β = glycogen synthase kinase-3β

GTP = guanosine-5'-triphosphate

GTPase = guanosine triphosphatase

h = hour

HIS3 = histidine gene and its promoter

HIV-1 = human immunodeficiency virus type 1

HTLV-1 = human T lymphotrophic virus type 1

HVR = hypervariable region

IAP = inhibitors of apoptosis protein

IKK = I-κB kinase

IL-x = interleukin-x

IRA1 = inhibitory regulator of the RAS-cAMP pathway

KanX = Kanamycin resistance cassette

KRAS = Kirsten rat sarcoma viral oncogene

LacNAc = N-acetyllactosamine

LGALS3 = lectin, galactoside-binding, soluble 3

LPG = lipophosphoglycan

LPS = lipopolysaccharide

MAPK = mitogen-activated protein kinase

MEK = MAPK/ERK kinase

MoBY-ORF = molecularly barcoded yeast open reading frame

mRNA = messenger RNA

mTOR = mammalian target of rapamycin

MTS1 = multiple tumour suppressor 1

MUC1 = mucin 1

NF-κB = nuclear factor κ-light-chain-enhancer of activated B cells

NF1 = neurofibromatosis type 1

$O.D._{600}$ = optical density at 600 nm

PAMP = pathogen-associated molecular pattern

PARP = poly (ADP-ribose) polymerase

PBS = phosphate buffered saline

PCD = programmed cell death

PCR = polymerase chain reaction

PVDF = polyvinylidene difluoride

PFA = paraformaldehyde

PGK1 = phosphoglycerate kinase 1

PH = pleckstrin homology

PI = propidium iodide

PI3K = phosphatidylinositol 3-kinase

PIP_2 = phosphatidylinositol-4,5-diphosphate

PIP_3 = phosphatidylinositol (3,4,5,)-triphosphate

PKA = protein kinase A

PKCζ = protein kinase C ζ

PLCε = phosphatidylinositol-4,5-diphosphate phosphodiesterase ε

PolIII = Polymerase III

PRR = pathogen recognition receptor

PTEN = phosphatase and tensin homolog deleted in chromosome ten

RA = Ras association

RAF = rapidly accelerated fibrosarcoma kinase

RAL = RAS-like

RALBP1 = CDC42/RAC-GAP-RAL binding protein 1

RALGDS = RAL guanine nucleotide dissociation stimulator

RAS = rat sarcoma viral oncogene

Rb = retinoblastoma

RbA = ribonuclease A

RbB = ribonuclease B

rDNA = ribosomal DNA

RIPA = radioimmunoprecipitation assay

RNA = ribonucleic acid

RNAi = RNA interference

ROS = reactive oxygen species

rpm = revolutions per minute

RPMI = Roswell Park Memorial Institute medium

rRNA = ribosomal RNA

S phase = synthesis phase

S6K = S6 kinase

SD = standard deviation

SDS-PAGE = sodium dodecyl sulphate- polyacrylamide gel electrophoresis

SGA = synthetic gene array

siRNA = small interference RNA

Smad3 = small mothers against decapentaplegic 3

snRNPs = small nuclear ribonucleo proteins

SOS = son of sevenless

SRB = sulforhodamine B

STRE = stress response elements

TBS = tris-buffered saline

TCA = trichloroacetic acid

TFD = Thomsen-Friedenreich disaccharide

TGF-β = transforming growth factor β

TKR = tyrosine kinase receptor

TOR = target of rapamycin

TRAIL = TNF-related apoptosis-inducing ligand

VEGF = vascular endothelial growth factor

w/v = weight per volume

WB = western blot

wt = wild type

yEGFP = yeast enhanced green fluorescent protein

YNB = yeast nitrogen base

YPD = yeast extract peptone dextrose

General Introduction

Part of this chapter was adapted from:
Cazzanelli G., Moreira T., Ferro S., Azevedo-Silva J., Nogueira E. and Preto A. (2016) Colorectal cancer therapeutic approaches: from classical drugs to new nanoparticles. *Frontiers in Anti-Cancer Drug Discovery*. Rahman, A. and Choudhary, M.I., Bentham Science Publishers. **7**: 3-82

1. The sugar code and its translator: the lectin superfamily

It is well known that two classes of molecules provide the information in a living organism: the nucleotides, components of the DNA and RNA, and the amino acids in which they are translated. However, it has become evident that these two instruments of transmitting information are not sufficient to explain all the molecular events, even taking into account all the possible modifications to which they can be subjected. A third class of molecules is present in the cells, known mainly for its role in energy metabolism and for being a major component of cell walls: carbohydrates (von der Lieth *et al.*, 1997). In the early '70s, it appeared clear that carbohydrates could have a role also in the communication of information inside a cell. Winterbun and Phelps wrote that these molecules are actually "*ideal for generating compact units with explicit informational properties*" (Winterburn and Phelps, 1972). Carbohydrates have many properties that make them very good messengers. First, they have multiple possibilities of variability. The basic unit of the sugar code is a furanose or a pyranose ring. One ring is linked to the other, forming polysaccharides, offering different hydroxyl groups and therefore creating different glycosidic linkages. Moreover, every monomeric ring can be presented in two anomeric constellations, α or β (Rüdiger and Gabius, 2011a). With the growing in size of polysaccharides, branching is not only possible, but very likely, and the possibilities of different branching are multiple. Moreover, sugars can be modified by substitutions, such as phosphorylation and sulfation. All these factors contribute to make the coding ability of carbohydrates order of magnitude higher than the one of nucleic acids and proteins (Laine, 1996). Accordingly, the enzymatic machinery necessary for glycans synthesis and modification accounts for a much higher number of genes compared to nucleic acids and proteins biosynthesis (Gabius *et al.*, 2011; Andre *et al.*, 2015). For example, around 200 genes are encoded in the genome for glycosyltransferases, the enzymes responsible for fucose or sialic acid addition and for specific chain elongation (Oriol *et al.*, 1999; Harduin-Lepers *et al.*, 2001; Ma *et al.*, 2006; Togayachi and Narimatsu, 2012). In addition, glycan structures are not genetically strictly coded, but they are influenced by environmental factors

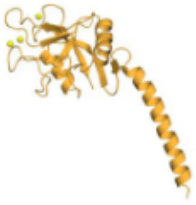
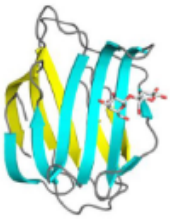
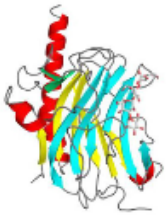
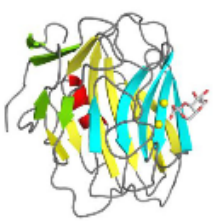
and by the availability of the enzymes necessary to assembly them. This makes them even more suitable to transmit information that can change depending on the time and the place (Pavelka, 1996; Abeijon *et al.*, 1997; Varki, 1998).

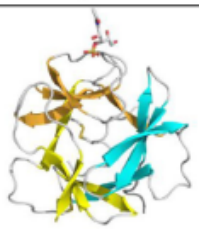
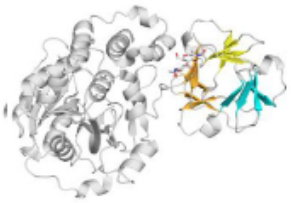
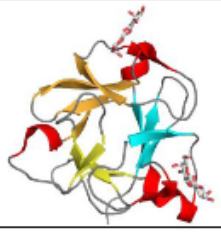
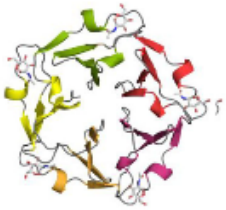
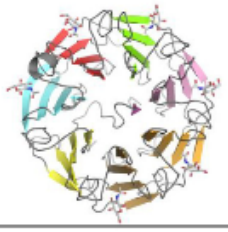
The wide array and specificity of information transmitted by sugars must have proper decoders in the cells, with equivalent degree of sophistication. There are different classes of proteins able to bind to sugars: the enzymes responsible for assembling and modifying their structures, specific types of antibodies reactive with carbohydrate epitopes (Schwarz and Dorner, 2003) and the lectin superfamily of proteins (Boyd, 1963; Gabius *et al.*, 2002).

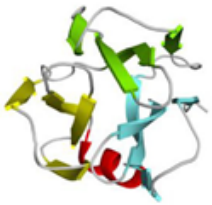
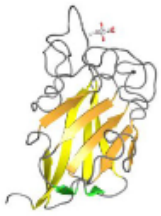
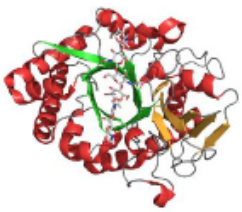
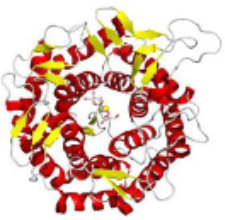
Lectins are the proteins responsible for translating the message carried by carbohydrates. The first lectins identified were plant lectins, isolated because of their specificity for histo-blood group determinants and their consequent ability to agglutinates erythrocytes of donors with different ABH(0) status (Renkonen, 1948; Kilpatrick and Green, 1992; Khan *et al.*, 2002). Nowadays, every sugar binding protein that is not an enzyme or a sensor/transport protein is named lectin (Rüdiger and Gabius, 2011b). Lectins proteins are subdivided into different classes, based on the structural definition of the folding pattern and on the architecture of the carbohydrate-binding domain. Up to now around 12 different folds have been characterized for lectins, including the best-known C-type lectins, galectins, I-type lectins, P-type lectins and pentraxins (Gabius *et al.*, 2011; Solis *et al.*, 2015) (**Table 1**). Some of the folding modules, such as β-sandwich, can be shared by different groups of lectins, with a different location of contact site for the ligand in the common fold (Loris, 2002; Murphy *et al.*, 2013; Solis *et al.*, 2015). As seen, there is a large number of lectins and their variability can be seen both in the number of families and in the number of members in each family. Such a broad set of lectins reflects the large variability among the glycan structures and allows a proper recognition of each glycan by a specific carbohydrate-binding domain. There are six levels of recognition between a lectin (receptor) and its specific glycan ligand (counter-receptor) (Gabius *et al.*, 2011). The first levels correspond to the specificity of recognition for mono- or disaccharides (level 1) and oligosaccharides (level 2). Subsequently, level 3 corresponds to the selection of the preferred oligosaccharide conformation among the several possible found in solution. Glycans are not highly flexible like peptides in solution, but they are fixed

in a limited number of conformers defined by their structural nature, in particular by the impossibility of free rotation around the glycosidic linkage imposed by the pyranose ring (Carver, 1991; von der Lieth *et al.*, 1998; Imberty and Perez, 2000). Next, the lectin recognizes its glycan ligand by the spatial presentation of the glycan chain, determined, among other factors, by the orientation of the branches and by the formation of clusters of headgroups in bi- to penta-antennary glycans (level 4). With their ability to form multimers, many lectins can bind specifically to the ligands when organized in clusters of different glycans chains found close on the same glycoprotein or glycoprotein/glycolipid complex (level 5). Finally, lectins can discriminate among glycan presentation in microdomains, composed by different glycoconjugates placed in spatial vicinity on the cell surface (level 6) (Gabius *et al.*, 2011; Andre *et al.*, 2015). Therefore, lectins not only recognize a sequence of different letters (monosaccharides) that compose different words (oligosaccharides), but they also have the ability to interpret entire texts (glycans clusters and microdomains). In addition to their glycans binding ability, many lectins are associated with other functional domains, such as membrane spanning sections, regions for oligomerization, sites for recognition of non-sugar type of ligands and even with other types of CRDs (Barondes, 1988; Cooper, 2002; Gready and Zelensky, 2009). Lectins have been identified to have a physiological role in many cellular processes, such as post-binding signalling and promoting adhesion between cells. They are also involved in a number of diseases: they help pathogens to enter the host by promoting adhesion and they are expressed in malignancy, often with a prognostic value (Gabius, 2000; Gabius *et al.*, 2004; Gabius and Kayser, 2014; Andre *et al.*, 2015). For this reason, lectins are also used as tools to detect specific glycan patterns in various tissues, in order to determine the physiological glycome and the variations correlated with various diseases formation, such as tissue fibrosis and malignancies (Gabius and Kayser, 2014). When used as tools, lectins are often labelled with enzymes like alkaline phosphatase or horseradish peroxidase, which help their recognition in the tissues (Hardonk and Scholtens, 1980; Straus, 1981).

Table 1: List of different types of CRD folding, associated to the 3D structure and the main lectins presenting that CRD structure (adapted from Solis *et al.*, 2015)

Type of fold	Overall fold	Example for lectin
C-type		asialoglycoprotein receptors, collectins, selectins
I-type		N-CAM, TIM-3, siglecs
P-type		mannose-6-phosphate receptors (MR) and erlectin (proteins with MR homology domain)
β-sandwich (galectins)		animal, fungal and human galectins
β-sandwich (chaperone)		calnexin, calreticulin
β-sandwich (transport-mediating proteins; ER and Golgi)		ERGIC-53, ERGL, VIPI, VIP36
β-sandwich (pentraxin)		C-reactive protein, serum amyloid P component

continued		
β-sandwich (N-glycanase)		CRD of Fbs1 in SCF E3 ubiquitin ligase and peptide-N-glycanase (PNGase)
β-trefoil (mannose receptor family)		cysteine-rich domain of C-type macrophage mannose receptor; phospholipase A2 receptor; ENDO180; DEC-205
β-trefoil (Gal*N*Ac transferases)		lectin domain in Gal*N*Ac transferases
β-trefoil (invertebrates lectins)		haemolytic lectin CEL-III of sea cucumber and lectin EW29 of earthworm
β-propeller (5-bladed)		tachylectin-2
β-propeller (6-bladed)		tachylectin-1
β-propeller (7-bladed)		lectin from mushroom *Psathyrella velutina*

continued		
β-prism I		ZG16p and ZG16b
β-prism II		pufferfish (*Fugu rupribes*) lectin
jelly-roll barrel		horseshoe crab tachylectin-4; eel (*Anguilla anguilla*) agglutinin; *Xenopus* X-epilectin
fibrinogen-like		ficolectins (H, L and M); intelectins (mammalian, *Xenopus*); tachylectin-5; slug (*Limax flavus*) lectin
α + β		lectin domain of mouse latrophilin-1; one G-protein coupled receptor (GPCR)
TIM barrel		YKL-40 (human cartilage glycoprotein-39, chitinase-like lectin)
$(\alpha\alpha)_7$ barrel		EDEM1, 2, 3 (ER-associated degradation-enhancing-α-mannosidase-like proteins); Mnl1

In conclusion, the sugar code is an underestimated, but yet very important part of the system of transmitting information in the cells and lectins, its major decoders, are a suitable object of further investigation.

1.1 The galectin family

The galectin family of lectins, or S-type lectins, is composed by several proteins characterized by a conserved peptide sequence element of about 130 amino acids in the carbohydrate-binding domain. Many of the galectins identified so far have been recognized by sequence similarity. These proteins emerged early in the metazoan evolution and are now found in different types of cells within an organism and in different organisms and species, including non-mammalian species such as birds, amphibians, fish, worms, sponges and fungi (Hughes, 1997; Cooper and Barondes, 1999).

The CRD (carbohydrate recognition domain) of galectins is formed by two antiparallel β sheets creating a sandwich like structure (Rini and Lobsanov, 1999) and is characterized by a high binding affinity for β-galactosides (Barondes *et al.*, 1994), with the exception of gal-10, which demonstrated a higher affinity for mannose-related sugars compared to β-galactosides, and gal-11, which lacks sugar-binding activity (Rabinovich and Toscano, 2009). Even though the binding affinity for β-galactosides is high, often the interaction with the monosaccharide galactose is weak, compared with the interaction with the disaccharide lactose, which is up to 100-fold more effective (Leffler and Barondes, 1986), or even bigger with some longer oligosaccharides (Brewer, 2004).

Until now, 15 mammalian galectins have been discovered and they are subdivided into three groups based on their structure and CRDs number. P*roto-type* galectins are characterized by a unique CRD, with the ability to act as monomer or homodimer (gal-1, -2,-5,-7,-10,-11,-13,-14,-15); *tandem-repeat* type galectins are defined by the presence of two distinct CRDs, not necessarily with the same binding properties, connected by a linker polypeptide (gal-4,-6,-8,-9,-12); *chimera* type is composed by a CRD at the C-terminal and a non-carbohydrate-binding domain and the N-terminal (gal-3) (**Fig. 1**). Galectins do not have

a redundant function in the cells, since they bind glycans with different specific affinities and each galectin is expressed in a tissue-specific or developmentally regulated fashion (Colnot *et al.*, 1996; Compagno *et al.*, 2014) (**Table 2**).

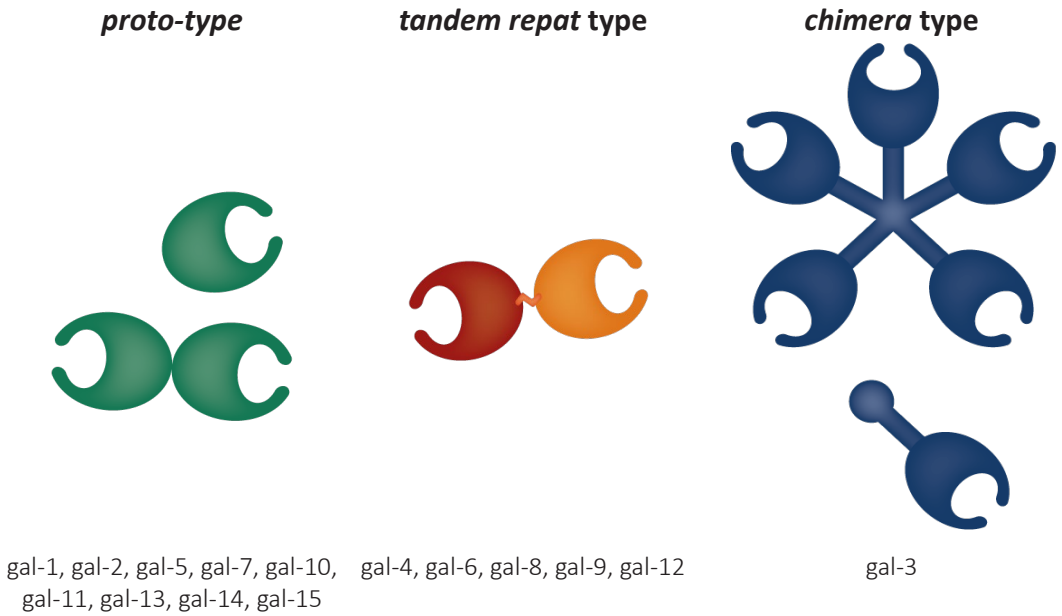


Figure 1: *Galectins subdivision in groups based on the structure and number of CRDs*. *Proto-type* galectins have a single CRD and can be found in solution as monomers or dimers. *Tandem repeat* galectins have two CRDs joined by a linker peptide. *Chimera* type galectin has a single CRD and a long N-terminal domain, which is necessary for multimerization, specifically, pentamers formation.

Galectins are synthetized in the cytoplasm and segregated to prevent premature binding to their ligands. They can then be secreted outside the cell through the alternative secretory pathway or be retained inside the cell to perform different tasks (Yang *et al.*, 2008). Some galectins, such as gal-1, -3, -4, are found both intra- and extracellularly, while some others have a preferential localization, like gal-7, which is mainly intracellular (Liu *et al.*, 2002; Yang and Liu, 2003). Extracellular galectin functions depend on their ability to oligomerize and cross-link their ligands. All galectins, regardless their subtype, are able to cross-link. *Tandem repeat* types use their two CRDs, while *proto-type* galectins and the *chimer*a type gal-3 form multivalent complexes (Boscher *et al.*, 2011). When galectins cross-link to their ligands they form supramolecular complexes called *lattices* (**Fig. 2**) and they promote the redistribution or the segregation of the bound ligands at the cellular membrane, increasing the avidity

and half-life of galectin-glycans interactions (Yang *et al.*, 2008; Rabinovich and Croci, 2012; Compagno *et al.*, 2014).

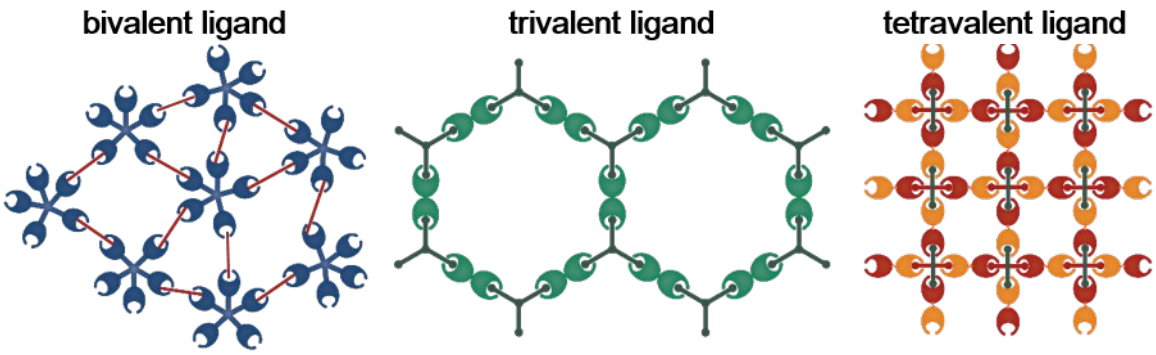


Figure 2: *Structures of the main types of lattices formed by galectins-glycans interaction. Chimera* type galectins can form pentamers that bind bivalent ligands; *proto-type* galectins, when in dimers, can bind trivalent ligands; *tandem repeat* type galectins can bind tetravalent ligands.

The glycome of a cell is subjected to continuous variations determined by endogenous and microenvironmental signals, which influence the expression and activity of enzymes involved in glycans processing, such as glycosyltransferases and glycosidases (Compagno *et al.*, 2014). The entire set of galectins is particularly fit to translate and induce signals in the cells. Due to high specialization of each member, they can sense variations in the saccharides specificity and glycans availability (Hirabayashi *et al.*, 2002; Rabinovich and Toscano, 2009; Vokhmyanina *et al.*, 2012) and they transduce these differences into the activation of different signalling pathways. However, galectins are not only involved in extracellular processes, such as signalling, immune cell activation, adhesion, receptor of pathogen glycans and DAMPs (damage-associate molecular patterns) functions (Garner and Baum, 2008; Rabinovich and Toscano, 2009; Vasta *et al.*, 2012; Compagno *et al.*, 2014), but they also act on multiple intracellular processes, such as cell signalling, splicing regulation, modulation of apoptosis and cell cycle progression (Liu *et al.*, 2002; Yang and Liu, 2003; Compagno *et al.*, 2014; Vladoiu *et al.*, 2014). Interestingly, most of the extracellular functions of galectins relate on their ability to bind to the glycans of glycoconjugates present in the extracellular matrix (ECM) or on plasma membrane, while their intracellular functions also depend on protein-protein and protein-nucleic acids interaction (Rabinovich and Croci, 2012).

Table 2: Different galectins affinity for glycans and main tissue expression of galectins (adapted from Rabinovich and Toscano (2009) and integrated).

Structure	Member	Carbohydrate specificity	Tissue expression
Proto-type (one single CRD that can form dimers)	**gal-1**	- High affinity for complex-type N-glycan (tri>bi>mono) - Non-reducing terminal N-acetyllactosamine (LacNAc) units (Hirabayashi *et al*., 2002; Brewer, 2004; Stowell *et al*., 2008; Cummings and Liu, 2009)	- Adult muscles (skeletal and smooth) - Thymus - Lymph nodes - Prostate - Spleen - Liver - Placenta - Endothelial cells - Skin - Olfactory neuron - Developing brain (Perillo *et al.*, 1998)
	gal-2	- Lower overall affinity compared to gal-1 - Non-reducing terminal LacNAc units - High affinity for blood groups A and B antigens (Hirabayashi *et al.*, 2002; Stowell *et al.*, 2008; Cummings and Liu, 2009)	- Epithelial cells of the stomach (rat) (Oka *et al*., 1999) - Endothelial cells (human) (Saal *et al.*, 2005)
	gal-5	- Multiantennary N-glycans with terminal LacNAc units - Enhanced binding to β1,3galactose extended LacNAc - Blood group A and B antigens (Wu *et al.*, 2006)	- Rat erythrocytes (Gitt *et al.*, 1995) - Surrounding erythroblasts (Rabinovich and Vidal, 2011) - Endosomal lumen of rat reticulocytes (Barres *et al.*, 2010)
	gal-7	- Relatively low affinity compared to gal-1 - Non-reducing terminal and internal LacNAc units - Affinity increases with branching number (tri>bi>mono) and LacNAc repeating units (type >type 2) (Hirabayashi *et al.*, 2002; Brewer, 2004)	- Keratinocytes at all stages of epidermal differentiation (Magnaldo *et al*., 1995; 1998)

	gal-10	- β-mannosides (Yang *et al*., 2008; Cummings and Liu, 2009)	- Eosinophils and basophils (Dvorak *et al*., 1991; 1992)
	gal-13	- High affinity for LacNAc and mannose (Than *et al*., 2004)	- Human placenta (Than *et al*., 2004)
	gal-14	- Non-modified α1,2 fucosylated and 2,6 sialylated poly-LacNAc structures (type 2>type 1) (http://www.functionalglycomics.org; Young *et al*., 2009)	- Ovine eosinophils (Dunphy *et al*., 2002; Young *et al*., 2009) - Chicken intestine (Stierstorfer *et al*., 2000)
***Tandem repeat* type** (two CRDs united by a linker peptide)	**gal-4**	- High affinity for α1,3 galactose- and α1,2 fucose-linked LacNAc and 3-*O*-sulfated LacNAc - High affinity for blood group A and B saccharides (Blixt *et al.*, 2004) - Glycosphingolipids carrying 3-*O*-sufated galactose residues (Ideo *et al.,* 2005) - Cholesterol 3-sulfate with no β-galactoside moiety (Ideo *et al.*, 2005, 2007)	- Epithelium of the gastrointestinal tract (Ideo *et al.*, 2005)
	gal-8	- 3-O-sulfation and α2,3 sialylation increase affinity for LacNAc (N-terminal CRD) - Preferential recognition of blood group B antigen - Preference for some glycosphingolipids including GM3 ad GD1a (Hirabayashi *et al*., 2002; Ideo *et al*., 2003; Carlsson *et al*., 2007)	- Vascular endothelial cells - Microvasculature of normal and tumoral prostatic and mammary tissue (human) (Delgado *et al*., 2011) - Endometrium (increased during lutheal phase) (Nikzad *et al*., 2013)
	gal-9	- Branched N-glycans and repeated LacNAc units - Preference for some glycosphingolipids including GM3 and GM1 (Hirabayashi *et al*., 2002)	- Liver and thymus of embryonic mice (Wada *et al*., 1997) - Monocytes (Matsuura *et al.*, 2009) - Lipid rafts of osteoblast (Tanikawa *et al.*, 2010)
	gal-12	- Lactose (Hotta *et al*., 2001; Yang *et al.*, 2001)	- Human adipocytes (Hotta *et al.*, 2001)

***Chimera* type** (one CRD and a non-lectin domain)	**gal-3**	- Non reducing terminal and internal LacNAc, affinity increases with multiple LacNAc units - A2,6 sialylated poly-LacNAc units - 3-O-sulfation enhances LacNAc recognition - High affinity for blood group A and B saccharides (Hirabayashi *et al*., 2002; Brewer, 2004; Stowell *et al.*, 2008; Cummings and Liu, 2009) - β-1,2-Linked oligomannosides (Fradin *et al*., 2000; Jouault *et al.*, 2006; Kohatsu *et al.*, 2006)	- Epithelial cells - Cartilage - Dendritic cells and inflammatory cells, especially macrophages (Wang *et al*., 1995)

1.1.1 *Intracellular functions of galectins*

As mentioned above, galectins can be found both in the extracellular environment and intracellularly, but also inside the cells most of the galectins can locate in different subcellular spaces, such as nucleus, cytoplasm and plasma membrane (Rabinovich and Croci, 2012; Compagno *et al.*, 2014). Galectins localization depends on several factors, such as the proliferative state of the cells and the post-translational modifications of the galectin (Compagno *et al.*, 2014). Gal-3, for example, is found both in the nucleus and in the cytoplasm in proliferative cells, while it is only cytoplasmic in arrested fibroblasts (Hamann *et al.*, 1991; Openo *et al.*, 2000). Moreover, this protein exists in two different states with specific localization. Gal-3 can be phosphorylated and in this case is located both in cytoplasm and nucleus, while the unphosphorylated form is only found in the nucleus (Cowles *et al.*, 1990). In addition to these specific cases, most of the galectins found in different subcellular compartments can shuttle between them, having only a temporarily preferential localization. Examples of galectins performing this nucleus/cytoplasm shuttle are gal-1 and gal-3 (Compagno *et al.*, 2014). As their intracellular localization, also the roles of galectins inside the cells change, among different galectins, for the same galectin in different intracellular locations, when expressed in different cell types and depending on the ligand availability inside the cells and therefore on the cell status (Boscher *et al.*, 2011; Compagno *et al.*, 2014).

Among the galectins found in the nucleus, the most characterized are certainly gal-3 and gal-1. These two galectins have been found to distribute in the nucleus in a punctate fashion, in structures referred as speckles, which contain several components of the splicing machinery, such as the Sm core polypeptides of snRNPs and the serine- and arginine-rich family of splicing factors (Vyakarnam *et al.*, 1997; Vyakarnam *et al.*, 1998; Park *et al.*, 2001). Moreover, they co-localize with another protein important for the splicing process, Gemin4 (Park *et al.*, 2001), confirming their involvement in the processing of mRNA. Supporting this notion, depletion of either galectin from nuclear extracts inhibits splicing activity (Laing and Wang, 1988; Dagher *et al.*, 1995; Wang *et al.*, 1995; Vyakarnam *et al.*, 1997). Interestingly, gal-1 and gal-3 presence seems to be mutually exclusive in the spliceosome (Wang *et al.*,

2006). Nuclear galectin-1 and gal-3 have also been found diffuse in the nucleosome and they can interact with different transcription factors, such as thyroid-specific transcription factor 1 and OCA-B, in a carbohydrate-independent manner (Paron *et al.*, 2003; Yu *et al.*, 2006). Other galectins that localize in the nucleus are galectin-7 (Inagaki *et al.*, 2008; Demers *et al.*, 2010), which influences the expression of TGF-β-(transforming growth factor β) responsive genes by promoting the nuclear export of the transcriptional co-activator Smad3 (Inagaki *et al.*, 2008), galectin-9, which is related to the activation of inflammatory cytokines in monocytes, probably through its association with nuclear factor interleukin 6 and/or AP-1 (activator protein 1) transcription factors (Matsuura *et al.*, 2009), galectin-2 (Dvorankova *et al.*, 2008), galectin-8 (Delgado *et al.*, 2011), galectin-10 (Dvorak *et al.*, 1992), galectin-11 (Dunphy *et al.*, 2000), galectin-12 (Hotta *et al.*, 2001) and galectin-14 (Dunphy *et al.*, 2002), whose roles in the nucleus are less defined.

Cytoplasmic galectins have a major impact on fundamental cellular processes, such as cell growth and apoptosis. Some of the galectins roles have been extensively studied and characterized, while some others are still poorly known. The influence of galectins on cell growth and apoptosis is often opposite, depending on the cell type and condition and on the galectin type (Liu *et al.*, 2002; Yang and Liu, 2003). Gal-3, the most extensively studied member of the galectin family, has mostly been characterized as anti-apoptotic and pro-survival protein. This protein exerts its role through the interaction with both anti-apoptotic factors (Yang *et al.*, 1996; Chen *et al.*, 2006) and pro-apoptotic proteins (Missotten *et al.*, 1999; Vito *et al.*, 1999), stabilizing mitochondria membrane (Yu *et al.*, 2002) and promoting cell adhesion (Matarrese *et al.*, 2000) (the role of gal-3 in cell growth and apoptosis will be discussed in detail in following sections). Galectin-1, on the other side, have been shown to have either positive or negative role on proliferation and cell death. Gal-1 blocks cell cycle at G2 phase (Blaser *et al.*, 1998) or before entry G2 phase (Wells *et al.*, 1999), it prevents quiescent cells to re-enter cell cycle (Blaser *et al.*, 1998) and it inhibits the growth of neuroblastoma cells in a carbohydrate independent manner (Kopitz *et al.*, 2001). It triggers apoptosis in activated human T-cell (Perillo *et al.*, 1998), probably by binding to CD45, CD43 and CD7 (Walzel *et al.*, 1999), and in certain types of thymocytes (Perillo *et al.*,

1997). Moreover, gal-1 can induce the phosphorylation of Bcl-2 (B cell lymphoma 2) and the induction of pro-apoptotic Bad (Rabinovich *et al.*, 2000; Brandt *et al.*, 2008). However, low doses of gal-1 induce cell proliferation (Adams *et al.*, 1996) and gal-1 levels correlate with states of malignancies of glioma, from low grade astrocytoma to glioblastoma (Yamaoka *et al.*, 2000), suggesting a role in cell growth promotion. Galectin-7 role in cell death induction is more defined, since this protein triggers apoptosis in a caspase dependent manner and increasing the release of cytochrome *c* (cyt *c*) from the mitochondria (Bernerd *et al.*, 1999; Kuwabara *et al.*, 2002). Gal-7 induces apoptosis also interacting with Bcl-2 through its CRD. If this interaction is disrupted, for example by UV irradiation or chemotherapeutic agents, gal-7 dissociated from Bcl-2 and recovers its pro-apoptotic effect (Villeneuve *et al.*, 2011). Cells overexpressing gal-7 showed a significant increase in apoptosis rate and the increased gal-7 expression correlates with the simultaneous overexpression of several genes involved in the apoptotic process (Kuwabara *et al.*, 2002). Of particular interest is the enhanced activity of c-Jun N-terminal kinase, an important regulator of apoptosis, upon gal-7 mediated apoptosis induction. Also gal-12 seems to be correlated with apoptosis induction. This *tandem repeat* type galectin shows some peculiar characteristics among the galectin family, especially its highly divergent C-terminal CRD. Moreover, it presents several AU-rich elements in the 3'-end of the untranslated region (Kozak, 1987). These elements are known to confer instability to mRNA and are not very diffused among eukaryotic mRNAs. However, their frequencies among genes involved in the control of cellular growth and differentiation, such as proto-oncoproteins, transcription factors and cytokines, is considerable (Kozak, 1987, 1991, 1992; Geballe and Morris, 1994; Chen and Shyu, 1995; Morris, 1995; Xu *et al.*, 1998; Morris and Geballe, 2000), suggesting an involvement of gal-12 in these processes. As support of a role of gal-12 in cell death and control of proliferation, an increased level of gal-12 mRNA in adipose tissue causes an increase in the number of apoptotic cells (Hotta *et al.*, 2001) and gal-12 expression has been found upregulated in cells blocked at G1 phase or at the G1/S boundary of the cell cycle (Yang *et al.*, 2001).

The variety of the roles of galectins inside the cells has been examined and from this analysis it appears clear that a role of galectins in cancer formation and progression is

more than conceivable. Indeed galectins have been implicated in a variety of tumours. As observed above for physiological cellular conditions, also in pathogenic conditions the role of galectins depend on the galectin type, its subcellular localization, the cell type and its proliferative state (Danguy *et al.*, 2002; Balan *et al.*, 2010; Vladoiu *et al.*, 2014) (**Table 3**). Not only different galectins can functions both as pro- and anti-apoptotic factors, but also the same galectin can have different roles depending on the type of cancer and its intracellular sub-localization, thus their targeting in cancer treatment must be extremely well studied and considered carefully (Vladoiu *et al.*, 2014). In addition to the above-mentioned roles in apoptosis and cell growth, galectins influence cancer progression through other important cellular processes. For example, it has been discovered that extracellular galectins have a great impact on cell adhesion, migration and invasion. Alterations in the interaction between galectins and transmembrane receptors have often been found in malignancies and late stage of carcinomas (Vladoiu *et al.*, 2014). Also in this case the effect of the same protein is often opposite. For example, gal-1, gal-7 and gal-8 are able to promote invasion and migration in hepatic and lung cancer (Hittelet *et al.*, 2003; Spano *et al.*, 2010; Chung *et al.*, 2012; Hsu *et al.*, 2013), breast cancer and T-cell lymphoma (Moisan *et al.*, 2003; Demers *et al.*, 2005; Demers *et al.*, 2007; Park *et al.*, 2009; Demers *et al.*, 2010), and glioma (Camby *et al.*, 2001), respectively, and to decrease the same processes in colorectal, gastric and urothelial (Matsui *et al.*, 2007; Kim *et al.*, 2013), and colon cancer cells (Nagy *et al.*, 2002). Gal-9 has divergent roles in cell adhesion, promoting it in melanoma, oral and colon cancer cell, but reducing it in breast cancer (Kageshita *et al.*, 2002; Irie *et al.*, 2005; Kasamatsu *et al.*, 2005; Yamauchi *et al.*, 2006; Nobumoto *et al.*, 2008; Zhang *et al.*, 2009). However, some galectins have a more well defined role in promoting invasion, like gal-3 (Bresalier *et al.*, 1998; Inufusa *et al.*, 2001; Ellerhorst *et al.*, 2002; O'Driscoll *et al.*, 2002; Jiang *et al.*, 2008; Braeuer *et al.*, 2012; Radosavljevic *et al.*, 2012; Song *et al.*, 2012), or in enhancing cell adhesion and reducing migration, like gal-4 (Belo *et al.*, 2013). Besides adhesion, migration and invasion, extracellular galectins act on cancer progression through other common mechanisms, such as angiogenesis and tumour immune escape. Galectin-1 and gal-3 promote immune evasion (O'Driscoll *et al.*, 2002; He and Baum, 2006; Daroqui *et al.*, 2007; Peng *et al.*, 2008;

Radosavljevic *et al.*, 2011; Tang *et al.*, 2012), and new vessels formation (Clausse *et al.*, 1999; Califice *et al.*, 2004a; Le Mercier *et al.*, 2008; Mourad-Zeidan *et al.*, 2008; Ito *et al.*, 2011), while gal-7 has an opposite effect on angiogenesis, inhibiting it (Ueda *et al.*, 2004).

In conclusion, galectins have a deep impact on cell proliferation and cell death and their role in these cellular processes is especially relevant during tumorigenesis. However, when studying the roles of galectins, special precautions must be taken, due to the high variety of their roles and their differentiation among different cell types in specific proliferative states, different cellular and sub-cellular localizations, available binding partners and their differential glycosylation and, of course, different galectins. Therefore, every system should be examined carefully and specific galectin functions in that system should not be generalized.

1.1.2 *Extracellular functions of galectins*

Galectins can influence cell growth and apoptosis also when localized extracellularly, interacting with glycans exposed on the external leaflet of the plasma membrane. In particular, several galectins can localize in lipid rafts, where they interact with gangliosides, glycosphingolipids and glycosylated transmembrane receptors (Fajka-Boja *et al.*, 2008; Tanikawa *et al.*, 2008; Hsu *et al.*, 2009; Kopitz *et al.*, 2012). The formation of clusters of galectins bound to their receptors enhances the avidity of the binding, stabilizing the downstream signalling. In particular, it has been discovered that gal-1 causes T-cells apoptosis when binding to membrane glycoconjugates (Walzel *et al.*, 2006; Earl *et al.*, 2010), and gal-8 has a pro-apoptotic effect on several cell types, such as synovial leukocytes, by interacting with CD44 and ECM proteins (Eshkar Sebban *et al.*, 2007), Jurkat cells, through a strong ERK1/2 activation and a consequent expression of death factor Fas ligand (Norambuena *et al.*, 2009), immature thymocytes (Tribulatti *et al.*, 2007) and activated T-cells (Cattaneo *et al.*, 2011). Also extracellular gal-2 induces apoptosis in a caspase dependent manner, associated with the release of cyt *c* and the increase Bax/Bcl-2 ratio (Sturm *et al.*, 2004).

Table 3: Main galectins expressed in different tumours (adapted from Vladoiu *et al.*, 2014).

CANCER TYPE	GALECTIN	EFFECT
BREAST	gal-1	Angiogenesis, evasion, progression (Daroqui *et al.*, 2007; Jung *et al.*, 2007; Ito *et al.*, 2011; Dalotto-Moreno *et al.*, 2013)
	gal-2	Adhesion (Barrow *et al.*, 2011)
	gal-3	Cell cycle arrest in response to anoikis, increases adhesion, tumour growth and protects from apoptosis (Kim *et al.*, 1999; Matarrese *et al.*, 2000; Honjo *et al.*, 2001; Moon *et al.*, 2001; Shekhar *et al.*, 2004)
	gal-7	Increases invasion, reduces chemosensitivity (Demers *et al.*, 2010)
	gal-9	Increases cell aggregation and reduces adhesion (Irie *et al.*, 2005)
COLORECTAL	gal-1	Reduces cell migration, induces adhesion and apoptosis (Hittelet *et al.*, 2003; Horiguchi *et al.*, 2003; Zhao *et al.*, 2010)
	gal-3	Metastasis formation, immune evasion, reduces apoptosis (Bresalier *et al.*, 1998; Shi *et al.*, 2007; Peng *et al.*, 2008; Mazurek *et al.*, 2012; Wu *et al.*, 2013)
	gal-4	Promotes adhesion, reduces cell migration and motility, induces cell cycle arrest (Ideo *et al.*, 2005; Satelli *et al.*, 2011; Kim *et al.*, 2013d)
COLON	gal-2	Adhesion (Barrow *et al.*, 2011)
	gal-7	Increases chemosensitivity and reduces cell growth, anchorage-independent cell growth and angiogenesis (Ueda *et al.*, 2004)
	gal-8	Reduces tumour growth and cell migration (Nagy *et al.*, 2002)
	gal-9	Increases adhesion in vitro but reduces metastasis formation in vivo (Nobumoto *et al.*, 2008; Zhang *et al.*, 2009)
PROSTATE	gal-1	Adhesion and apoptosis, reduces growth rate of LnCaP cells, immune evasion and tumour vascularization, heterotypic cell-cell adhesion (Clausse *et al.*, 1999; Ellerhorst *et al.*, 1999b; Ellerhorst *et al.*, 1999a; He and Baum, 2006; Laderach *et al.*, 2013)
	gal-3	Chemoresistance, cell proliferation, angiogenesis, migration and invasion (van den Brule *et al.*, 2000; Ellerhorst *et al.*, 2002; Califice *et al.*, 2004; Fukumori *et al.*, 2006; Wang *et al.*, 2009)

THYROID	gal-1	Associated with malignant transformation (Chiariotti *et al*., 1995; Xu *et al*., 1995)
	gal-3	Anchorage-independent growth and motility (Yoshii *et al*., 2001; Takenaka *et al*., 2003; Lavra *et al.*, 2009; Lin *et al*., 2009a; Lin *et al.*, 2009b; Shankar *et al*., 2012)
CERVICAL	gal-1	Radioresistance, proliferation and invasion (Huang *et al.*, 2012; Kim *et al*., 2013b)
	gal-7	Invasion and chemoresistance (Park *et al.*, 2009; Zhu *et al*., 2013)
	gal-12	Reduces cell growth (Yang *et al*., 2001)
PANCREAS	gal-1	Proliferation, invasion and immune evasion (Xue *et al*., 2011; Tang *et al*., 2012)
	gal-3	Increases invasion and proliferation, reduces chemosensitivity (Jiang *et al*., 2008; Kobayashi *et al*., 2011; Merlin *et al*., 2011; Song *et al*., 2012)
	gal-4	Reduces migration and metastasis formation (Belo *et al*., 2013)
OVARIAN	gal-1	Increases proliferation and invasion (Kim *et al*., 2012)
	gal-3	Reduces cell proliferation and increases apoptosis resistance (Oishi *et al*., 2007; Kim *et al.,* 2011a)
	gal-7	Cell proliferation (Kim *et al*., 2013a)
LUNG	gal-1	Chemoresistance, migration and invasion (Chung *et al.*, 2012; Hsu *et al*., 2013)
	gal-3	Adhesion, motility, invasion and immune evasion (O'Driscoll *et al*., 2002)
MELANOMA	gal-1	Cell aggregation (Tinari *et al.*, 2001)
	gal-3	Metastasis formation, immune evasion and angiogenesis (Mourad-Zeidan *et al*., 2008; Srinivasan *et al*., 2009; Radosavljevic *et al.*, 2011; Braeuer *et al*., 2012; Wang *et al*., 2012)
	gal-7	Chemoresistance (Biron-Pain *et al.*, 2013)
	gal-9	Cell aggregation and apoptosis (Kageshita *et al*., 2002; Nobumoto *et al*., 2008; Wiersma *et al.*, 2012)
NEUROBLA-STOMA	gal-1	Cell growth, immune evasion (Kopitz *et al.*, 2001)
	gal-3	Reduces apoptosis (Veschi *et al*., 2012)
	gal-7	Reduces cell growth (Kopitz *et al*., 2003)

GLIOMA and GLIOBLASTO-MA	gal-1	Cell growth, invasion, angiogenesis and chemotherapy resistance (Yamaoka *et al.*, 2000; Rorive *et al.*, 2001; Camby *et al*., 2005; Strik *et al*., 2007; Jung *et al*., 2008; Le Mercier *et al*., 2008a; 2008b)
	gal-3	Decreases cell motility and adhesion (Debray *et al*., 2004)
	gal-8	Cell migration (Camby *et al*., 2001)
LYMPHOMA	gal-1	Decreases viability and cell growth (Fouillit *et al.*, 2000; Poirier *et al*., 2001)
	gal-3	Resistance to Fas-induced apoptosis, chemoresistance or induces apoptosis (Hoyer *et al.*, 2004; Suzuki and Abe, 2008; Li *et al*., 2010; Clark *et al*., 2012)
	gal-7	Metastasis formation (Moisan *et al*., 2003; Demers *et al*., 2005; Demers *et al*., 2007)
GASTRIC	gal-3	Cell motility and chemoresistance (Cheong *et al*., 2010; Kim *et al*., 2010; 2011b)
	gal-7	Reduces cell proliferation, migration and invasion (Kim *et al*., 2013c)
HEPATIC	gal-1	Migration and invasion (Spano *et al.*, 2010)
	gal-3	Metastasis formation (Inufusa *et al*., 2001)
LEYDIG TUM. CELLS	gal-1	Cell proliferation and apoptosis (Biron *et al.*, 2006)
MYELOID LEUKAEMIA	gal-3	Reduces chemosensitivity (Cheng *et al*., 2011; Yamamoto-Sugitani *et al*., 2011)
BLADDER	gal-3	Protects cells against TRAIL-induces apoptosis (Oka *et al*., 2005)
TONGUE	gal-3	Cell proliferation, migration and invasion (Wang *et al*., 2013; Zhang *et al*., 2013)
RENAL	gal-3	Reduces chemosensitivity (Xu *et al.*, 2013)
UROTHELIAL	gal-7	Increases chemosensitivity (Matsui *et al*., 2007)
ORAL	gal-9	Increases adhesion (Kasamatsu *et al*., 2005; Yamauchi *et al.*, 2006)
MYELOMA	gal-9	Reduces cell growth and induces apoptosis (Kobayashi *et al.*, 2010; Kuroda *et al.*, 2010)
T CELL LEUKAEMIA	gal-12	Reduces cell growth (Yang *et al*., 2001)

In addition to cell death control, extracellular galectins have roles in cellular differentiation and physiology. Gal-9 promotes osteoblast differentiation when present in the lipid rafts, interacting with CD44 and the bone morphogenetic protein (Tanikawa *et al.*, 2010), while gal-1 has a fundamental role in determining the properties of antigen presenting cells through the activation of MAP kinases (Barrionuevo *et al.*, 2007; Malik *et al.*, 2009; Ortner *et al.*, 2011). Additional and specific roles of extracellular galectins are angiogenesis induction by gal-1, which interacts with several glycosylated receptors leading ultimately to vascular modulation of endothelial cells (Hsieh *et al.*, 2008) and control of intracellular cholesterol level by gal-4 in the epithelium of the gastrointestinal tract (Ideo *et al.*, 2005, 2007).

Besides interacting with glycoconjugates on the cellular wall, galectin can also bind to glycoprotein in the ECM, such as laminin (Zhou and Cummings, 1990; Sato and Hughes, 1992), fibronectin (Sato and Hughes, 1992; Ozeki *et al.*, 1995), and tenascin (Probstmeier *et al.*, 1995). These interactions, similarly to the ones happening on the cell wall, activate important signalling cascades that have a pivotal role in cell adhesion and motility. Variations in adhesion and motility mainly depend on reorganization of actin cytoskeleton and formation of adhesion plaques (Levy *et al.*, 2003; Goetz *et al.*, 2008). As described in table 3, the effect on the different galectins on adhesion, invasion and migration can change to a high degree, based on the concentration of galectin present in the media (Matarrese *et al.*, 2000), on the biological properties of the galectin, especially its binding ability and number of CRDs (Mehul *et al.*, 1995; van den Brule *et al.*, 1998), and on the activation of proteins that are involved in the interaction between galectins and ECM, such as integrins (Larsen *et al.*, 2006).

Extracellular galectins have a fundamental role also in cell immunity. Innate immunity is the first and faster level of defence of an organism. It is not specific for the particular antigen of a pathogen, but in most of the cases it is able to stop or defeat the infection. The cells responsible for the activity of the innate immunity mainly respond to two mechanism. First, they need signals able to tell them if a molecule belongs to the pathogen or to the organism itself, according to the self/non-self model (Janeway, 1989; Medzhitov and Janeway, 2002), in order to activate the immune response only in the presence of an actual invader. Second, they evaluate the degree of danger of the invading pathogen, according to the danger model

(Matzinger, 1994; 2002a; 2002b). Both models require specific proteins able to recognize self and non-self molecular motifs and proteins that can carry information about the degree of invasiveness and risk of the microorganism (Mushegian and Medzhitov, 2001).

As explained before, carbohydrates are important instruments to transmit information inside and outside the cells. Glycosylation is a ubiquitous modification of proteins and lipids, interesting about 50-70% of human proteins. Importantly, cells from different origins are able to synthetize different glycoconjugates, making the glycomic profile of a cell a specific and unique characteristic. In particular, mammalian cells have an array of glycoconjugates distinct from the one of pathogenic microorganisms, such as viruses, bacteria, fungi and parasites (Reuter and Gabius, 1999; Gabius *et al.*, 2002) (**Fig. 3**). Thus, the features of glycoconjugates specific to a particular class of organisms may serve as basis for recognition by host proteins (Buzas *et al.*, 2006). Considering this, it is clear the major role of lectins in immunity, helping the cells recognizing specific glycans on their own molecules or on pathogen molecules.

A confirmation of this could be seen in the fact that galectins are highly expressed in the cells of the innate and adaptive immune system and that they can be found both in the intracellular and the extracellular environment. Some lectins, such as selectins and siglects, are specialized in recognizing self-glycans, while others bind specifically to glycans found on pathogens. Galectins cannot be exclusively grouped in either category. Indeed galectins have been found to be involved in recognition of glycans from different origins and to act on different levels in innate immunity. They can serve as pathogen recognition receptors (PRRs), which specifically bind to pathogen-associated molecular patterns (PAMPs); they can bind to self glycans, stopping or attenuating the immune response; they can work as DAMPs, specific molecules released from the cells upon non-programmed cell death, which regulate innate immunity by activating immune cells such as macrophages and neutrophils and promote the reconstruction of tissues (Matzinger, 2002a; 2002; 2007; Kono and Rock, 2008). Upon binding to the glycans, the effects on the cells vary depending on different factors, such as glycans density, glycan clustering, glycan presentation on specific glycoproteins and glycolipids and interaction with other cell surface molecules. The interaction between galectins and glycans

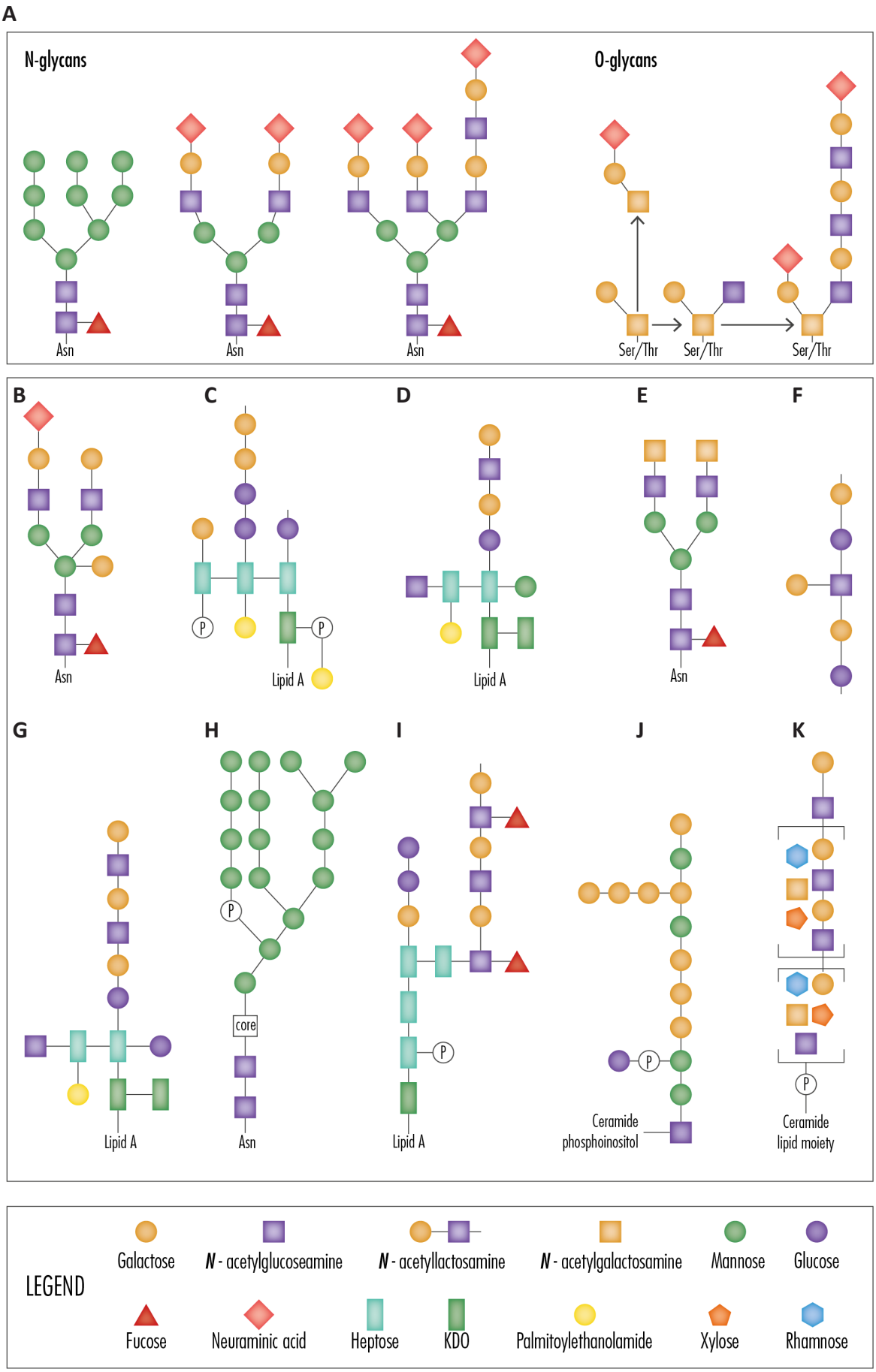

A
N-glycans
O-glycans
Asn
Asn
Asn
Ser/Thr
Ser/Thr
Ser/Thr
B
Asn
C
P
P
Lipid A
D
Lipid A
E
Asn
F
G
Lipid A
H
P
core
Asn
I
P
Lipid A
J
P
Ceramide
phosphoinositol
K
P
Ceramide
lipid moiety
LEGEND
Galactose
N - acetylglucoseamine
N - acetyllactosamine
N - acetylgalactosamine
Mannose
Glucose
Fucose
Neuraminic acid
Heptose
KDO
Palmitoylethanolamide
Xylose
Rhamnose

Figure 3: *Structure and composition of the main glycans patterns present on the surface of various types of cells*. N- glycans and O-glycans on human cells (**A**) and antennary glycans on microorganism cells that can be recognized by galectins (**B-K**). Complex type N-linked oligosaccharide from the HIV-1 gp120 envelope glycoprotein (**B**); *Haemophilus influenzae* LPS (**C**); *Neisseria meningitidis* LPS (**D**); *Schistosoma mansoni* LacdiNAc (LDN) (**E**); *Streptococcus pneumoniae* polysaccharide (**F**); *Neisseria gonorrhoeae* lipooligosaccaride (**G**); *Candida albicans* oligomannan (**H**); *Helicobacter pylori* LPS O-antigen side chain (**I**); *Leishmania major* LPG (**J**); *Trichomonas vaginalis* LPG (**K**) (based on information from Rabinovich and Toscano, 2009; Vasta, 2009).

could either promote or block the infection. Galectins can block invasion either directly killing the microorganism or stimulating the activation of the cells of the immune system (Buzas *et al.*, 2006; Sato *et al.*, 2009; Vasta, 2009; Baum *et al.*, 2014). For example, galectin-1 promotes the entry and attachment of HIV-1 (human immunodeficiency virus type 1) (St-Pierre *et al.*, 2011; Sato *et al.*, 2012), Nipah viruses (Levroney *et al.*, 2005) and human T-cell leukaemia virus type 1 (HTLV-1) (Gauthier *et al.*, 2008) by cross-binding glycans on both the pathogen cell wall and human cells, while galectin-4 impedes *Bordetella pertussis* and *Helicobacter pylori* to attach to glycolipids receptor occupying them (Ideo *et al.*, 2005; Danielsen and Hansen, 2006).

2. Galectin-3

Gal-3 is the only *chimera* type galectin in the galectin family (Hirabayashi and Kasai, 1993; Rabinovich, 1999) and the most studied member. It is characterized by the existence of an atypically long N-terminal domain, rich in proline and glycine, besides the C-terminal carbohydrate binding domain (Hsu *et al.*, 1992; Agrwal *et al.*, 1993). The N-terminal domain is essential for the biological activity of gal-3, since it is implicated in the secretion of gal-3 in the extracellular environment (Menon and Hughes, 1999) and it allows the oligomerization and therefore the cross-linking activity of the protein (Massa *et al.*, 1993). Gal-3 usually exists as a monomer, but it can form multimers up to pentamers (Ahmad *et al.*, 2004; Morris *et al.*, 2004) through either its N-terminal (Hsu *et al.*, 1992; Mehul *et al.*, 1994) or its C-terminal domain, when it is not occupied by glycan-binding (Yang *et al.*, 1998). Gal-3 size is between 29 and 35 kDa (Ho and Springer, 1982), depending on the specie.

Gal-3 has multiple ligands, which differ in both structure and function. The specificity of gal-3 toward one ligand instead of another depends on the sugar-binding affinity, on the phosphorylation status of gal-3 and on the time and space coordination of the ligand expression. Importantly, gal-3 can interact also with unglycosylated protein thanks to its N-terminal domain. Gal-3 shows a strong preference toward polylactosamine structures and the affinity increases with the increasing number of lactosamine units present on the glycan chain (Sato and Hughes, 1992; Hirabayashi *et al.*, 2002). Upon binding, the conformation of gal-3 changes, with the rearrangement of the backbone loops near the binding site (Agrwal *et al.*, 1993; Umemoto *et al.*, 2003).

Gal-3 coding gene is *LGALS3*, localized on the chromosome 14, *locus* q21-q22 (Raimond *et al.*, 1997). Gal-3 promoter region is particularly abundant in regulatory elements, which make its expression a complex process involving several transcriptions factors and signalling pathways. Some of the best-characterized regulatory elements are Sb1 binding site (GC boxes), cAMP dependent response element (CRE) motifs, NF-κB-like sites, among others (Kadrofske *et al.*, 1998). Gal-3 expression is modulated by various factors, including the proliferation and differentiation state of the cell, infections and tumour progression (Dumic *et al.*, 2006). For example, gal-3 was found at higher level in proliferating fibroblasts compared to quiescent cells (Moutsatsos *et al.*, 1987; Agrwal *et al.*, 1989) and its presence was detected in lymphocytes, otherwise expressing gal-3 to a null or minimal level (Flotte *et al.*, 1983; Liu *et al.*, 1985; Joo *et al.*, 2001), only following activation with Concanavalin A or CD3 (Joo *et al.*, 2001). Moreover, the change of gal-3 expression between resting macrophage and endocytic macrophage is so relevant that gal-3 is indicated as “macrophage activator marker” (Elliott *et al.*, 1991). Gal-3 expression is increased following transformation events, such as *RAS*-mediated transformation, and it is found at its peak when cells lose their growth anchorage-dependence (Hebert and Monsigny, 1994). Finally, gal-3 expression has been found elevated in cells infected by HTVL-1 (Hsu *et al.*, 1996) and by HIV-1 (Schroder *et al.*, 1995).

Gal-3 is ubiquitously expressed in adults, in particular in epithelial cells of various tissues and myeloid and amoeboid cells (Dumic *et al.*, 2006). It has been found significantly expressed also in cells of the immune response, such as neutrophils (Truong *et al.*, 1993a),

eosinophils (Truong *et al.*, 1993b), basophils and mast cells (Frigeri *et al.*, 1993; Craig *et al.*, 1995), Langerhans cells (Wollenberg *et al.*, 1993; Smetana *et al.*, 1999), dendritic cells (Flotte *et al.*, 1983; Dietz *et al.*, 2000), monocytes (Liu *et al.*, 1995) and macrophages (Flotte *et al.*, 1983; Liu *et al.*, 1995; Kasper and Hughes, 1996; Saada *et al.*, 1996; Maeda *et al.*, 2003). The type of cell that expresses gal-3 and the localization of the protein – intra- or extracellular, various sub-cellular compartments- define the biological role of the protein.

Gal-3 is synthetized in the cytoplasm, but it is found also on the cell membrane, in the nucleus and in the extracellular space. The way in which gal-3 is externalized is not completely understood yet. However, it is clear that it happens through an alternative secretory pathway, since gal-3 does not contain specific signal for translocation into the ER and subsequent externalization (Hughes, 1999). One of the proposed mechanism by which gal-3 is externalized is called *ectocytosis* and it is independent from the classical ER/Golgi pathway (Sato *et al.*, 1993; Sato and Hughes, 1994; Nickel, 2003; Krzeslak and Lipinska, 2004). As mentioned above, part of the N-terminal domain (aa 89 to 96) is considered necessary for the proper secretion of gal-3 (Menon and Hughes, 1999). According to the *ectocytosis* hypothesis, gal-3 is incorporated in small vesicles that invaginate from the plasma membrane and it is then quickly released in the extracellular space (Sato and Hughes, 1994; Bao and Hughes, 1995; Mehul and Hughes, 1997; Hughes, 1999; Menon and Hughes, 1999). Supporting the hypothesis that gal-3 is contained in vesicles and externalized through them is the finding that gal-3 was present in exosomes secreted by dendritic cells (Garin *et al.*, 2001; Thery *et al.*, 2001). Gal-3 can also be internalized by endocytosis in a lactose-dependent manner (Furtak *et al.*, 2001).

Due to the variety of cellular and subcellular locations and the amount of cell type in which it is expressed, gal-3 is one of the galectin with the highest degree of function diversification and broader involvement in cell physiology. As mentioned above, cytoplasmic gal-3 is involved in various intracellular events, such as the regulation of apoptosis, through the interaction of proteins such as Bcl-2 (Yang *et al.*, 1996; Fukumori *et al.*, 2006), CD95 (Fukumori *et al.*, 2004), Alix (Liu *et al.*, 2002), annexin VII (Yu *et al.*, 2002) and nucling (Liu *et al.*, 2004). It is also involved in cell proliferation and differentiation, interacting with the oncoprotein KRAS.

Considering the processes in which gal-3 is involved, it is evident its relevant role in human oncogenesis (Newlaczyl and Yu, 2011; Cay, 2012; Song *et al.*, 2014).

In the nucleus gal-3 is associated with the ribonucleoprotein complexes (Laing and Wang, 1988), being involved in the spliceosome assembly and in mRNA splicing (Dagher *et al.*, 1995). The role of gal-3 in splicing regulation has been previously described (*intracellular functions of galectins*), but the functions of gal-3 in the nucleus are not limited to such task. Indeed gal-3 participates in the activation of the transcription factor CREB (CRE binding protein) and Sp1 in the cyclin D1 promoter region, inducing cyclin D1 expression. Since CRE and Sp1 elements are very common among promoter regions, it is hypothesized that gal-3 could control also the expression of other important proteins, such as cyclin A, cyclin E, $p21^{Cip1,}$ and $p27^{Kip1}$ (Kim *et al.*, 1999). Moreover, gal-3 in the nucleus can bind to β-catenin and axin, two interacting proteins involved in the Wnt pathway, suggesting a role for gal-3 in Wnt pathway regulation (Shimura *et al.*, 2004; Shimura *et al.*, 2005).

When gal-3 is localized on the cell surface, it exerts functions such as adhesion, interacting with glycoprotein on the extracellular matrix, pathogen recognition through specific glycan patterns (Sato and Nieminen, 2004), and immune cell activation (Rabinovich and Toscano, 2009). Gal-3 involvement in adhesion is particularly important and it relays on its ability to bind to glycoconjugates on the cell and glycans in the ECM at the same time. As explained for all other members of galectin family, gal-3 can bind to several glycoproteins, including laminin (Massa *et al.*, 1993; van den Brule *et al.*, 1995; Kuwabara and Liu, 1996), fibronectin (Sato and Hughes, 1992), hensin (Hikita *et al.*, 2000), elastin (Ochieng *et al.*, 1999), collagen IV (Ochieng *et al.*, 1998) and tenascin-C and -R (Probstmeier *et al.*, 1995). In addition, gal-3 binds to certain type of integrins (Ochieng *et al.*, 1998) and is able to promote integrin activation through binding to CD98 (Hughes, 1999). The specific effect of gal-3 on adhesion depends, as usual, on the cell type and the glycoproteins that it expresses. For example, it enhances the adhesion of human neutrophils to laminin (Kuwabara and Liu, 1996) and endothelial cells (Sato *et al.*, 2002), while it acts as de-adhesion protein in the thymus (Villa-Verde *et al.*, 2002). Gal-3 role in immunity is complex and multiple. Further on it will be discussed gal-3 involvement in the recognition of self and non-self glycan and the

consequent response against pathogens, but gal-3 is also important to activate several cells of the immune system through the cross-linking of glycosylated membrane receptors. For example, gal-3 causes mediator release from mast cells (Frigeri *et al.*, 1993), induces phagocytic activity and CD66 surface expression on human neutrophils (Fernandez *et al.*, 2005), enhances the production of IL-1 following lipopolysaccharide (LPS) exposure (Jeng *et al.*, 1994), provokes the production of superoxide anion by human monocytes (Yamaoka *et al.*, 1995) and activate T-cell (Demetriou *et al.*, 2001). Considering the roles of these cell types in the onset of autoimmune disease, it is conceivable to suggest a role of gal-3 in the development of autoimmunity (Radosavljevic *et al.*, 2012; de Oliveira *et al.*, 2015). Indeed anti-gal-3 autoantibodies have been found in some autoimmune disorders such as Chron's disease (Jensen-Jarolim *et al.*, 2001) and in systemic lupus erythematosus and polymyositis/ dermatomyositis (Lim *et al.*, 2002). Moreover, the level of gal-3 was increased in sera and synovial fluid from patients with rheumatoid arthritis (Ohshima *et al.*, 2003).

In addition to autoimmune disease and cancer, gal-3 has been implicated in other pathological conditions. For example, a correlation between heart failure and gal-3 has been notice, to the point of suggesting the usage of gal-3 as predictive biomarker for mortality upon heart failure (de Boer *et al.*, 2012; Ho *et al.*, 2012; Srivatsan *et al.*, 2015). Gal-3 has also been implicated in a number of metabolic disorders, often related to chronic inflammation, such as obesity, diabetes, hypercholesterolemia and hypertension. The level of circulating gal-3 in patients affected by these disorders was significantly higher than in normal population. Several studies confirm the correlation between level of gal-3 and altered glucose homeostasis, with consequent arise of obesity and/or diabetes type 2 (Menini *et al.*, 2016).

2.1 Extracellular galectin-3 role in innate immunity

As mentioned, gal-3 is synthesized and accumulated in the cytoplasm of various cells. However, many of its functions are exploited only if gal-3 is secreted in the extracellular environment. Gal-3 may exit the cell upon breakage of the cell, following necrosis, or it can be secreted through the leaderless (or alternative) secretory pathway. Extracellular gal-3 is mainly involved in innate immunity, mainly binding to self-glycans expressed on the cell surface (Mandrell *et al.*, 1994). When gal-3 cross-links various glycans on the same cell, it forms two- and three-dimensional complexes called *lattices* (Lee and Lee, 2000; Sacchettini *et al.*, 2001). L*attices* cover great areas on the cell surface and have a big steric dimension. Thus they noticeably reduce the lateral mobility of the other proteins present on the cell surface, including the receptors responsible for activating the pathways of the innate immunity. In this way *lattices* raise the threshold for ligand-dependent receptor clustering and thus they can impede or reduce signal transduction (Pelletier and Sato, 2002). Therefore, the recognition of self-glycans by gal-3 could block the activation of the immune response and the triggering of potential autoimmune response. This could be verified by the progression of the infection by *Leishmania major*. Gal-3 can bind to the lipophosphoglycan (LPG) of this parasite, which in turn has a protease (gp63) able to cleave gal-3, separating the CRD from the oligomerization N-terminal domain. Gal-3 cannot cross-link without the N-terminal domain and it is not able to form *lattices*. It has been demonstrated that the absence of *lattices* causes an enhanced immune reaction and decreases the threshold for the initiation of signal transduction (Pelletier and Sato, 2002; Spath *et al.*, 2003).

Gal-3 has another role in innate immunity besides recognizing self-glycans. It is highly expressed in myeloid cells such as macrophages and stromal cells of the lymph nodes and in specific, its expression increases during inflammation. Also the release of gal-3 increases under stress condition: immature macrophages and neutrophils are unable to secrete gal-3, while after activation macrophages can release up to 50% of their total gal-3 (Sato and Hughes, 1994). It is conceivable to consider gal-3 as a DAMP, which helps in

the efficient and precise activation of the innate immune response. Gal-3 can function as neutrophil adhesion molecule, involved in the migration of neutrophils in the site of the infection (Hsu *et al.*, 1996; Haziot *et al.*, 2001; Sato *et al.*, 2002). This process seems to be correlated to the pathogenicity of the invading microorganism. In a model with gal-3 deficient mice the neutrophils migration was impaired only when the mice were infected with the pathogenic *Streptococcus pneumonia* and not with the non-pathogenic *Escherichia coli* (Sato *et al.*, 2002). Besides enhancing neutrophils migration, gal-3 causes neutrophils and macrophages activation and their oxidative burst (Liu *et al.*, 1995; Yamaoka *et al.*, 1995; Karlsson *et al.*, 1998). Gal-3 is involved in the clearance of *Schistosoma mansoni,* causing eosinophil-mediated cytotoxicity toward the parasite (Truong *et al.*, 1994) and inducing its phagocytosis by macrophages, after the binding to its eggs through the GalNAcβ1-4GlcNAc contained in the pathogen glycans (van den Berg *et al.*, 2004); it acts as a chemoattractant for macrophages (Sano *et al.*, 2000) and as opsonin for apoptotic neutrophils (Karlsson *et al.*, 2009), increasing the efficiency of phagocytosis of apoptotic cells by macrophages when present on their surface (Sato and Nieminen, 2004).

Gal-3 has the ability to bind to non-self glycans, producing different outcomes based on the microorganism they belong to. It can actively reduce the growth or even kill the pathogen, as observed for its direct bacteriostatic activity toward *Streptococcus pneumonia* (Farnworth *et al.*, 2008). Sometimes it enhances the recruitment of the immunity cells, such as when it binds to the O-antigen of *Helicobacter pylori* (Lim *et al.*, 2003; Huff *et al.*, 2004; Fowler *et al.*, 2006) or to the LacdiNAc glycans of *Schistosoma mansoni* (van den Berg *et al.*, 2004). Other times its defensive activity can be subverted by the microorganisms, which take advantage of its cross-linking ability to adhere and invade the host cells. Examples are the parasites *Trypanosoma cruzi* (Vray *et al.*, 2004), which uses gal-3 to mediate its adhesion to laminin of the coronary artery smooth muscle cells, *Herpex simplex* virus (Woodward *et al.*, 2013) and *Neisseria meningitis* (Quattroni *et al.*, 2012). Gal-3 is also able to bind to the LPS of various microorganism, such as *Klebsiella pneumoniae*, *Salmonella minnesota*, *Salmonella typhimurium*, *Escherichia coli,* using either its N- or C- terminal domain (Mey *et al.*, 1996), and to bind directly to both gram-negative - *Klebsiella pneumoniae, Neisseria meningitidis,*

Neisseria gonorrhoeae, Haemophilus influenzae, and *Pseudomonas aeruginosa*- and gram positive - *Streptococcus pneumoniae* – bacteria (Mandrell *et al.*, 1994; Mey *et al.*, 1996; Gupta *et al.*, 1997; Vinogradov and Perry, 2001; John *et al.*, 2002).

2.1.1 Focus on the effects of galectin-3 on Candida albicans

Gal-3 has a specific role in the recognition of the pathogen fungus *Candida albicans*. It was discovered that a 32 kDa macrophage protein was able to bind specifically to β-1,2-linked mannopyranose units present on the cell wall of *C. albicans* (Fradin *et al.*, 2000). Interestingly, these glycans are a specific feature of *C. albicans*, not being present in the cell wall of *Saccharomyces cerevisiae* or other species of *Candida.* The interaction between gal-3 and this type of glycan was considered unusual, since gal-3 binds specifically to β-galactosides. It was later verified that gal-3 was bound to *C. albicans* cell wall only in living yeast, and that after the pathogen phagocytosis, it translocates to inside the cell locating in particular in the phagocytic cups (Jouault *et al.*, 2006). Notably, the importance of gal-3 in tissue invasion by *C. albicans* is inferred from the fact that tissues affected by systemic candidiasis show a broader expression of gal-3, which is present throughout granulomata, in the macrophages and in stromal cells (Kohatsu *et al.*, 2006). In healthy tissues gal-3 is present almost only in macrophages. Moreover, it has been observed that the level of gal-3 in new-born infants is significantly lower than in adults, which appears to be the reason why new-borns are more sensitive to candidiasis than adults (Linden *et al.*, 2013a). Accordingly, the absence of gal-3 in model mice increased mortality and enlarged the area of the brain infected by *C. albicans* (Linden *et al.*, 2013a).

In a proteomic analysis of sub-cellular fractions (cytosol, organelle/membrane, nucleus) of murine macrophages (Reales-Calderon *et al*., 2013), it was found that gal-3 expression increased after infection with *C. albicans*. Specifically, gal-3 was detected in the phagocytic cups, as mentioned above (Jouault *et al.*, 2006), as well as outside the cells. Actually, an increase in the amounts of gal-3 present on the cell surface or in the extracellular space was verified, along with a concomitant decrease in the cytoplasmic location (Reales-Calderon

et al., 2012). The macrophage gal-3 increased secretion upon *C. albicans* invasion was also described in other models, like human gingival epithelial cell line Ca9-22, and human gingival fibroblasts (Tamai and Kiyoura, 2014). Also neutrophils secret a higher amount of gal-3 upon *C. albicans* infection and this causes an enhanced phagocytosis of the pathogens (Linden *et al.*, 2013b). Gal-3 seems to be necessary for appropriate tumour necrosis factor α production (Esteban *et al.*, 2011; Devillers *et al.*, 2013) and plays a relevant role in neutrophils activation, which in turns is crucial for fungal clearance (Linden *et al.*, 2013a). Importantly, it was shown that gal-3 has a direct fungicidal effect on *C. albicans*, since yeast cells treated with gal-3 had decreased viability. Gal-3, in addition, caused morphological changes in a fraction of yeast cells, namely decreasing their size (Kohatsu *et al.*, 2006).

2.2 Roles of galectin-3 in cancer

Gal-3 has been shown to be involved in several fundamental processes of cell life, including apoptosis, cell cycle regulation and immune escape, which are particularly relevant in tumour formation and progression (Califice *et al.*, 2004b; Krzeslak and Lipinska, 2004; Newlaczyl and Yu, 2011; Song *et al.*, 2014; Ahmed and AlSadek, 2015; Wang and Guo, 2016).

Gal-3 involvement in apoptosis has been extensively studied and gal-3 has been determined to be both a pro- and anti-apoptotic protein, based on its localization. Intracellular gal-3 mainly acts as anti-apoptotic protein through the interaction with several proteins involved in this process, while extracellular gal-3 can be also pro-apoptotic (**Fig. 4**) (Krzeslak and Lipinska, 2004; Dumic *et al.*, 2006; Ahmed and AlSadek, 2015). Gal-3 is highly homologous to Bcl-2, an anti-apoptotic protein belonging to the Bcl-2 proteins family. Gal-3 and Bcl-2 present 28% homology and 48% similarity (Akahani *et al.*, 1997).

In particular, their N-terminal domains are both rich in proline, glycine and alanine, and they both contain the NWGR motif – in gal-3 CRD-, which is a conserved short sequence in BH1 domain of Bcl-2 family proteins, fundamental for their anti-apoptotic activity. Gal-3 is the only galectin so far known to have an NWGR motif (Akahani *et al.*, 1997). This motif allows the

oligomerization of gal-3 through its CRD, in the absence of a glycan ligand (Yang *et al.*, 1998), but it also makes possible the formation of Bcl-2 homodimer and Bcl-2/Bax heterodimer (Hanada *et al.*, 1995). NWGR region seems to be involved also in the interaction between gal-3 and Bcl-2, which is lactose-dependent, even though Bcl-2 is not a glycoprotein. It is hypothesized that lactose presence may induce a conformational change in the CRD, which contains the NWGR motif, inhibiting gal-3/Bcl-2 interaction (Yang *et al.*, 1996). Modifications in this motif annul the anti-apoptotic activity of gal-3, highlighting its importance (Akahani *et al.*, 1997). The mechanism by which gal-3 exerts its anti-apoptotic function through Bcl-2 interaction implies gal-3 translocation from the cytoplasm to the mitochondria, where Bcl-2 is also present, and the consequent stabilization of mitochondria membrane and inhibition of cyt *c* release (Matarrese *et al.*, 2000; Moon *et al.*, 2001). The translocation is mediated by synexin, whose availability can thus become a limiting factor for gal-3 anti-apoptotic activity (Yu *et al.*, 2002). The pro-apoptotic activity of gal-3 can be mediated also by other proteins, such as CD95 receptor (APO-1/Fas), as happens in T-cells (Fukumori *et al.*, 2004). CD95 triggers in the cell the activation of several caspases, leading ultimately to the formation of the death-inducing signalling complex and the activation of caspase-3 (Dumic *et al.*, 2006). Gal-3 can modulate CD95 activity, determining the final level of intensity of its effects (Fukumori *et al.*, 2004). In a different cell model, human bladder cancer cells, gal-3 overexpression was shown to promote the activation of phosphatidylinositol 3-kinase (PI3K)/Akt pathway, inhibiting the loss of mitochondria potential and the activation of caspase-9 and-3, resulting ultimately in suppression of apoptosis (Oka *et al.*, 2005). The anti-apoptotic activity of gal-3 in the cytoplasm can be modulated by its interaction with nucling, a pro-apoptotic protein able to decrease both the mRNA and the protein level of gal-3 (Liu *et al.*, 2004). Interestingly, extracellular gal-3 seems to have an opposite effect on apoptosis compared to its intracellular localization. Indeed it has been observed that extracellular gal-3 induces T-cell apoptosis (Ochieng *et al.*, 1998; Fukumori *et al.*, 2003) through the interaction with CD29, CD7, CD95, CD98 and T-cell receptor (Scaffidi *et al.*, 1998; Fukumori *et al.*, 2004). This apparent discordant role of gal-3 in apoptosis is actually conceivable with the hypothesis that tumour cells externalized gal-3 to induce immune cell apoptosis in order to be able to escape

from the normal cells defence mechanisms (Nakahara *et al.*, 2005; Ahmed and AlSadek, 2015). In addition to inducing apoptosis of T-cell, gal-3 promotes immune evasion of cancer cells by suppressing the production of IL-5, impeding the differentiation of B-lymphocytes, increasing phagocytosis of neutrophils (Fernandez *et al.*, 2005) and enhancing the oxidative response of immune cells (Feuk-Lagerstedt *et al.*, 1999). Gal-3 also reduces the motility and functionality of tumour infiltrating lymphocytes by forming *lattices* with big steric volume (Demotte *et al.*, 2008).

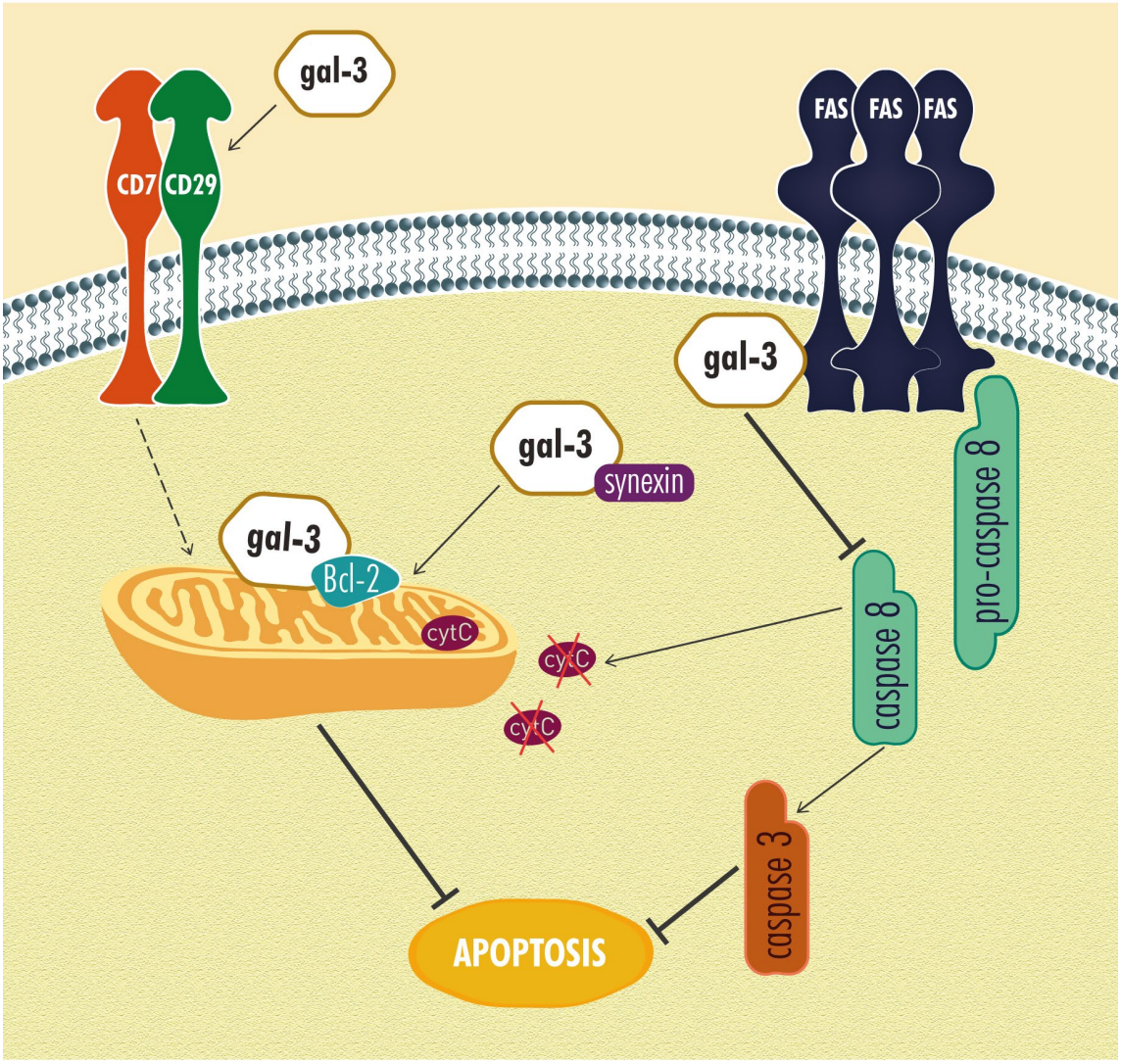


Figure 4: D*iverging role of intracellular and extracellular gal-3 in modulating apoptosis.* Inside the cells gal-3 binds to Bcl-2 and synexin, preventing the release of cytochrome c from the mitochondria, and it can interact with Fas receptor, impeding the activation of caspase 8 and the consequent caspases cascade that leads to apoptosis. On the other side, when outside the cell gal-3 can interact with CD7 and CD29, promoting the release of cyt *c* from mitochondria and apoptosis.

Gal-3 not only influences cell life by inhibiting apoptosis, but also by regulating cell cycle progression. It has been demonstrated in breast epithelial cells BT459 that gal-3 is capable to block cell cycle progression at various stages when cells are subjected to a pro-apoptotic stimulus, preventing them to die by apoptosis (Kim *et al.*, 1999; Lin *et al.*, 2000). For example, this cell line naturally undergoes anoikis, cell death provoked by loss of anchorage, but when gal-3 is overexpressed the cells are blocked in G1 phase instead, without any sign of apoptosis induction. This event is accompanied by a downregulation of cyclin A and E, responsible for cell cycle progression from G1 to S phase, and by the upregulation of their inhibitors, $p21^{Cip1}$ and $p27^{Kip1}$. Gal-3 causes also the hypophosphorylation of the retinoblastoma protein (Rb), which needs to be hyperphosphorylated to allow cell cycle progression, and the expression of cyclin D1, probably to help the cell pass the apoptosis-sensitive point in early G1 phase (Kim *et al.*, 1999). Another mechanism by which gal-3 help avoiding apoptotic death is the blockage of the cell cycle in G2/M phase following the treatment of the cells with genistein, which causes instead apoptosis in cells not expressing gal-3. Also in this case gal-3 regulates $p21^{/Cip1}$ and $p27^{Kip1}$ expression level (Lin *et al.*, 2000). As mentioned above, the influence of gal-3 on cell cycle is probably due to the transcription regulation of the genes involved, such as cyclins and their inhibitors (Kim *et al.*, 1999).

Gal-3 also acts on cell proliferation, mainly by activating or stabilizing other important pathways involved in this process. Gal-3 can stabilize KRAS nanoclusters, thus increasing and modulating KRAS signalling (Elad-Sfadia *et al.*, 2004; Shalom-Feuerstein *et al.*, 2005; Levy *et al.*, 2010) (gal-3 interaction with KRAS will be discussed further in detail). Gal-3 can also act on Akt activity, promoting the cell proliferation and apoptosis evasion induced by this protein (Oka *et al.*, 2005).

The above-mentioned processes in which gal-3 is involved are functioning in both normal and transformants cells, but gal-3 contributes also to activation of pathway relevant specifically for cancer progression. Gal-3 has been shown to promote angiogenesis in tumours. In particular, exogenous gal-3 enhances the formation of new capillaries in mice (Nangia-Makker *et al.*, 2000) by interacting with integrins or glycans expressed on the cell surface (Fukushi *et al.*, 2004). The binding triggers the clustering of gal-3 on the cell surface

and the activation of focal adhesion kinase, with the consequent activation of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor, which ultimately lead to new vessels formation (Markowska *et al.*, 2010). Confirming the role of gal-3 on VEGF secretion, it has been observed that a depletion of gal-3 strongly reduces VEGF level (Machado *et al.*, 2014). Gal-3 has also been reported to interact with the endothelial cell surface enzyme aminopeptidase N/CD13, regulating endothelial vascularization in early steps of angiogenesis (Yang *et al.*, 2007). In addition to promote the formation of new blood vessels, gal-3 is involved also in ensuring the survival of tumour cells circulating in the blood system and their vascularization, provoking tumour metastasis. Gal-3 can bind to oncofoetal Thomsen-Friedenreich (galactose β1,3 N-acetylgalactosaminea) disaccharide (TFD) (Glinsky *et al.*, 2001; Yu, 2007; Yu *et al.*, 2007), present in the core I structure of mucin-type *O*-linked glycan, in the transmembrane glycoprotein mucin-1 (MUC1) expressed by tumour cells. The interaction gal-3/MUC1 causes the redistribution of MUC1 on the cell surface, which allows the exposure of otherwise hidden small adhesion molecules. These adhesion molecules contribute to the heterotypic adhesion of tumour cells to the vascular endothelium (Zhao *et al.*, 2009) and to the homotypic adhesion of tumour cells between them, forming tumour micro-emboli, which allow a longer survival of circulating tumour cells by preventing anoikis (Zhao *et al.*, 2010). Gal-3 can also act as direct adhesion molecule for homo- and heterotypic adhesions by interacting with TFD (Glinsky *et al.*, 2001; Khaldoyanidi *et al.*, 2003; Zou *et al.*, 2005). To promote cancer cell adhesion, gal-3 binds to glycans present on the surface of tumour cells such as the Mgat5-modified N-glycans (Lagana *et al.*, 2006), laminin and lysosome associated glycoproteins (Dumic *et al.*, 2006; Barrow *et al.*, 2011). Gal-3 induces the expression of several pro-inflammatory cytokines, which indirectly promote metastasis formation. The effect of these cytokines is to increase the expression of endothelial cell surface adhesion molecules such as E-selectin, intercellular adhesion molecule 1 and vascular cell adhesion molecule 1, which lead to an enhanced cancer cell-endothelial adhesion, cell migration and tubule formation (Chen *et al.*, 2013). Besides contributing to adhesion between tumour cells and tumour and vascular cells, gal-3 is also important for adhesion of tumour cells to the ECM. Overexpression of gal-3 is associated to increased adhesion to

ECM and cell evasion from primary tumour sites (Ochieng *et al.*, 2004). Gal-3 overexpression correlates with increase invasiveness and one of the possible mechanism causing this effect is the binding of gal-3 to transmembrane receptors such as EGFR (epidermal growth factor receptor) and TGF-β receptor, which prevent their endocytosis and signalling (Dumic *et al.*, 2006; Barrow *et al.*, 2011). As support of the relevant role of gal-3 in metastasis formation and invasiveness (Funasaka *et al.*, 2014), the level of circulating gal-3 is higher in cancer patients with metastasis and this overexpression is correlated to increased potential to develop new metastasis and worst prognosis (Iurisci *et al.*, 2000).

Consistently with previously explained information indicating gal-3 as involved in multiple cellular and extracellular processes that ultimately lead to cancer formation, progression and metastasization (**Fig. 5**), gal-3 expression has been found upregulated in several cancer types, including colon, head and neck, gastric, endometrial, thyroid, liver, bladder cancers and breast cancers (Bresalier *et al.*, 1997; Hsu *et al.*, 1999; Shekhar *et al.*, 2004; Shimura *et al.*, 2004; Song *et al.*, 2009; Guha *et al.*, 2014). Moreover, gal-3 overexpression has been discovered to promote neoplastic transformation (Honjo *et al.*, 2001; Yoshii *et al.*, 2001; Elad-Sfadia *et al.*, 2004; Liu and Rabinovich, 2005). However, as often observed in regards to gal-3 functions, its relevance on cancer progression may vary among different cancer types, different cancer stages and different intracellular localization (Song *et al.*, 2014; Ahmed and AlSadek, 2015).

The multiplicity of gal-3 roles in tumour formation has made it a potential target for inhibitors, which could block cancer progression by decreasing gal-3 activity. In recent years, many synthetic inhibitors of gal-3 CRD have been developed, including peptide antagonists (Nangia-Makker *et al.*, 2008), lactulose amines (Glinskii *et al.*, 2012), and galactose-based inhibitors (John *et al.*, 2003; Glinskii *et al.*, 2005). In addition, it has been discovered that a natural product, pectin, can efficiently inhibit gal-3. In particular, modified citrus pectin inhibited cell adhesion *in vitro* (Pienta *et al.*, 1995) and metastasis formation *in vivo* (Glinskii *et al.*, 2005). Another pectin derivative, GCS-100, showed great potential for the treatment of multiple myeloma (Chauhan *et al.*, 2005; Streetly *et al.*, 2010). Also curcumin has been shown to decrease gal-3 basal level (Dumic *et al.*, 2002). However, it has to be noticed that

many of these inhibitors only act on extracellular gal-3 and its effects, allowing the pro-survival signalling caused by intracellular gal-3, since they mainly occupy gal-3 CRD (Ahmed and AlSadek, 2015).

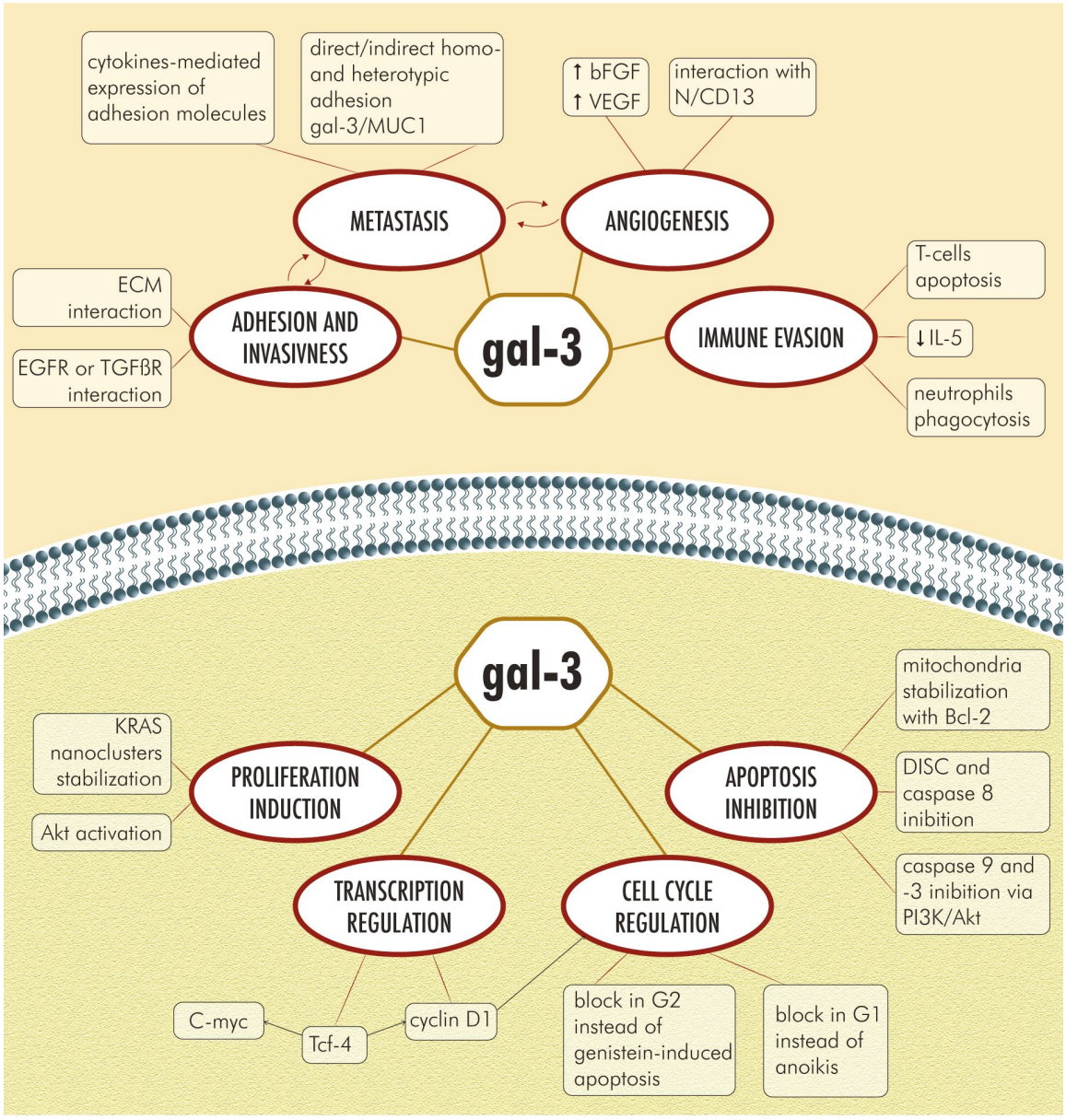


Figure 5: *Main roles of intra- and extracellular gal-3 in promoting cancer progression*. Intracellular gal-3 promotes cancer progression by interacting and activating oncoproteins such as KRAS, by inhibiting apoptosis through the interaction and inhibition of various pro-apoptotic proteins, by enhancing the transcription of genes involved in cell cycle progression and also by directly modulating cell cycle progression. Extracellular gal-3 is involved in facilitating adhesion and invasiveness of cancer cells, promoting and the same time the formation of metastasis and new vessels that bring oxygen and nutrient to the cancer cells. In addition, gal-3 promotes immune evasion of the cancer cells.

2.2.1 Focus on the role of galectin-3 in colorectal cancer

Gal-3 expression was detected in colorectal cancer (CRC) by immunohistochemical analysis and a correlation between gal-3 expression and poor prognosis was noticed (Arfaoui-Toumi *et al*., 2010). Moreover, gal-3 was found expressed at a higher level in colon tumours with lymph nodes metastasis compared to tumours without metastasis (Irimura *et al*., 1991; Wu and Gan, 2007; Liu *et al*., 2008a). In general, gal-3 is overexpressed in colorectal cancer compared to normal tissue (Lee *et al*., 1991; Ohannesian *et al*., 1994; Endo *et al*., 2005; Zaia Povegliano *et al*., 2011; Cay, 2012). Interestingly, gal-3 translocates from the nucleus to the cytoplasm following the progression from colorectal adenoma to carcinoma (Sanjuan *et al*., 1997; Andre *et al*., 1999), and gal-3 cytoplasmic localization is known to exert an anti-apoptotic role, in contrast to the pro-apoptotic role of nuclear gal-3 (Califice *et al*., 2004a). In addition to overexpression in CRC tissue, circulating gal-3 level in the blood stream was found increased up to 5-fold in CRC patients compared to healthy people, and this increase was even more emphasized in patients with metastasis (Iurisci *et al*., 2000). For these reasons gal-3 has been suggested as CRC prognostic marker (Legendre *et al.*, 2003; Arfaoui-Toumi *et al*., 2010).

3. Ras proteins

RAS genes are the founding members and prototypes of the RAS superfamily of small guanosine triphosphatases (GTPases). Ras superfamily accounts for more than 150 human genes, subdivided in five main branches on the basis of sequence and functional similarities: RAS, Rho, Rab, Ran and ARF (Colicelli, 2004; Wennerberg *et al.*, 2005; Cox and Der, 2010). *RAS* genes are highly conserved in a wide variety of species, including the yeasts *S. cerevisiae* and *S. pombe* (Dhar *et al.*, 1984; Powers *et al.*, 1984; Fukui and Kaziro, 1985; Fukui *et al.*, 1986), *Drosophila* (Neuman-Silberberg *et al.*, 1984), *Caenorhabditis elegans* (Beitel *et al.*, 1990; Han and Sternberg, 1990), zebrafish (Cheng *et al.*, 1997; Liu *et al.*, 2008b), *Dictostelium* (Reymond *et al.*, 1984) and, of course, mammals (Malumbres and Barbacid, 2003). All RAS proteins work substantially as binary molecular switches between an inactive

state, in which they are bound to GDP, and an active state, in which they are bound to GTP. The activation of RAS proteins leads to subsequent activation of a signalling cascade, which differs depending on the organism (Wennerberg *et al.*, 2005; Cox and Der, 2010). Even though the specific proteins that interact with RAS for the activation of downstream signalling are different in different organisms and for different RAS isoforms in the same organism, most of them share a conserved domain to interact with RAS proteins, the RAS-binding domain or RAS association (RA) domain (Cox and Der, 2010). Many RAS effectors have been identified by screening cDNA libraries for the presence of RA domain (Hofer *et al.*, 1994; Kikuchi *et al.*, 1994; Spaargaren and Bischoff, 1994). The conserved working mechanism of RAS proteins relies on their conserved sequence, in particular on the presence of a set (1 to 5) of G box GDP/GTP-binding motif elements beginning at the N-terminal domain, which together form a G domain of approximately 20 kDa (Bourne *et al.*, 1991) (**Fig. 6**).

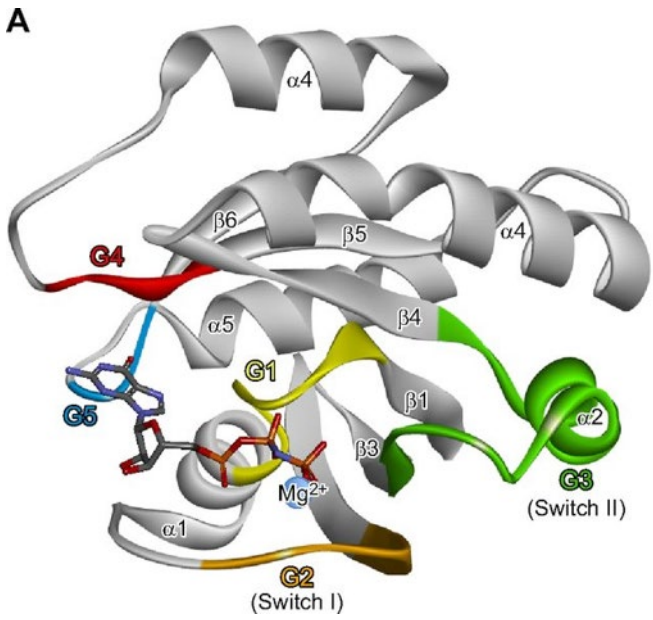


Figure 6: *Structure of human HRAS bound to GTP*. Human HRAS, similarly to all RAS proteins, is composed by 6 β-sheets and 5 α-helices. The conserved G motifs (G1-G5) belonging to the G domain of RAS, involved in GTP/GDP binding are showed (adapted from Loirand *et al.*, 2013).

Particularly relevant among the G box motifs are switch I and switch II, which regulate the conformational changes between GTP- and GDP-bound RAS protein (Milburn *et al.*, 1990; Schlichting *et al.*, 1990). The conformations of active and inactive state are very similar, besides the pronounced changes corresponding to these two regions, and exactly these variations lead to different affinities of active or inactive RAS proteins toward RAS regulators and effectors (Scheffzek *et al.*, 1996; Bishop and Hall, 2000; Repasky *et al.*, 2004).

The RAS proteins switching between GTP- and GDP-binding is regulated by two classes of proteins, GTPase activating proteins (GAPs) and guanine-nucleotide exchange factors (GEFs). GAPs enhance the intrinsically low GTPase activity of RAS proteins, up to 300-fold acceleration (Trahey and McCormick, 1987), in order to reinstate the GDP-bound form of RAS proteins (Bernards and Settleman, 2004), whereas GEFs promote the formation of GTP-bound form, by triggering the dissociation of GDP from RAS proteins, allowing the more abundant GTP to bind in its place (Schmidt and Hall, 2002) (**Fig. 7**).

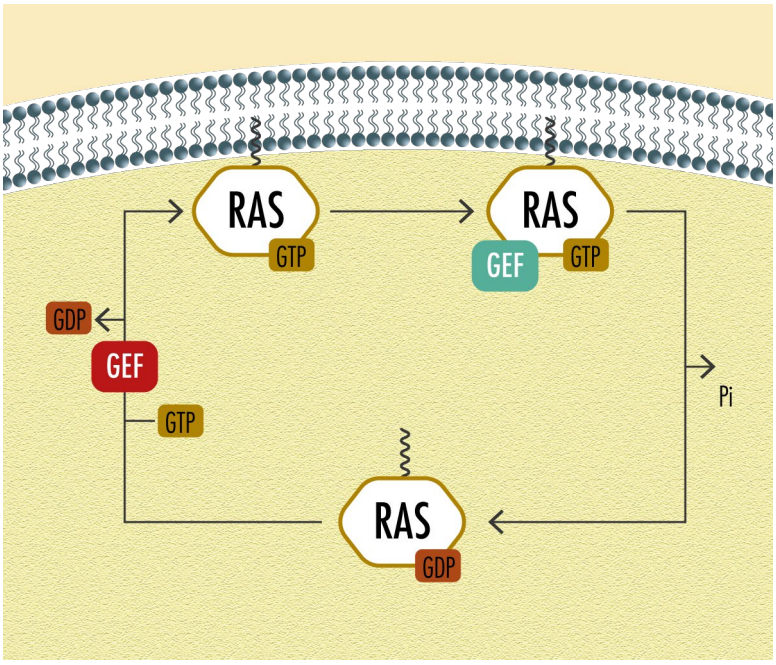


Figure 7: *Mechanism of activation/deactivation of RAS proteins*. Inactive RAS proteins bound to GDP are localized in the cytoplasm. GEFs catalyse the liberation of GDP from the binding site, allowing GTP, more abundant in the cell, to bind instead. GTP-bound RAS are translocated to the membrane and activated. GAPs enhance the endogenous GTPase activity, hydrolysing GTP to GDP. RAS is inactive again and goes back to the cytoplasm, where the cycle can begin again, upon proper stimulus.

RAS proteins activity is not regulated only by its GDP/GTP binding state, but also by post-translational modifications, which determine RAS sub-cellular localization. RAS proteins localization on plasma membrane or endomembranes establishes the class of effectors and regulators available, determining in turn the downstream signalling (Wennerberg *et al.*, 2005; Cox and Der, 2010). RAS proteins are synthetized as cytoplasmic proteins (Shih *et al.*, 1979) and then undergo different post-translation modifications that fully activate the proteins and target them on the inner leaflet of the plasma membrane (Cox and Der, 2010). The C-terminal domain is necessary for the proper sub-cellular localization, because it contains the CAAX (C = cysteine, A = aliphatic amino acid, X = terminal amino acid) motif indispensable for membrane targeting (Cox and Der, 2002; Wennerberg *et al.*, 2005; Cox and Der, 2010). The cysteine in the CAAX motif is the target of farnesyltransferase and geranylgeranyltransferase I, which catalyse the addition of a farnesyl or geranylgeranyl isoprenoid, respectively (Casey *et al.*, 1989; Hancock *et al.*, 1989; Schafer *et al.*, 1989; Wennerberg *et al.*, 2005; Cox and Der, 2010). This modification is the first step of RAS processing, followed by other modifications, such as palmitoylation, driven by a second signal contained in the hypervariable region (HVR) at the C-terminal, which, as suggested by the name, shows great diversification depending on RAS protein isoform, in contrast with the highly conserved G domain (Cox and Der, 2010). These two steps are the minimum signal required for transit to and tenure at the membrane, either plasmatic or of internal organelles. This specific differentiation, plasma or endo-membranes, depends exactly on the second step of RAS proteins processing.

3.1 Ras pathway in *Saccharomyces cerevisiae*

S. cerevisiae expresses two homologous isoproteins of the human RAS family, Ras1 and Ras2. They show a domain of 180 amino acids conserved with mammalian Ras proteins (G domain), and a region of divergence of 120 amino acids at the C-terminal (DeFeo-Jones *et al.*, 1983; Dhar *et al.*, 1984; Powers *et al.*, 1984). The yeast Ras proteins are ~40 kDa, almost the double of the size of the mammalian Ras, which are ~21 KDa (Papageorge *et al.*, 1984; Fujiyama and Tamanoi, 1986) (**Fig. 8**).

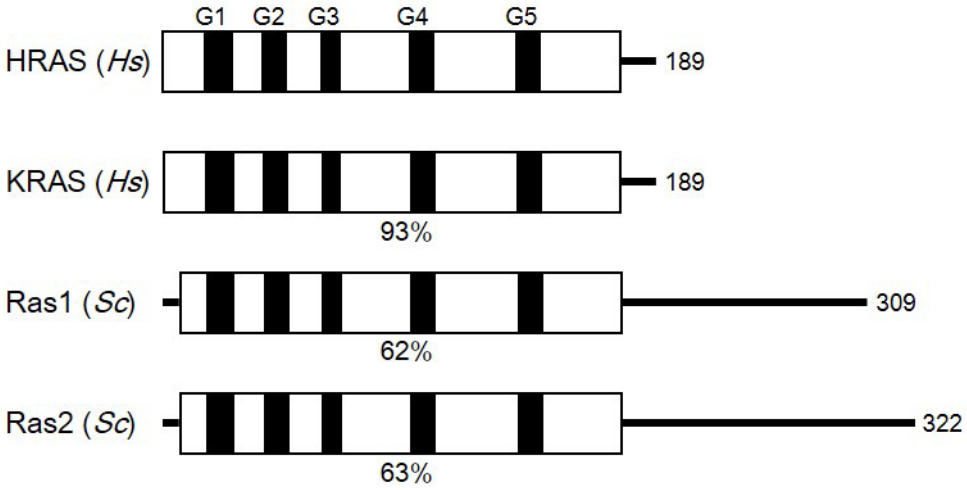


Figure 8: *Homology between human and yeast RAS proteins*. Yeast and human RAS proteins present conserved G motifs (G1-G5), which form the G domain for GTP/GDP binding. Human RAS proteins are highly homologous, KRAS showing 93% of homology to HRAS. Yeast Ras proteins are bigger, having more than 100 extra amino acids, but still present around 60% homology to human RAS (based on information from Tamanoi, 2011; Cox and Der, 2010).

As all RAS proteins, yeast Ras proteins act as molecular switches between an active and an inactive conformation and they transduce signals from outside to inside the cell about the nutritional conditions of the environment, especially glucose availability (Gibbs and Marshall, 1989; Busti *et al.*, 2010; Tamanoi, 2011) (**Fig. 9**). Ras proteins exert this function mainly controlling 3',5'-cyclic adenosine monophosphate (cAMP) metabolism. cAMP is essential for *S. cerevisiae* viability, being yeast mutants with impaired cAMP production unviable (Molenaar *et al.*, 1988). cAMP acts as second messenger in the regulation of several fundamental cellular processes. These include protein phosphorylation, accumulation of storage carbohydrates, mitochondrial functions (Ortiz *et al.*, 1983; Uno *et al.*, 1983; Francois *et al.*, 1984; Pohlig and Holzer, 1985; Rödel *et al.*, 1985; Lopez-Boado *et al.*, 1987; Müller and Bandlow, 1987; Rittenhouse *et al.*, 1987; Francois and Hers, 1988), sporulation (Cameron *et al.*, 1988), sensitivity to heat shock (Uno *et al.*, 1985) and cell cycle progression (Matsumoto *et al.*, 1982; Matsumoto *et al.*, 1983a, 1983b; Kataoka *et al.*, 1984). cAMP is produced by adenylate cyclase, which localizes at the plasma membrane and synthetizes cAMP from guanine nucleotides (Gibbs and Marshall, 1989; Thevelein and de Winde, 1999; Tamanoi,

2011). Adenylate cyclase is a large protein (~2000 aa) encoded by the *CYR1/CDC35* gene and formed by four different domains, the N-terminal, the middle repetitive, the catalytic and the C-terminal. The middle repetitive domain contains a LLR domain, which is constituted by a repeat of 23-residue amphipathic leucine rich motif and is the primary site of interaction with Ras proteins. Indeed, Ras proteins interact with adenylate cyclase through the RA domain present at the N-terminal of the LLR domain (Kido *et al.*, 2002) and, when active, stimulate cAMP production activating in turn adenylate cyclase (Toda *et al.*, 1985; Wigler *et al.*, 1988; Broach and Deschenes, 1990; Thevelein, 1992; Tatchell, 1993; Thevelein, 1994). Once produced, cAMP acts downstream as second messenger and the amount of cAMP is the results between its synthesis by adenylate cyclase and its hydrolysis by the high-affinity (Pde2) and low-affinity (Pde1) phosphodiesterase (**Fig. 9**) (Sass *et al.*, 1986; Nikawa *et al.*, 1987a).

The main target of cAMP is protein kinase A (PKA), which is composed by a catalytic subunit, encoded by the three genes *TPK1*, *TPK2* and *TPK3* (Toda *et al*., 1987a; Toda *et al*., 1987b), and a regulatory subunit encoded by *BCY1*. PKA exists in the cell as inactive heterotetrametric holoenzyme composed by two catalytic subunits and two regulatory subunits (Toda *et al.*, 1987a; Toda *et al.*, 1987b). The three isoforms of the catalytic subunits are largely redundant, even though some specific functions have been identified (Robertson and Fink, 1998; Robertson *et al.*, 2000; Pan and Heitman, 2002; Chevtzoff *et al.*, 2005; Palomino *et al.*, 2006). cAMP binds to the regulatory unit Bcy1, which relieves its inhibitory effect on the catalytic subunit, allowing the phosphorylation of several downstream targets (Thevelein and de Winde, 1999; Busti *et al.*, 2010; Tamanoi, 2011). It has been demonstrated that the deletion of *BCY1* causes the strongest downregulating effect on the Ras/cAMP/PKA pathway (Thevelein and de Winde, 1999) and that at least one out of three genes encoding the catalytic subunit has to be present in order to maintain cell viability (Toda *et al.*, 1987b). cAMP synthesis is one of several PKA targets, suggesting a strong negative feedback regulation (Nikawa *et al.*, 1987b; Mbonyi *et al.*, 1990). Indeed there are evidences that Pde1 is phosphorylated by PKA (Ma *et al.*, 1999) and adenylate cyclase itself has been proposed as PKA target (Thevelein and de Winde, 1999). PKA, as mentioned above, has a wide variety of substrates, whose

activation leads to a dramatic change in the transcriptional program, which helps the cells to adapt to new nutrient conditions. PKA regulates the metabolism of storage carbohydrates, ribosomal biogenesis, stress response (Gancedo, 2008; Zaman *et al.*, 2008), the polarity of actin cytoskeleton (Ho and Bretscher, 2001), spore morphogenesis (McDonald *et al.*, 2009), the synthesis of cyclins and the subsequent cell cycle progression control (Kataoka *et al.*, 1984; Toda *et al.*, 1985; Hubler *et al.*, 1993; Sun *et al.*, 1994), growth and cell size (Busti *et al.*, 2010). In particular, when glucose is available, Ras proteins are activated by binding to GTP and the cells adjust to favour cell growth and proliferation, upregulating genes involved in the glycolytic flux, repressing genes involved in stress response, and enhancing fermentation to the detriment of respiratory growth (Wang *et al.*, 2004; Garmendia-Torres *et al.*, 2007; Slattery *et al.*, 2008; Zaman *et al.*, 2008; Zaman *et al.*, 2009; Busti *et al.*, 2010).

PKA specifically inhibits Msn2 and Msn4 transcription factors, which mediate stress response by binding to stress response elements (STRE) in promoters. Msn2/4 are triggered by nutrients availability, in particular glucose, as well as stress stimuli (Martinez-Pastor *et al.*, 1996; Boy-Marcotte *et al.*, 1998; Moskvina *et al.*, 1998; Gasch *et al.*, 2000; Causton *et al.*, 2001). Nuclear Msn2/4 promote the activation of genes encoding for molecular chaperones, antioxidant proteins, enzymes involved in carbohydrates metabolism and proteolysis (Boy-Marcotte *et al.*, 1998; Gasch *et al.*, 2000; Causton *et al.*, 2001). The inhibition of Msn2/4 by PKA can be direct, or mediated by Rim15, a critical kinase for entry in quiescence (Reinders *et al.*, 1998; Pedruzzi *et al.*, 2003; Swinnen *et al.*, 2006) and target of PKA (Pedruzzi *et al.*, 2000; Cameroni *et al.*, 2004). PKA can also regulate other stress responsive genes, such as *HSP12* and *HSP26,* downregulating the activity of the transcription factor Hsf1 by inhibition of its phosphorylation (Ferguson *et al.*, 2005). Hsf1 controls the transcription of genes involved in a variety of processes, such as heat-stress response, protein folding and degradation, detoxification, energy generation carbohydrate metabolism and cell wall organization (Wiederrecht *et al.*, 1988; Smith and Yaffe, 1991; Zarzov *et al.*, 1997; Hahn *et al.*, 2004; Imazu and Sakurai, 2005; Eastmond and Nelson, 2006). Importantly, PKA regulates also the transcriptional repressor Rgt1, which, as part of the Snf3/Rgt2 pathway, controls the expression of the sugar transporters encoded by the *HXT* genes (Ozcan and Johnston, 1999;

Kim and Johnston, 2006).

Yeast Ras proteins, similarly to most of the proteins in the Ras family, are regulated by GAPs and GEFs. Two homologous GAPs are present in yeast, Ira1 and Ira2 (inhibitory regulator of the Ras/cAMP pathway), with similar functions. These two proteins contain a GAP domain of approximately 360 aa, which is responsible for the enhanced GTPase activity of Ras (Tanaka *et al.*, 1991). These genes were identified and their function was attributed thanks to the similarity with mammalian GAP protein (Tanaka *et al.*, 1990). On the other hand, yeast GEF Cdc25 was the first GEF to be identified (Broek *et al.*, 1987; Robinson *et al.*, 1987). Cdc25 promotes the release of GDP bound to Ras, which is then substituted by GTP. *CDC25* is an essential gene for yeast viability, whereas it homologous *SDC25* is dispensable (Damak *et al.*, 1991). Interestingly, mutations in *IRA* genes can suppress mutations in *CDC25*, making the double mutant *ΔiraΔcdc25* viable (Tamanoi, 2011).

As mentioned before, Ras proteins undergo several post-translational modifications in order to be fully active. Also in yeast, Ras proteins are synthetized in the cytoplasm and directed to and retain in the membrane thanks to the post-translational modifications. The C-terminal of Ras proteins contains the CAAX motif, which allows the farnesylation on the cysteine residue by the heterodimeric farnesyltransferase encoded by *RAM2* and *DPR1/RAM1* genes (Goodman *et al.*, 1990). Following processing steps imply the removal of the last three residues at the C-terminal (Fujiyama and Tamanoi, 1990) and the methylation of the cysteine (Fujiyama *et al.*, 1991). Finally, Ras proteins are palmitoylated by a heterodimeric palmitoyltransferase encoded by *ERF2* and *ERF4*, which uses palmitoyl-CoA as a donor of palmitoyl group (Lobo *et al.*, 2002; Swarthout *et al.*, 2005). Ras are ultimately located on the inner leaflet of the plasma membrane. Interestingly, yeast Ras proteins contain an extra C-terminal portion of around 120 aa, not present in Ras protein of other organisms (**Fig. 8**). It seems like this part of the protein acts as a regulatory domain for Ras, promoting its interaction with GDP and therefore the permanence of the inactive status (Gibbs and Marshall, 1989). As confirmation, yeast cells expressing mammalian Ras proteins, or truncated yeast Ras proteins lacking the C-terminal domain, do not require a functional *CDC25* gene product to be viable, while they do when expressing normal Ras proteins (Marshall *et al.*, 1987).

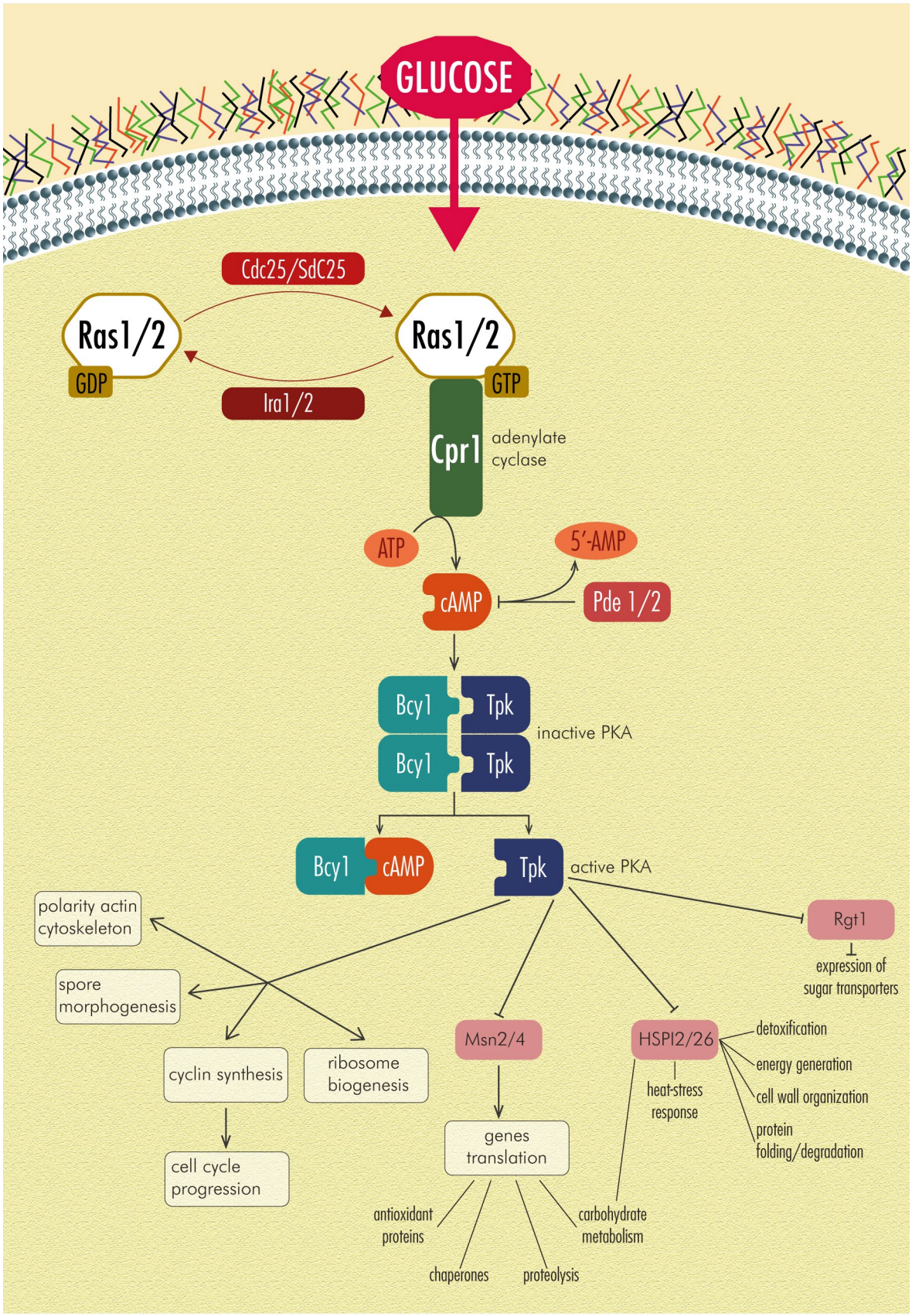

GLUCOSE
Cdc25/SdC25
Ras1/2
GDP
Ras1/2
GTP
Ira1/2
Cpr1
adenylate cyclase
ATP
5'-AMP
cAMP
Pde 1/2
Bcy1
Tpk
inactive PKA
Bcy1
Tpk
Bcy1
cAMP
Tpk
active PKA
polarity actin cytoskeleton
spore morphogenesis
cyclin synthesis
ribosome biogenesis
cell cycle progression
Msn2/4
genes translation
antioxidant proteins
chaperones
proteolysis
carbohydrate metabolism
HSP12/26
heat-stress response
detoxification
energy generation
cell wall organization
protein folding/degradation
Rgt1
expression of sugar transporters

Figure 9: *Schematic representation of Ras/cAMP/PKA pathway in* S. cerevisiae. Ras1 and Ras2 are activated upon nutrient availability signalling, glucose in particular. The GEF proteins Cdc25 or Sdc25 catalyse the liberation of GDP and the binding of GTP. Active Ras proteins activate in turn adenylate cyclase, which produces cAMP. This second messenger binds to the inhibitory unit of PKA, liberating the catalytic unit, which phosphorylates multiple downstream targets, leading to the activation of a variety of cellular processes or to the inhibition of transcription factors that control stress response. The pathway can be inactivated by the hydrolysis of cAMP by the phosphodiesterase Pde1 and Pde2, and by the GTPase activity of the GAPs Ira1 and Ira2.

RAS1 and *RAS2* are located in chromosome XV and XIV, respectively (Kataoka *et al.*, 1984; Tatchell *et al.*, 1984), and encode for two highly homologous proteins of 36 and 40 kDa (Papageorge *et al.*, 1984; Feuerstein *et al.*, 1987). The only variable region between *RAS1* and *RAS2* accounts for 120 aa at the C-terminal domain (DeFeo-Jones *et al.*, 1983; Dhar *et al.*, 1984; Powers *et al.*, 1984), with the exclusion of the last 4 residues constituting the CAAX motif. This divergence initially led to the hypothesis that Ras1 and Ras2 may have distinct functions (DeFeo-Jones *et al.*, 1983; Dhar *et al.*, 1984; Powers *et al.*, 1984), whereas they actually have the same function, but different expression regulation (Breviario *et al.*, 1986; Breviario *et al.*, 1988). The first idea was strongly suggested by the fact that Ras1 could not complement growth defects caused by the deletion of *RAS2*, such as reduced growth on non-fermentable carbon sources and low level of intracellular cAMP (Kataoka *et al.*, 1984; Fraenkel, 1985; Tatchell *et al.*, 1985), and that *RAS1* mutant did not present any clear phenotype. These effects are now explained by the different regulation of mRNA production and protein translation of *RAS1*. Indeed, the levels of both *RAS1* mRNA and protein synthesis are reduced as cells approach mid-logarithmic phase and when cells are grown on non-fermentable carbon sources (Breviario *et al.*, 1986). In opposition, *RAS2* mRNA levels are high during all the culture growth phases (Breviario *et al.*, 1988) on both fermentable and non-fermentable carbon sources (Breviario *et al.*, 1986). Another evidence that the only difference between *RAS1* and *RAS2* resides on differential expression was obtained by expressing *RAS1* under the constitutive *ADH1* promoter. In this case *RAS1* was able to fully suppress the phenotype of *Δras2* and the hyperactive mutant of *RAS1* showed the same effects as the constitutively expressed *RAS2* (Marshall *et al.*, 1987).

3.1.1 Ras proteins involvement in the control of yeast growth

Ras/cAMP/PKA pathway is the main player in sensing glucose availability and the responsible for the adaptation of the transcription profile to the presence of this fermentable carbon source (Slattery *et al.*, 2008; Zaman *et al.*, 2009). Yeast cells adapt rapidly to the addition of glucose when growing on non-fermentable carbon sources, changing the expression profile of more than 40% of their genes (Wang *et al.*, 2004; Zaman *et al.*, 2009). Nutrient availability, especially glucose, signals to the cells that the conditions are suitable for mass accumulation, sustained by increased ribosome production, and for accelerating the generation time, or growth rate (Zaman *et al.*, 2008; Busti *et al.*, 2010). These processes are actually strictly interdependent, being the growth rate determined by the rate of mass accumulation, which in turns depends on nutrient availability, and both growth rate and mass accumulation influence cell cycle progression and cell size (Zaman *et al.*, 2008) (**Fig. 10**).

Nutrients determine the amount of energy available for anabolic processes, in particular for ribosome biogenesis, considering that ribosome synthesis consumes around 90% of the total energy of the cell (Warner *et al.*, 2001). Yeast ribosomes are composed by 79 ribosomal proteins, encoded by 138 genes contained in the ribosomal protein (RP) regulon, and by 4 rRNAs encoded by around 150 rDNA repeats, and other 236 genes under the control of the Ribi regulon are involved in the ribosome assembly and translational capacity (Warner *et al.*, 2001). Ras/cAMP/PKA pathway, together with other pathways sensing different nutrients availability – especially TOR (target of rapamycin) pathway for nitrogen – controls the production of most of the above-mentioned factors necessary for complete ribosomal functionality (Zaman *et al.*, 2008; Busti *et al.*, 2010). PKA directly regulates the phosphorylation status of the Maf1 repressor (Moir *et al.*, 2006; Oficjalska-Pham *et al.*, 2006; Roberts *et al.*, 2006), which inhibits Polymerase III (PolIII) under nutrient limitations or other stress conditions. When PKA phosphorylates Maf1, the repressor is retained in the cytoplasm and cannot exert its usual function in the nucleus, allowing the transcription of 5S RNA, tRNAs

and various small nuclear RNAs by PolIII (Upadhya *et al.*, 2002; Desai *et al.*, 2005). Moreover, PKA exerts a major control on ribosomal proteins synthesis by modulating the activity of Sfp1, a zinc-finger transcription factor responsible for the translation of Rb and Ribi regulon (Zaman *et al.*, 2008; Busti *et al.*, 2010). Also in this case, the subcellular location of Sfp1 is the key regulator of its activity. When cells are actively growing and PKA is active, Sfp1 is found in the nucleus where it can exert its normal transcriptional activity, whereas under nutrient limitation or stress condition Sfp1 translocates in the cytoplasm (Fingerman *et al.*, 2003; Jorgensen *et al.*, 2004; Marion *et al.*, 2004). Sfp1 also relieves the inhibitory activity on Rb and Ribi regulons exerted by the complex formed by Fhl1, Ifh1 and Crf1 (Jorgensen *et al.*, 2004). PKA seems to have an additional direct repressive effect on this complex, probably by phosphorylating the repressor Crf1 and thus displacing it from Fhl1 and Ifh1, free to activate the transcription of Ribi and Rb regulons (Martin *et al.*, 2004).

Ribosome biogenesis is only one of the processes related to cell growth controlled by Ras/cAMP/PKA pathway. It has been demonstrated that this pathway strongly influences cell cycle and correlated cell size (Zaman *et al.*, 2008; Busti *et al.*, 2010). Cell cycle and cell size are tightly interconnected in yeast cells, being the progression through cell cycle dependent on the cell mass (Mitchison, 1971; Hartwell and Unger, 1977; Johnston *et al.*, 1979; Lord and Wheals, 1983; Vanoni *et al.*, 1983). Yeast cells match the amount of time necessary to prepare the duplication of the genetic material (cell cycle) with the time needed for the duplication of the non-genetic components of the cell (growth cycle) (Johnston *et al.*, 1977; Zaman *et al.*, 2008). The check point to assess the proper preparation of the cell for division is called START and is located at the burdens between G1 and S phase (Loewith *et al.*, 2002). Once the cell is able to pass the START point, it is committed to enter and progress through the remaining phases of the cell cycle (Busti *et al.*, 2010). One of the requirements to pass the START point is exactly the achievement of the proper cell size. One hypothesized mechanism by which cells sense their size is the protein content at the onset of DNA replication, defined Ps value (Porro *et al.*, 2003). In order to commit into S phase, the cells have to pass two consecutive thresholds, and the time taken to overcome them is function of nutrients availability and consequent growth rate (Alberghina *et al.*, 2004; Vanoni *et al.*, 2005; Alberghina *et al.*, 2009).

When a mother cell divides, the daughter cell receives a fixed amount of the two cyclin-dependent kinase inhibitors Far1 and Sic1, and of Whi5. The formation of functional complexes of cyclins (Clns 1, 2 and 3) and cyclin dependent kinases (Cdks) is necessary to phosphorylate Whi5, which acts as inhibitor of the SBF and MBF transcription factors. Phosphorylation of Whi5 relieves it from its inhibitory function, allowing the activation of MBF and SBF and the consequent transcription of genes involved in the S phase (Costanzo *et al.*, 2004; de Bruin *et al.*, 2004). As described before, the amount of Far1, Sic1 and Whi5 is fixed, while the amount of cyclin 3 (Cln3) is constant per unit mass, thus it is growing proportionally to the growing of the cell, giving a measure of cell mass accumulation (Mendenhall and Hodge, 1998; Rupes, 2002). When the amount of Cln3 overcomes the one of Far1, the cell has passed the first threshold. A second threshold, directly dependent on the first, is passed after the cell has performed various biochemical activities necessary for the actual onset of S phase, including Whi5 phosphorylation (Busti *et al.*, 2010). The involvement of Ras/cAMP/PKA pathway in the above-mentioned processes has been demonstrated by the analysis of mutants in proteins fundamental for the pathway. Inactivation of essential component can induce cell cycle arrest in G1, even in the presence of adequate nutrients (Zaman *et al.*, 2008) and reduced cAMP signalling causes a decrease in cell size (Baroni *et al.*, 1989), similarly to a weak PKA activity due to *Δtpk1tpk2tpk3bcy1* quadruple null mutant (Cameron *et al.*, 1988; Tokiwa *et al.*, 1994). On the other hand, hyperactivation of the pathway caused by constitutively active *RAS2*^{val19}, by deletion of both *PDE1* and *PDE2* or by inactivation of *BCY1* and *IRA2,* leads to a strong increase in cell volume (Baroni *et al.*, 1994; Mitsuzawa, 1994; Jorgensen *et al.*, 2002), caused as well by an increase in the intracellular cAMP content (Tokiwa *et al.*, 1994; Verwaal *et al.*, 2002). Some of the effects of Ras/cAMP/PKA pathway on cell size can be explained by the strong influence on ribosomal biogenesis, but the effects on cell cycle progression are too quick to derive from altered biosynthetic capacity (Zaman *et al.*, 2008). Ras/cAMP/PKA pathway has been shown to negatively influence the expression of Cln1 and Cln2, but not Cln3 (Baroni *et al.*, 1994; Tokiwa *et al.*, 1994) by suppressing their translation. Cln3 in this way counteracts the inhibition of the other Clns mediating their growth-dependent expression (Baroni *et al.*, 1994). More recently, PKA has been found to directly phosphorylate Whi3,

a negative regulator of G1 cyclins, inhibiting its functions and thus promoting the passage through START (Mizunuma *et al.*, 2013).

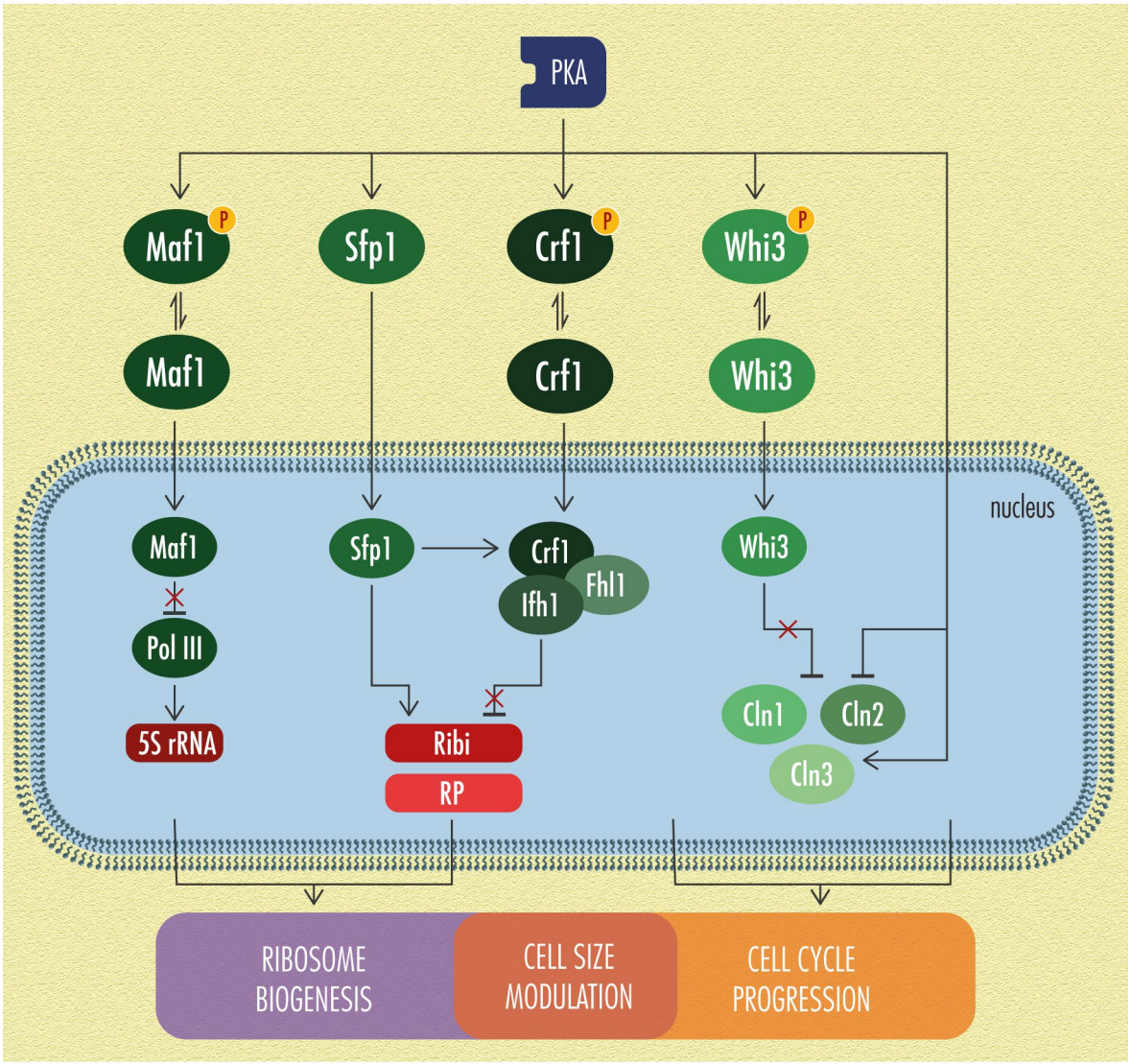


Figure 10: *Ras/cAMP/PKA involvement in promoting yeast cell growth*. Active PKA can phosphorylate multiple targets that ultimately lead to ribosome biogenesis and cell cycle progression, both controlling cell size. PKA-mediated phosphorylation of Maf1, Crf1 and Whi3 blocks these proteins in the cytoplasm, impeding their translocation into the nucleus, where they exert their inhibitory roles on various proteins. Maf1 would not inhibit PolIII, allowing the transcription of th5e 5S ribosomal RNA; Crf1 would not form an inhibitory complex together with Ifh1 and Fhl1, making this way possible the transcription of the Ribi and the RB regulons, which control ribosome biogenesis; Whi3 would not block the activity of the cyclins, allowing their complexation with CDK and the consequent progression of the cell cycle. PKA can also directly activate Sfp1, which promotes Ribi and RB transcription, and can modulate cyclins activity, promoting Cln3 activation and inhibiting Cln2.

3.1.2 Yeast Ras proteins involvement in stress response and ageing

As mentioned above, Ras/cAMP/PKA pathway controls the activity of two transcription factors fundamental for stress response, Msn2 and Msn4. Lack of essential nutrients can be seen as a stress inducer, therefore it is not surprising that Ras/cAMP/PKA pathway is involved in the signalling of stress response (**Fig. 11**). Msn2 and Msn4 are zinc-finger DNA-binding proteins that recognize specifically STRE elements in the genes promoter, activating them upon stress induction (Gorner *et al.*, 1998). PKA directly phosphorylates Msn2 nuclear localization signal, making the transcription factor inactive by translocating it into the cytoplasm (Gorner *et al.*, 2002). When environmental conditions are good for yeast cells, PKA phosphorylates Msn2, which becomes inactivated, and stress response genes are repressed. The fact that deletion of both *MSN2* and *MSN4* suppresses the lethality of loss-of-function mutations in Ras/cAMP/PKA pathway confirms the tight connection between Ras/cAMP/PKA pathway and Msn2 and Msn4 (Smith *et al.*, 1998). PKA can modulate the activity of other stress response genes, through the inhibition of the transcription factor Hsf1 (Ferguson *et al.*, 2005). When active, Hsf1 promotes the expression of heat shock responsive genes such as *HSP12* and *HSP26* by binding to the heat shock element in their promoters (Ferguson *et al.*, 2005). The modulation of genes such as *HSP12* and *HSP26* can happen also through the activation of Gis1, which binds to specific elements in the promoter called "post-diauxic shift" (Flattery-O'Brien *et al.*, 1997; Pedruzzi *et al.*, 2000). Gis1 is activated by Rim15, which in turn is inhibited by Ras/cAMP/PKA activity (Pedruzzi *et al.*, 2000). Finally, Ras/cAMP/PKA pathway is also involved in adaptation to hyperosmotic stress together with the Hog1 MAPK (mitogen activated protein kinase) pathway (Zaman *et al.*, 2008). Hog1 is responsible for the modulation of most transcription factors needed for hyperosmotic stress adaptation, including the repressor Sko1, which becomes active after being phosphorylated by Hog1 (Zaman *et al.*, 2008). PKA can also phosphorylate Sko1, contributing to the regulation of the response to hyperosmotic stress (Proft *et al.*, 2001; Pascual-Ahuir and Proft, 2007).

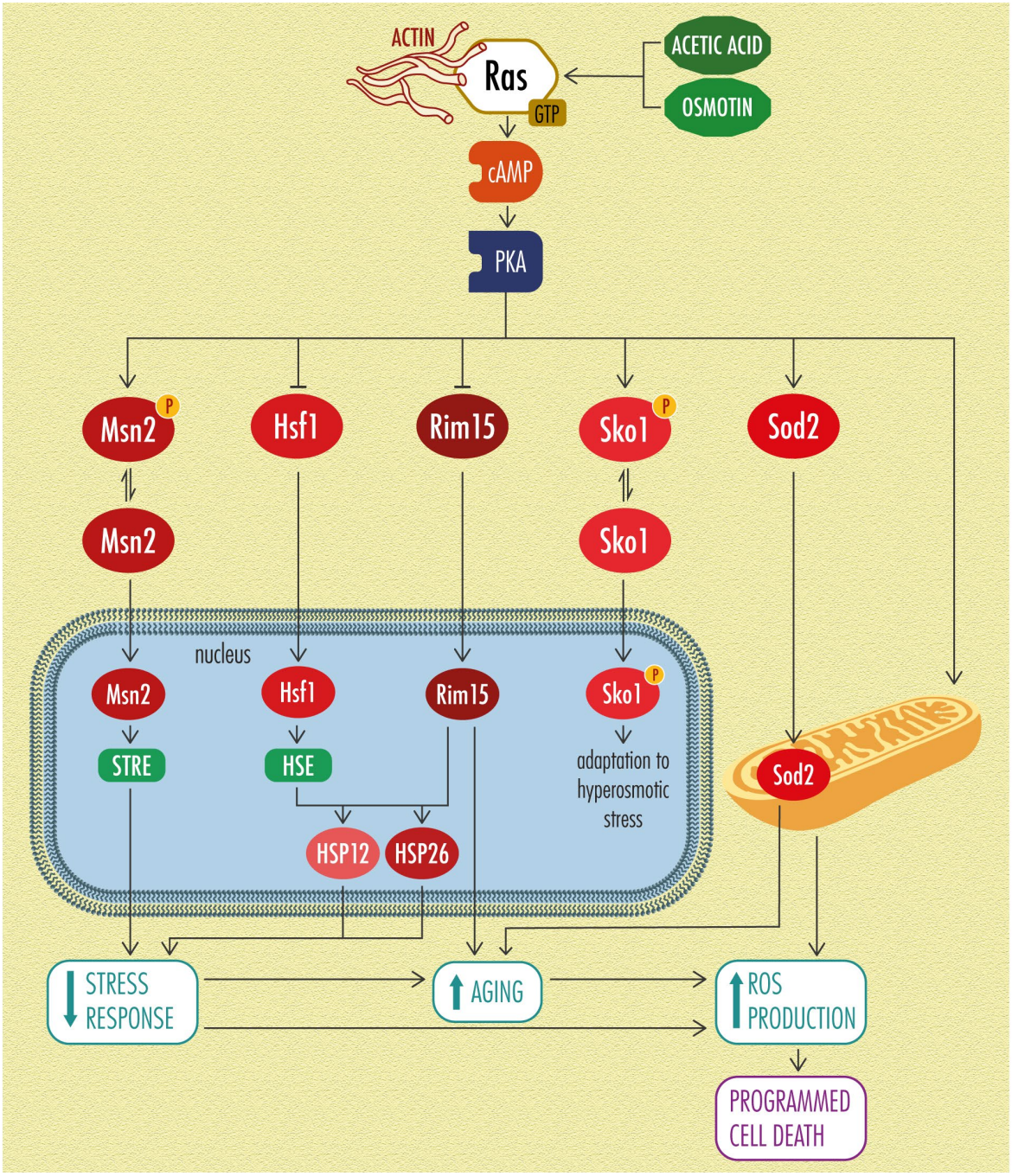


Figure 11: *Ras/cAMP/PKA involvement in promoting yeast stress response, aging and PCD*. Active PKA inhibits Hsf1 and Rim15, downregulating the transcription of HSE into HSP12 and HSP26, thus decreasing stress adaptability. PKA phosphorylates the transcription factors Msn2, impeding its translocation in the nucleus and the consequent activation of STRE, also leading to decreased stress response adaptation. Sko1 is another phosphorylation target of PKA, which mediated adaptation to hyperosmotic stress, when phosphorylated. PKA also modulates aging and PCD, mainly triggered by accumulation of ROS. PKA regulates mitochondria stability through Sod2 activity, also causing ROS accumulation. Both decreased stress response and aging lead to ROS accumulation, which in turn is the main trigger of PCD in yeast. External stimuli of PCD is actin rearrangement, acetic acid and osmotin.

Stress response is strongly interconnected with chronological ageing in yeast, which is defined by the period of survival of non-dividing cells in culture (Burtner *et al.*, 2009; Longo and Fabrizio, 2012). As expected, ageing cells in a batch culture are subjected to several stress stimuli, such as nutrient depletion over time, acidification of the medium and accumulation of reactive oxygen species (ROS). Therefore, it is conceivable that the pathways involved in stress response and in the regulation of chronological ageing overlap (Longo, 2003, 2004; Longo and Fabrizio, 2012). In particular, Ras/cAMP/PKA has been suggested having a pro-ageing role in yeast (**Fig. 11**), by downregulating the stress response genes that usually help controlling critical factors, and by shifting energy from protection to growth (Longo and Fabrizio, 2012). Accordingly, both loss-of-function and gain-of-function mutants in this pathway show altered phenotypes in regard to chronological life span (CLS). Constitutively active *RAS2^{val19}* and constitutive activation of PKA shortens the chronological life span of yeast, whereas *Δras2* was the first identified long-lived mutant (Fabrizio *et al.*, 2003; Hlavata *et al.*, 2003). As confirmation, also the deletion of *CYR1* gene causes an increase in CLS (Fabrizio *et al.*, 2003).

As mentioned, Ras/cAMP/PKA pathway influences the duration of survival mainly by downregulating Msn2, Msn4 and Gis1 (Wei *et al.*, 2008), but it also seems to modulate the activity of the mitochondrial superoxide dismutase Sod2, which is as well necessary to protect from stress and slow down ageing (Fabrizio *et al.*, 2003). Even though the role of Ras/cAMP/PKA pathway as pro-ageing has been well established, it is import to remind that different studies produced contrasting results. Sun *et al*. (1994) reported that disruption of *RAS2* causes a decrease in CLS instead of the increase described by previous authors, similarly to two more recent high throughput studies, in which deletion of *RAS2* caused a decreased in CLS (Marek and Korona, 2013; Garay *et al.*, 2014). Moreover, there are evidences that overexpression of *RAS2* causes increase in CLS, instead of decrease (Sun *et al.*, 1994). The differences may be due to variations in the yeast genetic backgrounds used or in different experimental conditions.

3.1.3 Yeast Ras proteins involvement in programmed cell death

Stress response and ageing, besides being tightly interconnected between them, are also linked to another important cellular process in yeast, programmed cell death (PCD). Ageing yeast cells show signs typical of apoptotic cells, such as phosphatidylserine externalization, chromatin condensation, DNA breakage and accumulation of ROS (Fabrizio *et al.*, 2004; Herker *et al.*, 2004). It is therefore conceivable that the same signalling pathways regulating these processes, as well as others, such as cell cycle regulation and membrane and lipid cell wall synthesis (Laun *et al.*, 2005), may be involved in both PCD and ageing. Consequently, Ras/cAMP/PKA pathway is a major intracellular player in PCD process (Gourlay *et al.*, 2006; Frohlich *et al.*, 2007; Carmona-Gutierrez *et al.*, 2010) (**Fig. 11**). It has been discovered that increased activation of Ras signalling induces in yeast cells, both *S. cerevisiae* and *C. albicans*, the formation of markers typical of apoptosis (Gourlay and Ayscough, 2006; Phillips *et al.*, 2006). Two stimuli have been identified so far that lead to Ras/cAMP/PKA hyperactivation and subsequent cell death. (i) It has been observed that osmotin, a protein produced by plants in defence of pathogenic fungi, induces apoptosis by increasing Ras2 activity (Narasimhan *et al.*, 2001). Osmotin binds to Pho36, a G-protein-like homologous of the mammalian adiponectin receptor, causing the inappropriate inactivation of Ras signalling that ultimately leads to PCD (Narasimhan *et al.*, 2001). (ii) Actin cytoskeleton stimulates Ras-mediated apoptosis in yeast. Mutation or addition of drugs can change actin dynamics, causing the formation of F-actin aggregates, which in turn trigger the constitutive activation of Ras2 and apoptosis (Gourlay and Ayscough, 2006). Interestingly, F-actin aggregates are also often observed in ageing yeasts (Gourlay *et al.*, 2006) and actin dynamics regulated by Ras2 plays an important role in both PCD and ageing (Pichova *et al.*, 1997; Ho and Bretscher, 2001; Gourlay *et al.*, 2004; Gourlay and Ayscough, 2005a). This highlights once again the similarities between apoptotic and ageing yeast cells.

The Tpk3 subunit of PKA exerts an important function as linker between actin dynamics and yeast PCD, regulating the enzymatic content of mitochondria (Chevtzoff *et al.*, 2005).

The role of mitochondria is indeed fundamental in yeast PCD, being the main responsible of ROS production when dysfunctional (Gourlay *et al.*, 2006). ROS accumulation is the central and first event in yeast PCD, regulating the following events that ultimately lead to death (Madeo *et al.*, 1999; Ludovico *et al.*, 2001; Mitsui *et al.*, 2005; Silva *et al.*, 2005; Weinberger *et al.*, 2005), and in both osmotin- and actin-induced PCD, accumulation of ROS seems to be the main responsible for cell death, since the addition of antioxidants could suppress the apoptotic phenotype (Narasimhan *et al.*, 2001; Gourlay and Ayscough, 2005b). Besides the above mentioned possible involvement of Tpk3 (Chevtzoff *et al.*, 2005), the precise mechanism by which Ras/cAMP/PKA pathway produces ROS is still not completely disclosed, but it has been showed that ROS production is decreased when cAMP level is lowered by *PDE2* overexpression (Gourlay and Ayscough, 2005b), whereas ROS level increases when the constitutively active $RAS2^{ala18val19}$ allele is expressed (Hlavata *et al.*, 2003).

Ras/cAMP/PKA pathway regulates also cell death in acidic environment (Lastauskiene *et al.*, 2014). Intracellular acidification caused by acetic acid, a known inducer of apoptosis in yeast (Ludovico *et al.*, 2001; Fannjiang *et al.*, 2004), leads to Ras/cAMP/PKA activation, which causes consequent cell death (Thevelein and de Winde, 1999; Mollapour *et al.*, 2006). Supporting this notion, deletion of *RAS* genes suppresses the apoptotic phenotype of the cells and leads to necrosis instead, while, in acidic environment, hyperactivation of Ras pathway by constitutively active allele $RAS2^{val19}$ or by deletion of *PDE2* increases apoptotic cell death (Lastauskiene *et al.*, 2014). Similarly, the chronologically aged *RAS2* deletion mutant was shown to be resistant to acetic acid-induced death (Burtner *et al.*, 2009). This is a further evidence of the connection between PCD, ageing and Ras/cAMP/PKA pathway.

3.2 Mammalian RAS proteins

RAS proteins family in mammalian cells accounts for 36 members, among which the most extensively studied isoforms are KRAS, HRAS and NRAS (Bar-Sagi and Hall, 2000; Malumbres and Barbacid, 2003; Wennerberg *et al.*, 2005).

RAS proteins work as transducer of pro-survival signals from the extracellular space to the intracellular compartment, through different tyrosine kinases receptors (TKRs), such as the ones belonging to the ErbB family of related cell membrane receptor, among which the most studied is EGFR. The dimerization of the receptors upon ligand binding triggers a conformational change that activates the catalytic tyrosine kinase domain, enabling the autophosphorylation of the intracellular carboxyl-terminal domain and therefore its activation. Once phosphorylated, it provides the ideal docking sites for multiple signalling proteins (Martinelli *et al.*, 2009; Normanno *et al.*, 2009; Saif, 2010). The phosphorylated intracellular domain of EGFR recruits guanine tyrosine exchange factors and these proteins remove GDP from RAS proteins, which consequently bind to GTP, resulting in activated RAS able to signal downstream (Schulze *et al.*, 2005). Activated RAS proteins can bind and activate at least 20 different effectors, among which the best known and characterized are RAF kinases, phosphatidylinositol 3-kinase and RAL guanine nucleotide dissociation stimulator (RALGDS). Other less studied effectors of RAS proteins are $p120^{GAP}$, RIN1, Tiam, Af6, Nore1, PLCε and PKCζ (Shields *et al.*, 2000; Downward, 2003; Herrmann, 2003; Rajalingam *et al.*, 2007) (**Fig. 12**).

Briefly, activated RAF kinases phosphorylate and activate MEK (MAPK/ERK kinase), leading to the activation of MAPK (also called ERK, extracellular signal-regulated kinase). MAPK regulates several transcriptional factors that influence cell cycle progression, proliferation and survival (Malumbres and Barbacid, 2003). In the other pathway, activated PI3K catalyses the production of PIP_3 (phosphatidylinositol-3,4,-triphosphate) by phosphorylating PIP_2 (phosphatidylinositol-4,5-diphosphate), a process reversed by the phosphatase PTEN. PIP_3 activates phosphatidylinositol dependent kinase 1, which recruits AKT to the plasma membrane and activates it (Efferth, 2012). Activated AKT main downstream effector is mTOR

(mammalian target of rapamycin), which is an atypical serine/threonine kinase that can form two distinct complexes, depending on which proteins interact with it. The mTOR complex 1 mainly acts by activating ribosomal S6 kinase and IF4B binding protein 1 (4EBP1), which promote the transcription of genes involved in cell growth, cell cycle progression and energy metabolism.

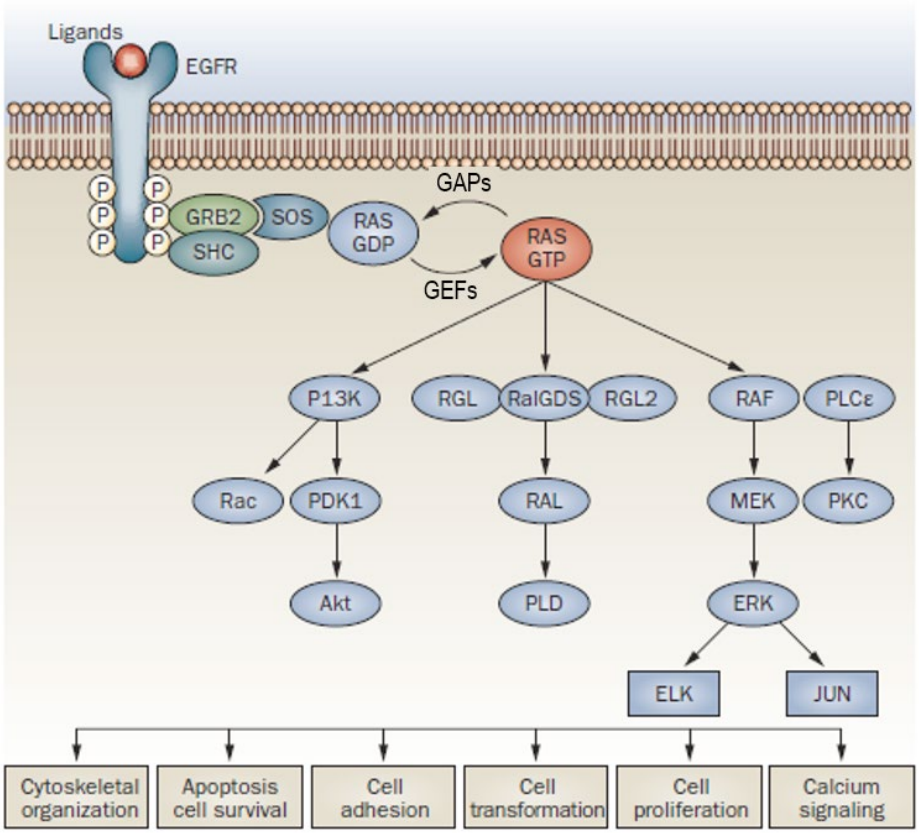


Figure 12: *RAS signalling pathway and main RAS effectors in mammals*. RAS proteins are activated upon growth stimulation. They are recruited to the cell membrane, where they interact with several adaptor proteins. On the membrane they can be activated by GEF and inactivated by GAP. Active RAS can interact with multiple downstream effectors, including RAF, PI3K, and RalGDS, among the most well-known. The various signalling cascades leads to various responses, including cytoskeleton organization, pro-survival signalling, cell adhesion, cell proliferation and calcium signalling (adapted from Normanno *et al.*, 2009).

The mTOR complex 2 mainly phosphorylates AKT, originating an auto-sustaining positive feedback loop, which results in cell survival and proliferation. Moreover, AKT activation enhances telomerase activity, triggers inhibition of apoptosis, inhibiting the release of cyt *c* from mitochondria and inactivating pro-apoptotic factors such as BAD and pro-caspase 9, and regulates modulators of angiogenesis through the activation of nitric oxide synthase (Yan *et al.*, 1998; Scheid and Woodgett, 2001a, 2001b; Wang and Zhang, 2014). RAS, when interacting with RALGDS, one of four RAS-related RAL proteins, stimulates RAL (RAS-like) GTPases, inducing the activation of phospholipase D1 and CDC42/RAC-GAP-RAL binding protein 1 (RALBP1). These, among other pro-survival functions, promote the progression of the cell cycle, inhibiting transcription factors implicated in cell cycle arrest such as the FORKHEAD transcription factors (Downward, 2003; Rajalingam *et al.*, 2007). Therefore, RAS proteins have a critical role in human oncogenesis, not only providing crucial signalling in proliferation and survival pathways like autophagy (Furuta *et al.*, 2004; Elgendy *et al.*, 2011; Guo *et al.*, 2011; Lock *et al.*, 2011; Alves *et al.*, 2015), but also promoting related processes such as angiogenesis and invasiveness (Shields *et al.*, 2000; Downward, 2003). Importantly, RAS proteins are frequently found mutated in tumour cells (approximately 20% of tumours) and KRAS is the most frequently mutated among all RAS proteins, accounting for about 85% of the total.

Similarly to all RAS proteins, also mammalian RAS are helped in the switch between GTP- and GDP-bound form by GEFs and GAPs (Rajalingam *et al.*, 2007). So far, three classes of mammalian GEFs are known, which share the common CDC25 catalytic domain and an N-terminal RAS exchange motif. The most extensively studied class in the one composed by SOS1 and SOS2 (son of sevenless), mammalian homologues of the first GEF *son of sevenless,* discovered in *Drosophila* (Rogge *et al.*, 1991; Bonfini *et al.*, 1992). SOS proteins contain the catalytic domain that promotes the formation of GTP-bound RAS and a pleckstrin homology (PH) domain that facilitates SOS membrane localization (Downward, 1994). SOS proteins are recruited to the plasma membrane by the adaptor protein Grb2 upon TKRs activation. The complex Grb2-SOS is formed through the Src homology 3 domain of Grb2 and is localized in the cytoplasm in resting cells. When the TKRs are activated, Grb2 can interact with the

phosphorylated tyrosine residues on the receptors through its SH2 domain, dragging with it on the plasma membrane SOS. In alternative, Shc adaptor protein can mediate the interaction between Grb2-SOS and tyrosine kinase receptors (Downward, 1994; Daub *et al.*, 1996). When SOS is placed on the plasma membrane, it is localized in the vicinity of RAS proteins and it can exert its activity of promoting the formation of RAS-GTP (Downward, 1994). Other classes of GEFs are RAS-Guanine Nucleotide Releasing Factors (Mitin *et al.*, 2005) and RAS-Guanine Nucleotide Releasing Proteins (Ebinu *et al.*, 1998; Tognon *et al.*, 1998). Also GAPs have been found numerous in mammalian. $p120^{GAP}$ was the first GAP identified, and also the first protein found to interact with the effector domain of RAS. $p120^{GAP}$ contains the catalytic domain, which facilitates the hydrolysis of GTP, and three adaptor domains, SH2, SH3 and PH (Adari *et al.*, 1988). Another important GAP is neurofibromin, which has been identified as tumour suppressor gene and is particularly relevant in some diseases, such as the neurofibromatosis type 1 (NF1) (Cichowski *et al.*, 2003). Two GAPs activated by Ca^{2+} intracellular level are CAPRI (Ca^{2+}-promoted RAS inactivator) and RASAL (RAS GTPase-activating like) (Allen *et al.*, 1998; Lockyer *et al.*, 2001).

As mentioned above, three main isoforms of RAS proteins are studied in mammals, HRAS, NRAS and KRAS, and two different splicing variants of KRAS are possible, KRAS4A and KRAS4B (Schubbert *et al.*, 2007). The three isoforms share approximately 85% of homology and their divergence is noticed in particular in the 25 aa of the hypervariable region at the C- terminal (Hancock and Parton, 2005), which is responsible for the specific membrane targeting (**Fig. 13**).

All three isoforms undergo an initial farnesylation, like every other RAS protein (Casey *et al.*, 1989; Hancock *et al.*, 1989; Schafer *et al.*, 1989), but after this first step, their processing depends specifically on their HVR. HRAS is palmitoylated on C181 and C184, NRAS is palmitoylated only on C181 and KRAS4B, the most diffuse between the two KRAS splicing variants, is not palmitoylated at all (Casey *et al.*, 1989; Hancock *et al.*, 1989; Hancock *et al.*, 1990). KRAS4B, instead, shows a polybasic domain, a stretch of lysines, which probably interacts with the head groups of plasma membrane lipids (Rajalingam *et al.*, 2007). These variations influence the internal trafficking of RAS proteins. The palmitoylated isoforms

HRAS, NRAS and KRAS4A enter the exocytic pathway though the Golgi and are subsequently addressed to the various microdomains of the plasma membrane (Choy *et al.*, 1999), whereas KRAS4B bypasses the Golgi and directly interacts with the microtubules through the polybasic domain (Rajalingam *et al.*, 2007). In addition to different post-translational modifications and intracellular trafficking, the three isoforms are differentially expressed in tissues and during development (Muller *et al.*, 1983; Leon *et al.*, 1987). Moreover, only KRAS has been discovered to be strictly necessary in mouse development, whereas HRAS and NRAS seem to be dispensable (Umanoff *et al.*, 1995; Johnson *et al.*, 1997; Koera *et al.*, 1997; Esteban *et al.*, 2001). All these data, together with the indications that mutations of specific RAS isoforms are found preferentially in certain types of cancer (Bos, 1989), strongly suggest that the three isoforms perform distinct functions in distinct tissue, cell type and sub-cellular localization.

Figure 13: *Human RAS isoforms homology and hypervariable regions*. Human RAS isoforms present a high degree of homology in the G domain, appointed to GTP/GDP binding, and a high degree of variability in the hypervariable region. The differences present in this region are responsible for the different processing and membrane localization of each RAS isoform (adapted from Schubbert *et al.*, 2007; Cox and Der, 2010).

3.2.1 Role of mammalian RAS proteins in cell cycle

As described above, RAS proteins have multiple effectors in mammals and can regulate various processes in the cells. Among RAS functions, a fundamental role in both physiological and transformant conditions is covered by the regulation of cell cycle progression (Peeper *et al.*, 1997; Pruitt and Der, 2001; Coleman *et al.*, 2004), similarly to what happens for yeast Ras proteins (Zaman *et al.*, 2008; Busti *et al.*, 2010).

RAS pathway in mammals controls the cell cycle through a variety of different proteins, resulting ultimately to the inactivation of the Rb family of pocket proteins (Mittnacht *et al.*, 1997; Peeper *et al.*, 1997) (**Fig. 14**). Rb proteins are found in a hypophosphorylated state in resting cells, which confers them the ability to sequester members of the E2F transcription-factor family. These transcription factors, once free from Rb inhibition, modulate the transcription of genes involved in the progression of cell cycle from G1 to S phase, especially in the regulation of DNA synthesis (Coleman *et al.*, 2004). What free the E2F transcription factors from inhibition is the phosphorylation of Rb by cyclin-CDK complexes. Cyclins and CDKs regulate the progression of the cell cycle by associating in active complexes. In particular, D-type cyclins D1, D2 and D3 can associate with CDK4 and CDK6 to promote the passage from G1 to S phase (Pruitt and Der, 2001; Coleman *et al.*, 2004). The activities of cyclin-CDK complexes are regulated by two classes of inhibitors, the INK4 CDKI (CDK inhibitor) that only inhibits CDK activity by 1:1 protein interaction, and the CIP/KIP family CDKIs that act on the whole CDK-cyclin complex, (Pruitt and Der, 2001; Coleman *et al.*, 2004). The connection between RAS-driven pathways and the cell cycle is confirmed by experiments that utilized RAS neutralizing antibodies (Yang *et al.*, 1995; Kang and Krauss, 1996; Pruitt *et al.*, 2000). Cells subjected to this type of treatment stopped growth in G1 and presented hypophosphorylated Rb. On the other hand, when oncogenic RAS was expressed in the cells, they were able to enter the cell cycle independently from growth factor (Feramisco *et al.*, 1984; Mulcahy *et al.*, 1985).

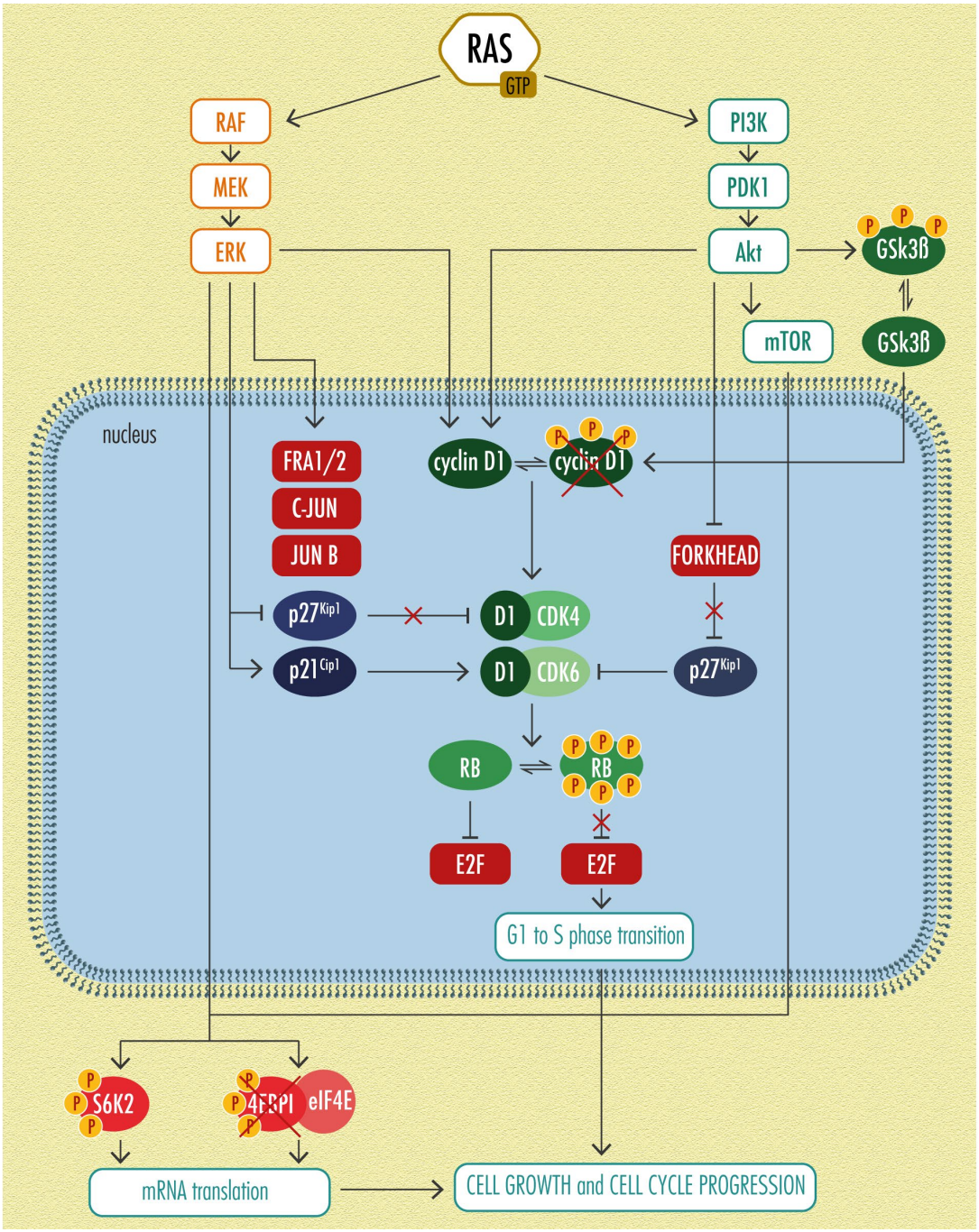


Figure 14: *Human RAS proteins role in cell cycle progression*. Active RAS proteins contribute to cell cycle progression activating both the main branches of their signalling cascades, RAF/MEK /ERK and PI3K. Their signalling converge on the activation of cyclin D1, which can form active complexes with CDK4 and CDK6, promoting the hyperphoshporylation of Rb, the consequent release of Rb inhibition on E2F transcription factor and the transcription of genes necessary for G1 to S phase transition. In addition, both branches contribute to mRNA translation, inhibiting 4EBPI and phosphorylating S6K2. They also regulates cyclin/CDK inhibitors, PI3K branch by inhibiting the FORKHEAD transcription factors, which in turn release their inhibition on $p27^{Kip1}$, Raf branch by directly inhibiting $p27^{Kip1}$ and activating $p21^{Cip1}$. Moreover, the Raf branch directly activates several transcription factors, including FRA1, FRA2, c-JUN and JUNB.

RAS pathway influences cell cycle progression increasing the expression of cyclin D1 through the RAF/MEK/ERK pathway (Albanese *et al.*, 1995; Lavoie *et al.*, 1996; Winston *et al.*, 1996; Aktas *et al.*, 1997), which activates a number of transcription factors such as FRA1, FRA2, c-JUN and JUNB (Balmanno and Cook, 1999; Treinies *et al.*, 1999), and through the PI3K branch of RAS effectors (Gille and Downward, 1999). Besides activating cyclin D1 transcription, PI3K also modulates its activity influencing its post-translational modifications. PI3K, through its downstream effector Akt, causes the phosphorylation of glycogen synthase kinase-3β (GSK-3β), which in turn phosphorylates cyclin D1, promoting its degradation. If phosphorylated by Akt, GSK-3β is inhibited, allowing a longer half-life of cyclin D1 (Diehl *et al.*, 1998). Besides directly promoting cyclin D1 expression, RAS pathway also modulates the activity of two CDKIs of the CIP/KIP family, $p27^{Kip1}$ and $p21^{Cip1}$. In particular, RAS enhances $p27^{Kip1}$ degradation and decreases its protein synthesis through the RAF/MEK/ERK cascade, diminishing $p27^{Kip1}$ inhibition on cyclin-CDK complexes (Rivard *et al.*, 1999; Treinies *et al.*, 1999; Delmas *et al.*, 2001). In addition to RAF-mediated inactivation of $p27^{Kip1}$, RAS can modulate $p27^{Kip1}$ activity also via PI3K, enhancing $p27^{Kip1}$ proteasome degradation (Takuwa and Takuwa, 1997; Mamillapalli *et al.*, 2001) and inactivating its transcription through the inhibition of the FORKHEAD transcription factor family (Kops *et al.*, 1999; Medema *et al.*, 2000; De Ruiter *et al.*, 2001). On the other hand, RAS acts on $p21^{Cip1}$ increasing its transcription levels (Liu *et al.*, 1996), which may sound counterintuitive. However, $p21^{Cip1}$, besides inhibiting cyclin-CDK complexes, can function as a positive regulator of the cell cycle, especially promoting cyclin D1-CDK assembly and their nuclear retention (LaBaer *et al.*, 1997; Cheng *et al.*, 1999; Alt *et al.*, 2002). The actual function of CDKIs may depend on their protein levels in the cell, being merely inhibitory at high levels or promoting cell cycle progression at lower level (Coleman *et al.*, 2004).

RAS pathway also exerts its effect on other two key regulators of cell cycle, p70 ribosomal S6 kinase (S6K1 and S6K2) and 4EBPI, mostly known to be modulated by mammalian target of rapamycin (mTOR), which can be activated by PI3K branch of RAS cascade through Akt. S6K1 and S6K2 function is to phosphorylate the S6 protein of 40D ribosomal complex. This protein promotes mRNA translation (Dufner and Thomas, 1999), while 4EBPI usually

segregates eIF4E, inhibiting the formation of the translation-initiation complex eIF4E (Kleijn *et al.*, 1998; Qin *et al.*, 2016). RAS/RAF/ERK/MEK pathway can phosphorylate both S6K2 (Martin *et al.*, 2001; Iijima *et al.*, 2002) and 4EBPI (Lin *et al.*, 1995; von Manteuffel *et al.*, 1996; Herbert *et al.*, 2002), promoting S6K2 activation and 4EBPI dissociation from eIF4E. There are evidences that RAS pathway decrease 4EBPI level also downregulating its transcription (Rolli-Derkinderen *et al.*, 2003).

3.2.2 Role of mammalian RAS proteins in apoptosis

In addition to regulate cells proliferation promoting cell cycle progression, RAS pathways control cell survival modulating apoptosis (Downward, 1998; Chang *et al.*, 2003; Cox and Der, 2003) (**Fig. 15**).

RAS-mediated survival signals promote apoptosis evasion, especially through PI3K pathway. One of the downstream targets of PI3K is Akt, a kinase able to phosphorylate a number of substrates, among which several are important apoptosis regulators. In particular, Akt can phosphorylate Bad, a pro-apoptotic member of the Bcl-2 family, and this phosphorylation causes Bad to bind to 14-3-3 in an inactive complex, instead of sequestering the anti-apoptotic proteins Bcl-2 and Bcl-X_L (Datta *et al.*, 1999). To confirm the importance of PI3K for apoptosis evasion, experiments showed that activated PI3K or Akt can abrogate apoptosis, whereas a dominant-negative Akt can enhance it (Cox and Der, 2003). Moreover, it has been shown that activation of PI3K is necessary to suppress apoptosis induced by oncogenic RAS (Gire *et al.*, 2000). In addition to Akt, PI3K can activate another important factor for survival, NF-κB, through the activation of Rac, in a redox-dependent manner (Sulciner *et al.*, 1996; Irani *et al.*, 1997; Joneson and Bar-Sagi, 1999). Rac, and consequently NF-κB, can also be activated by RAS directly through Tiam1, in a PI3K independent manner (Lambert *et al.*, 2002). A third RAS-mediated way to activate NF-κB is the phosphorylation of I-κB kinase (IKK) by Akt. Once Akt phosphorylates IKK, this releases NF-κB from cytoplasmic sequestration, allowing it to enter the nucleus, where NF-κB exerts its function as transcription factor (Romashkova and Makarov, 1999). NF-κB is a potent transcription factor that induces the transcription

of several anti-apoptotic genes, such as inhibitors of apoptosis proteins (IAPs) (Mayo and Baldwin, 2000).

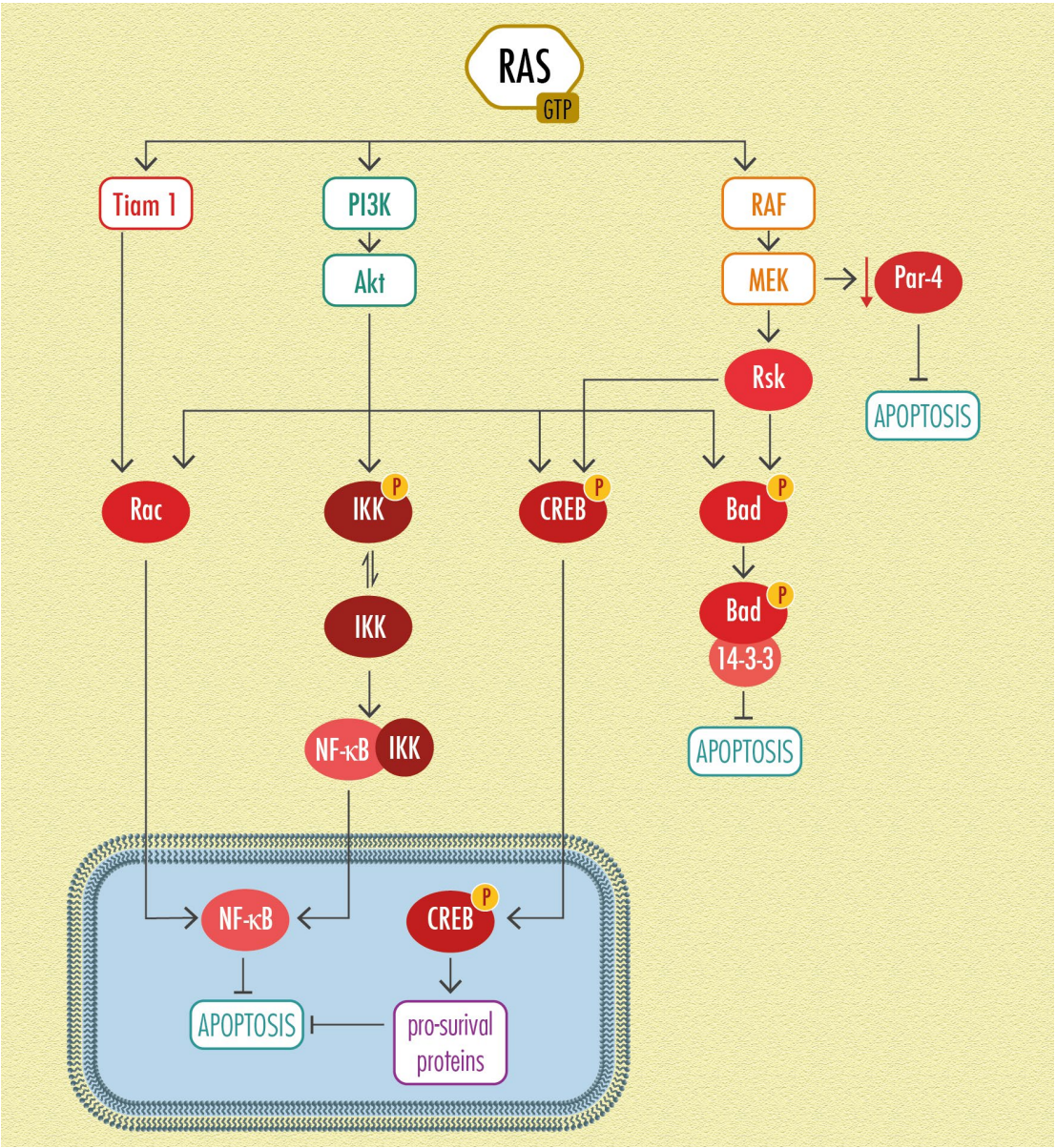


Figure 15: *Human RAS roles as anti-apoptotic proteins*. Human RAS proteins defend the cells from apoptosis mainly through the activation of PI3K and the consequent activation of Akt. Among Akt multiple targets there are Rac, IKK, CREB and Bad. Rac can be activated also by Tiam1 and activates in turn NF-κB, an important pro-survival factor. Akt-mediated phosphorylation of IKK allows the release of NF-κB from cytoplasmic sequestration and its consequent nucleus translocation and activation. Akt phosphorylates also Bad, promoting the inhibition of the pro-apoptotic factor 14-3-3 and the consequent inhibition of apoptosis, and CREB, promoting the transcription of pro-survival genes. CREB is phosphorylated also by Rsk, activated by Raf/MEK/ERK signalling cascade. In addition, MEK promotes the downregulation of the pro-apoptotic protein Par-4, contributing to apoptosis inhibition.

RAS pathway is also important to control a particular type of apoptosis, anoikis, specifically induced by cell loss of anchorage. Usually loss of attachment causes the translocation of Bax, a pro-apoptotic member of the Bcl-2 family, the release of cyt *c* from the mitochondria and the consequent caspases activation (Rytomaa *et al.*, 2000). It has been discovered that oncogenic RAS promotes resistance to anoikis (Rak *et al.*, 1995) through two distinct mechanisms. It downregulates Bak, a pro-apoptotic member of Bcl-2 family, in a PI3K-dependent manner (Rosen *et al.*, 1998), and it avoids the downregulation of the anti-apoptotic protein Bcl-X_L in a PI3K- and RAF-independent manner (Rosen *et al.*, 2000). Moreover, ectopic expression of activated RAF of MEK, downstream targets of RAS, allows the cells to overcome anoikis (Le Gall *et al.*, 2000). In addition, Akt can prevent the release of cyt *c* caused by anoikis (Rytomaa *et al.*, 2000), and BRAF can stop caspases activation, even if cyt *c* is released from mitochondria (Erhardt *et al.*, 1999).

The other branch of RAS signalling, the RAF/ERK/MEK signalling cascade, also contributes to the regulation of apoptosis, sometimes converging its signals to the same targets as the PI3K branch. The pro-apoptotic protein Bad can be phosphorylated, and thereby inactivated, by both Akt kinase (activated by PI3K) and Rsk kinases (activated by MEK) (Blume-Jensen *et al.*, 1998; Bonni *et al.*, 1999; Fang *et al.*, 1999; Tan *et al.*, 1999). These same kinases can phosphorylate also CREB transcription factor, which induces expression of pro-survival proteins (Du and Montminy, 1998; Bonni *et al.*, 1999). In addition to these common targets, the RAF/ERK/MEK branch can also act alone for the inhibition of apoptosis in specific situations. For example, RAS has been shown to help escaping apoptosis by downregulating Par-4, a pro-apoptotic transcription repressor, through MEK activity (Nalca *et al.*, 1999), and by inducing p53 degradation, thereby annulling p53-mediated apoptosis induction (Ries *et al.*, 2000). Moreover, RAS signal activity through the RAF/ERK/MEK cascade modulates the expression level of several proteins belonging to the Bcl-2 family. The loss of KRAS can provoke downregulation of the pro-apoptotic Bcl-2 (Tsuchida *et al.*, 2000), and dominant-negative RAF causes decreased level of Bcl-2 and increased level of Bcl-X_S (Navarro *et al.*, 1999).

However, defining the role of the RAF/ERK/MEK branch in apoptosis induction has been

more complicated than expected. This RAS-regulated cascade has been shown to be both anti- and pro-apoptotic, depending on the situation, whereas PI3K branch has always been defined as pro-survival. This apparent contradiction is highlighted by the fact that oncogenic RAS can promote pro-apoptotic signalling, which can in turn be suppressed by anti-apoptotic signalling induced by RAS itself (Mayo *et al.*, 1997; Joneson and Bar-Sagi, 1999). It is thought that the anti-apoptotic function of RAS is more common in transformed cells, in which oncogenic RAS promotes survival and, , apoptosis evasion, while an anti-apoptotic role is more suitable in normal cells, in which activated RAS may induce a pro-apoptotic response, in order to protect the cells from oncogenic transformation (Cox and Der, 2003). In particular, RAS can induce apoptosis through the protein RASSF1, a RA domain-containing tumour suppressor (Dammann *et al.*, 2001; Pfeifer *et al.*, 2002). RASSF1 protein is found complexed with the closely related protein Nore1 (Ortiz-Vega *et al.*, 2002) and with the pro-apoptotic protein Mst1, which is a caspase-3 enhancer (Lee *et al.*, 2001; Khokhlatchev *et al.*, 2002). Activated RAS can bind to RASSF1 (Vos *et al.*, 2000) and stimulate apoptosis. Another way for RAS to induce apoptosis is through $p120^{GAP}$, which is both a regulatory GAP and a RAS effector (Tocque *et al.*, 1997). $p120^{GAP}$ is a substrate of caspase cleavage and its N-terminal domain, after been separated from the C-terminal domain by low caspase activity, can be further cleaved in N1 and N2 fragments, which have a strong pro-apoptotic activity (Yang and Widmann, 2001). This cleavage can be triggered by various stimuli, such as Fas, TRAIL and DNA-damaging agents (Wen *et al.*, 1998; Widmann *et al.*, 1998; Clarke *et al.*, 2000). $p120^{GAP}$ can also regulate other proteins activity in order to induce apoptosis, such as Survivin, a member of the IAP family protein (Gigoux *et al.*, 2002), and mTid-1, a DNAJ-type chaperone protein (Trentin *et al.*, 2001).

3.2.3 Role of mammalian RAS proteins in cancer

As mentioned above, *RAS* genes are often found mutated in cancer. 20% of human tumours presents activating point mutation in *RAS* genes, with *KRAS* being the most frequently mutated among *RAS* isoforms (~85%), followed by *NRAS* (~15%) and *HRAS* (less than 1%) (Downward, 2003). The mutations of a specific RAS isoform appear to be preferential depending on the type of cancer (Bos, 1989), so then, for example, high percentage of mutated *KRAS* were described in pancreatic and colorectal cancers and more frequent *HRAS* mutations were found in hematopoietic tumours (Forbes *et al.*, 2011).

RAS mutations are single nucleotide point mutations, occurring mostly on codon 12, 13 and 61, and the relative frequency of one of those mutations depends on RAS isoform. In the case of *KRAS*, the majority of the mutations happens at codon 12, followed by codon 13 and 61 (**Fig. 16**), while the opposite trend is observed for *NRAS* (Prior *et al.*, 2012). The possible mutations could convert the codons 12, 13 and 61 in 6 other amino acids via single-base substitutions, but more than 60% of spotted mutations corresponds only to three of the 18 possible mutations. In particular, the majority of mutations are substitution of a glycine with another amino acid, the most frequent of which is an aspartate (32.5%), followed by a valine (22.5%) (Andreyev *et al.*, 2001; Normanno *et al.*, 2009; Prior *et al.*, 2012).

All the mutations of RAS proteins disrupt the proper endogenous GTPase activity of the protein or render RAS-GTP insensitive to GAP activity, leading to the accumulation of the activated GTP-bound form of RAS and therefore to a constant pro-survival signalling in the cells, which leads to cancer progression (Prior *et al.*, 2012). In particular, mutations at codon 12 make impossible for RAS to form the transition-state complex with GAPs (Franken *et al.*, 1993; Gremer *et al.*, 2008). Similarly, substitution of glutamine at codon 61 disrupts the transition-state mimic usually formed between RAS and GAPs, thereby impeding GAP to enhance GTP hydrolysis (Gibbs *et al.*, 1988; Gremer *et al.*, 2008). Mutations at codons 12, 13 and 61 also impaired the intrinsic GTPase activity, with mutations at codon 13 and 61 causing a 10-fold reduction of hydrolysis rate compared to normal RAS (Buhrman *et al.*, 2010; Prior *et al.*, 2012).

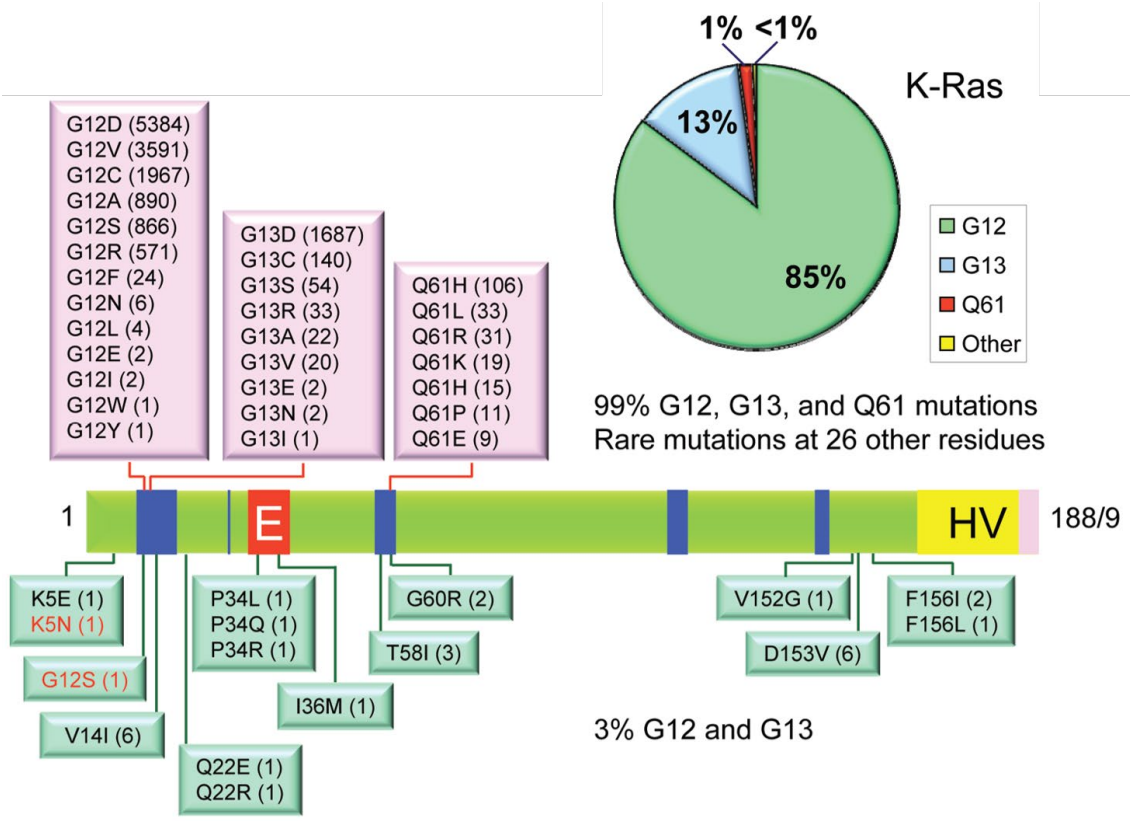


Figure 16: *Possible KRAS point mutations with their relative frequencies*. Point mutations of KRAS mainly happen at residue G12, G13 and Q61 and in most cases they are substitutions of a glycine with an aspartic acid (G12 and G13) or of a glutamine with a histidine (adapted from Cox and Der, 2010).

3.2.3.1 Focus on the role of RAS gene mutations in the development of colorectal cancer

The molecular changes underlying CRC development have been extensively studied and the specific gene mutations involved in the progress and carcinogenesis of CRC have been already characterized (**Fig. 17**). The primary genetic alteration in CRC carcinogenesis is the adenomatous polyposis coli (APC) gene mutation. Secondary genetic alterations occur with activation of the tyrosine kinase receptor EGFR, as well as of KRAS or BRAF oncogenes, followed by inactivation of some tumour suppressor genes, along with microsatellite and chromosomal instability (Tannapfel *et al.*, 2010; Mudassar *et al.*, 2014).

APC is a tumour suppressor gene mutated both in inherited and sporadic CRC forms (Smith *et al.*, 2002; Waldner and Neurath, 2010). Germline mutations in this gene lead to the development of a high number of premalignant polyps in the colon of individuals affected

with familial adenomatous polyposis (Aoki and Taketo, 2007; Waldner and Neurath, 2010; Raskov *et al.*, 2014).

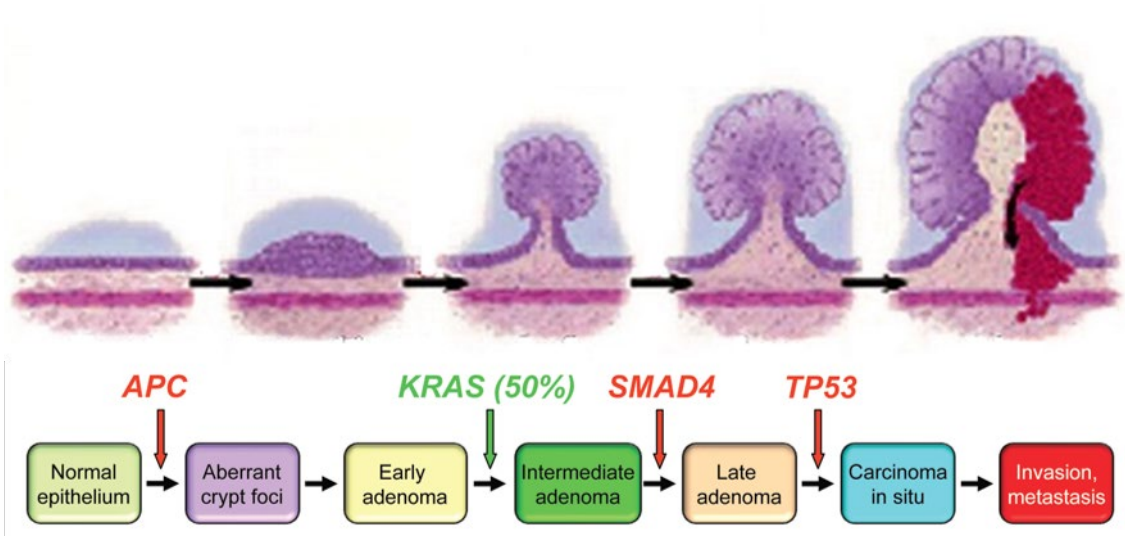


Figure 17: *Colorectal carcinogenesis from normal epithelium to colon cancer.* The main mutations in CRC development are represented here: APC, EGFR, KRAS, BRAF, p53 (adapted from Cox and Der, 2010; Sandouk *et al.*, 2013).

In cases of sporadic CRC, APC mutations are present in microscopic adenomas (~50-60%) (Smith *et al.*, 2002; Aoki and Taketo, 2007). The APC gene product is a protein (~312 kDa) that functions as a "gatekeeper" that regulates the entry of intestinal epithelial cells into the adenoma-cancer progression (Smith *et al.*, 2002). APC is a multi-domain protein that contains binding sites for numerous other proteins, including microtubules components, Wnt pathways regulators- axin, β-catenin and the cytoskeleton regulator EB1 (Smith *et al.*, 2002; Aoki and Taketo, 2007). EGFR is found overexpressed in CRC (Spano *et al.*, 2005). EGFR activity is de-regulated due to several reasons, such as EGFR overexpression and hyperactivation by the overproduction of the ligand (Lin *et al.*, 2001), constitutive activation of receptor, for example by dimerization with the constitutive active HER2/neu receptor (Graus-Porta *et al.*, 1997), or as a result of autocrine loops (Prenzel *et al.*, 1999), and epigenetic changes such as DNA methylation within the gene promoter (Derks *et al.*, 2008). EGFR hyperactivity seems crucial for CRC, being overexpressed in a high percentage of colorectal cancers (65-70%)

(Salomon *et al.*, 1995; Messa *et al.*, 1998) and being EGFR overexpression correlated with more aggressive disease and worst prognosis (Amador and Hidalgo, 2004). Other mutations implicated in CRC development are activation of *KRAS* or *BRAF* oncogenes, frequently found in sporadic CRC- approximately 35-40% and 5-10% of the CRC cases, respectively (Velho *et al.*, 2008; Yokota, 2012; Gonsalves *et al.*, 2014; Raskov *et al.*, 2014; Sridharan *et al.*, 2014). Mutated KRAS is found both in CRC and in large colorectal adenomas, but less frequently in small adenomas, which suggests that the mutation appears before the acquisition of the malignant state and that its presence confers a growth advantage (Leslie *et al.*, 2002). Similarly, to *KRAS*, mutated *BRAF* is also involved in the alteration of RAS/RAF/MEK/ERK pathway in CRC cases (Velho *et al.*, 2008). An important step in colon carcinogenesis is the loss of p53 function, which has been reported to occur relatively late in the development of CRC tumours (Smith *et al.*, 2002). The *TP53* gene, located on chromosome 17p, is mutated in 70% of all CRC tumours (Smith *et al.*, 2002). The gene encodes the p53 protein, a well-known "guardian of the genome" (Raskov *et al.*, 2014), responsible for inhibiting cell proliferation whenever DNA damage occurs. If the repair mechanisms fail, p53 initiates an apoptotic program, preventing cells from proliferate (Hollstein *et al.*, 1991). However, if p53 is mutated and DNA is not repaired, the damage spreads to the daughter cells. In this case, it has been demonstrated that mutation in the *TP53* gene is crucial for the progression from non-invasive to invasive disease, found in the following frequencies: adenomas (5%), malignant polyps (50%) and invasive CRC (75%), with an increasing frequency correlated to the extent of malignancy (Raskov *et al.*, 2014). *SMAD4* is another tumour suppressor gene often mutated in colorectal cancer. The SMAD4 protein is part of the TGF-β signalling pathway, which is responsible for controlling the expression of cell cycle regulators, differentiation factors and cell adhesion factors (Massaous and Hata, 1997). SMAD4 forms heterodimers with other SMAD proteins, acting as transcription factors (Imamura *et al.*, 1997; Hahn *et al.*, 2004). However, mutation of SMAD4 impairs its regulatory activity, blocking the transcription of important genes necessary for cell cycle control and apoptosis (Woodford-Richens *et al.*, 2001). As cancer progresses, the mutation rate in this gene increases; it is found mutated in about 31% of the metastatic colorectal cancer cases (Miyaki *et al.*, 1999; Maitra *et al.*, 2000),

suggesting that loss of this gene is important for the development of the metastatic state. Following these key genetic alterations, a number of acquired genetic mutations in other genes, including PIK3CA, FBXW7, SMAD2, TCF7L2, NRAS, FAM123B CTNNB1, have also been described to contribute to colorectal carcinogenesis (Keku *et al.*, 2015).

As described above, *KRAS* mutations are particularly relevant for the onset of CRC. In addition, it is important to consider *KRAS* mutations for prognostic and therapeutic implications. *KRAS* mutations, especially at codon 12, seem to indicate an increased risk of recurrence and death (Andreyev *et al.*, 1998; Andreyev *et al.*, 2001), are associated with a shorter 3-year survival, together with mutations in *BRAF* and *PI3KCA* (Barault *et al.*, 2008), and *KRAS* status of lymph nodes is linked to a higher risk of recurrence in patients with stage II CRC (Belly *et al.*, 2001). *KRAS* mutations must also be examined before treatment with anti-EGFR monoclonal antibody cetuximab and panitumumab. Indeed, patients expressing mutated *KRAS* have been found only moderately responsive or not responsive at all in a variety of clinical trials (Moroni *et al.*, 2005; Lievre *et al.*, 2006; Benvenuti *et al.*, 2007; Di Fiore *et al.*, 2007; Frattini *et al.*, 2007; Khambata-Ford *et al.*, 2007; Amado *et al.*, 2008; Karapetis *et al.*, 2008; Lievre *et al.*, 2008; Bibeau *et al.*, 2009), to the point that the European Medicines Evaluation Agency allowed the usage of panitumumab only in patients with wild type *KRAS.*

Due to the importance of KRAS in CRC and to the impossibility to block the signalling pathway upstream KRAS, when it is mutated, several attempts to block directly KRAS have been made. Attempts to inhibit KRAS protein were made using molecules capable of blocking farnesylation. This post-translational modification is necessary for proper KRAS activity, therefore blocking it may be a way to indirectly stop KRAS action (James *et al.*, 1993). Two different molecules were developed: tipifarnib and lonafarnib. Unfortunately, neither of them showed significant antitumor activity, mainly because KRAS can be activated also via gerenyl-geranylation (Caponigro *et al.*, 2003). The main transducer of KRAS signalling is BRAF, thus RAF proteins inhibitors were also developed. The first and most well-known is sorafenib, which is able to inhibit ARAF and both mutant and wild-type BRAF, showing antitumor activity in human xenograft models (Wilhelm *et al.*, 2004). A more specific BRAF inhibitor is vemurafenib, but it only showed a modest clinical activity due to a rapid feedback

activation of EGFR (Johnson *et al.*, 2009; Prahallad *et al.*, 2012). Several MEK inhibitors were designed, such as CI-1040, a small molecule orally administered that can bind to a site other than the ATP-binding site, which is common to many human kinases. It caused decrease in phospho-MAPK, but only modest clinical response in a phase II clinical trial (Lorusso *et al.*, 2005).

3.2.4 Nanocluster organization of mammalian RAS proteins

As mentioned above, RAS isoforms are differentially processed after being synthetized in the cytoplasm, and the different post-translational modifications determine the intracellular trafficking, the membrane interaction and the final sub-cellular localization of each isoform (Hancock, 2003; Omerovic *et al.*, 2007; Prior and Hancock, 2012). In particular, after an initial farnesylation on the cysteine of the common CAAX motif and the subsequent cleavage of the last three residues (Omerovic *et al.*, 2007), the route of the four isoforms – KRAS4A, KRAS4B, HRAS and NRAS – takes different directions. NRAS and KRAS4A are mono-palmitoylated, NRAS is di-palmitoylated and KRAS4B contains a polylysine sequence (Choy *et al.*, 1999; Apolloni *et al.*, 2000). These second signals ensure the membrane localization of RAS proteins, with different affinity and duration. As described above, also the protein trafficking is different. The traffic of palmitoylated proteins occurs through the Golgi/exocytic pathway up to the plasma membrane, whereas KRAS4B bypasses the Golgi and reaches the plasma membrane through an unknown mechanism (Choy *et al.*, 1999; Apolloni *et al.*, 2000). There are three hypotheses about the trafficking mechanism of KRAS4B to the plasma membrane. It is thought that the polybasic domain allows the simple diffusion of KRAS4B down an electrostatic gradient, attracted by the negatively charged membrane (Roy *et al.*, 2000), or that a chaperone protein similar to RhoGPD dissociation inhibitor may exist also for KRAS4B, or that KRAS4B is driven by a microtubule-dependent process (Hancock, 2003). Indeed KRAS4B seems to be the only RAS isoform able to bind to TAXOL-stabilized microtubules *in vitro* (Thissen *et al.*, 1997; Chen *et al.*, 2000).

The duration of the protein-membrane interactions also depends on the post-translational modifications. The electrostatic interaction of KRAS4B with the membrane due to the hexalysine motif has a very short half-life (Silvius *et al.*, 2006), while the palmitoylation allows a more stable association with the membrane (Misaki *et al.*, 2010). Even if longer compared to the electrostatic interaction, also interaction mediated by palmitoylation is short, due to the short half-life of palmitoylation itself (10-20% of N- and HRAS half-life), and this causes the cycling of RAS proteins back and forth the plasma membrane (Magee *et al.*, 1987; Baker *et al.*, 2003). The dynamics of acylation/deacylation of RAS and their consequent trafficking from plasma membrane to cytosol and *vice versa*, several times in a protein life, is important for ensuring the correct localization of the palmitoylated isoforms (Goodwin *et al.*, 2005; Rocks *et al.*, 2010). Palmitoylation also influences the orientation of RAS proteins and their consequent association with specific effectors and partition in specific microdomains, by varying the depth of insertion of the palmitoyl group in the membrane (Bergeron *et al.*, 1988; Vogel *et al.*, 2007; Abankwa *et al.*, 2008a; Abankwa *et al.*, 2008b; Brunsveld *et al.*, 2009). In conclusion, RAS post-translational modifications address different RAS isoforms to different localization, specifically different intracellular organelles, and the relative proportion of each isoform in each location varies. Typically, the relative contribution on endomembranes is NRAS > HRAS and KRAS4A > KRAS4B (Wittinghofer *et al.*, 1993; Prior and Hancock, 2012), and KRAS4B is more often associated with plasma membrane, whereas NRAS is found more frequently on endomembranes (Prior and Hancock, 2012).

As mentioned above, RAS HVR drives the microdomains localization of specific RAS isoforms (Hancock *et al.*, 1990; Apolloni *et al.*, 2000; Hancock, 2003; Omerovic *et al.*, 2007; Quinlan and Settleman, 2008; Prior and Hancock, 2012). The plasma membrane, where RAS proteins exert their major functions, is not a homogeneous bilayer of lipids, but rather a complex and dynamic mixture of phospholipids, cholesterol and proteins, controlled from the inside by an actin network (Murase *et al.*, 2004; Kusumi *et al.*, 2005; Morone *et al.*, 2006). RAS proteins, similarly to other membrane proteins, are strictly organized in the domains formed on the plasma membrane by the assembling of these different factors, while being at the same also found as free monomers (Prior and Hancock, 2012). RAS microdomains

are small in diameter (12 to 24 nm) and therefore called nanoclusters, comprise 6-7 RAS proteins and have a short half-life of about 0.1-1 sec (Hancock and Parton, 2005; Plowman and Hancock, 2005; Plowman *et al.*, 2005). As resulted clear from their short half-life, RAS nanoclusters are highly dynamic, continuously assembling and disassembling. Due to the fact that HVR leads the positioning of RAS isoform in the nanocluster and that each isoform has a different HVR, also the nanoclusters are different for each RAS isoform (Omerovic *et al.*, 2007; Prior and Hancock, 2012). For example, KRAS is mostly found in actin-dependent, cholesterol independent nanoclusters (Prior *et al.*, 2003b; Plowman *et al.*, 2008), whereas HRAS and NRAS are localized in cholesterol-dependent nanoclusters (Niv *et al.*, 2002; Prior *et al.*, 2003a; Plowman and Hancock, 2005; Roy *et al.*, 2005; Eisenberg *et al.*, 2006). In particular, HRAS has been positioned in specific plasma membrane domains called lipid raft when GDP-bound, and outside these domains when activated (Chen and Resh, 2001; Prior *et al.*, 2001). This change in nanocluster positioning between the active and inactive state seems to be due to conformational changes. The binding to GTP causes the rearrangements of switch I and switch II, and of a third region called switch III (Abankwa *et al.*, 2008a; Abankwa *et al.*, 2010). These variations cause the G-domain to rotate and to change the affinities for specific elements in the membrane, for its stabilizing proteins and its effectors (Abankwa *et al.*, 2008a; Abankwa *et al.*, 2010). On the other side, KRAS was found to attract phospholipids such as PIP_2, an important substrate of PI3K, thanks to its polybasic domain (Murray *et al.*, 1999; Wang *et al.*, 2002a). This differential segregation of RAS isoforms is thought to be responsible for the lack of functional redundancy and to be the cause for different affinities for the effectors, triggering different activation cascades (Hancock and Parton, 2005; Omerovic *et al.*, 2008). In addition to diversification for RAS isoforms, the same isoform can segregate differently depending on its activation state, as described above for the case of HRAS (Prior *et al.*, 2001; 2003a). All these data indicate the fundamental role of nanoclusters formation for RAS signalling, highlighted by the fact that when nanocluster formation is inhibited, signal output is reduced to the 3% of maximal signalling (Tian *et al.*, 2007). Moreover, it was discovered that when a smaller percentage of RAS proteins is clustered, the released signal decreased, even if the same amount of RAS proteins was present (Harding and Hancock, 2008; Kholodenko *et*

al., 2010). This indicate that RAS activation is not sufficient to ensure proper RAS signalling, but also correct nanoclustering is necessary. This notion opens new therapeutic possibilities that aim at impeding nanoclusters formation in oncogenically transformed cells, rather than blocking RAS activation or enhancing GTP hydrolysis. Importantly, RAS nanoclusters may be stabilized by cytosolic proteins. A well-studied case is the one of HRAS stabilization by cytoplasmic galectin-1. Gal-1 was discovered to be able to cross-link to active HRAS, but not inactive HRAS or other RAS isoforms (Paz *et al.*, 2001). To further uncover the role of gal-1 in regard to HRAS nanocluster distribution, the downregulation of gal-1 annuls HRAS clustering at the plasma membrane, with a simultaneous mislocalization in the cytosol (Paz *et al.*, 2001; Elad-Sfadia *et al.*, 2002; Prior *et al.*, 2003a). This kind of galectin-RAS interactions was also observed for gal-3 and KRAS (discussed in detail in following chapter). In light of the importance of RAS nanoclustering mentioned above and of the role of galectins in preserving this process, galectins also could be considered as potential therapeutic target to impede abnormal RAS signalling and therefore cancer progression and, on the other hand, galectins could also be seen as putative responsible for non-mutated RAS oncogenic activation, by increasing their nanocluster segregation and therefore their signalling.

4. Interaction between intracellular galectin-3 and KRAS

Similarly, to what was described above for HRAS/gal-1 interaction, KRAS has been found to interact with intracellular gal-3. The interaction occurs between gal-3 hydrophobic pocket and the farnesyl group on the cysteine in the CAAX motif of KRAS (Elad-Sfadia *et al.*, 2004; Ashery *et al.*, 2006; Shalom-Feuerstein *et al.*, 2008) and it is highly specific. Gal-3 interacts with other RAS proteins only to a much lower level (Elad-Sfadia *et al.*, 2004; Shalom-Feuerstein *et al.*, 2005). Moreover, gal-3 preferentially binds to activated KRAS (Elad-Sfadia *et al.*, 2004). The interaction was firstly demonstrated by co-immunoprecipitation and co-localization experiments, and it was noticed that, in addition to merely interact, gal-3 modulate KRAS activity and *vice versa* (Elad-Sfadia *et al.*, 2004; Shalom-Feuerstein *et al.*, 2005; Song *et al.*, 2012). Activated KRAS seems to mediate gal-3 translocation to the plasma membrane, where the two proteins co-localize in specific punctuated patterns (Elad-Sfadia *et al.*, 2004; Shalom-Feuerstein *et al.*, 2008; Levy *et al.*, 2011). On the other hand, phosphorylation of gal-3 by casein kinase 1 or gal-3 overexpression mediates KRAS localization on the plasma membrane, proved by the fact that ablation of gal-3 or inhibition of its phosphorylation causes KRAS to mislocalize in the cytoplasm (Levy *et al.*, 2010; Song *et al.*, 2012). The membrane localization is therefore reciprocally regulated, and the same happens for the distribution in nanoclusters on the membrane, since gal-3 levels control the amount of clustered KRAS. Indeed reduction in gal-3 levels decreases also nanoclustered KRAS, whereas an increase in gal-3 causes a consequent increased in both KRAS and gal-3 nanoclusters, which overlap (Shalom-Feuerstein *et al.*, 2008; Abankwa *et al.*, 2010; Levy *et al.*, 2011). On the other side, the RAS inhibitor *trans*-farnesylthiosalicylic acid disrupts KRAS-GTP/gal-3 nanoclusters and co-localization on the plasma membrane (Levy *et al.*, 2010). To further show the specific association of gal-3 only with activated KRAS, no alterations of nanoclusters containing KRAS-GDP were noticed upon variations of gal-3 levels (Shalom-Feuerstein *et al.*, 2008). This concurs with KRAS forming distinct nanoclusters based on its activation state.

Besides modulating KRAS nanoclustering, gal-3 seems to strongly contribute to its activation status. Overexpression of gal-3 resulted in increased amount of GTP-bound KRAS, which contrarily decreased upon gal-3 downregulation, without altering the global KRAS level, in several cancer types and normal cells, such as fibroblasts (Elad-Sfadia *et al.*, 2004), breast cancer cell line (Shalom-Feuerstein *et al.*, 2005), thyroid cancer (Levy *et al.*, 2010) and pancreatic cancer (Song *et al.*, 2012). Mutation in residue 125 of gal-3 from a valine to an alanine (V125A), which is enclosed in the hydrophobic pocket fundamental for KRAS binding, does not alter the binding ability of gal-3 to KRAS, but alters the activation capability of KRAS and the consequent downstream signalling, together with the amount of KRAS-GTP nanoclusters (Shalom-Feuerstein *et al.*, 2008), further proving the role of gal-3 in ensuring KRAS activation. In addition, gal-3 promotes KRAS association with the plasma membrane, another important factor for KRAS activity (Bhagatji *et al.*, 2010) and reduces the efficiency of $p120^{GAP}$ in hydrolysing GTP, stabilizing the active state of KRAS (Elad-Sfadia *et al.*, 2004). Gal-3 expression in a cell seems to increase not only the activation, but also the expression of KRAS in thyroid cancer cells (Levy *et al.*, 2010). Conversely, in neural PC12 cells is gal-3 expression to be induced by RAS pathway (Kuklinski *et al.*, 2003) and in mouse embryonic fibroblast lacking gal-3 reduced KRAS levels are present, probably because gal-3 modulates the transcription level of the tumour suppressor *let-7c*, which in turn inhibits KRAS transcription (Levy *et al.*, 2011). Other evidences indicated that gal-3 stabilize KRAS protein by decreasing its degradation level (Levy *et al.*, 2011). In conclusion, gal-3 can enhance KRAS activity both by stabilizing its nanocluster organization and by favouring its active state over its GDP-bound state.

Gal-3 expression also modulates the specific branch activated by active KRAS, in a way that seems to depend on cell type. In breast cancer cells a switch from PI3K and Akt activation to ERK/MEK cascade has been noticed (Shalom-Feuerstein *et al.*, 2005; Shalom-Feuerstein *et al.*, 2008), whereas in fibroblasts PI3K stimulation resulted enhanced to the detriment of ERK/MEK (Elad-Sfadia *et al.*, 2004). Moreover, when KRAS interacts with gal-3, the cell starts using preferentially KRAS at the expenses of the other RAS isoforms present. In particular, when KRAS is preferred over NRAS, the anti-apoptotic effect of gal-3 is increased (Shalom-

Feuerstein *et al.*, 2005).

In view of the important individual role of KRAS and gal-3 in promoting cell proliferation and inhibition of apoptosis, and since gal-3 appears to enhance KRAS activity through different mechanisms, it is conceivable to expect that this interaction could cause worsening in cancer progression (**Table 4**). It has been shown that gal-3 interaction with KRAS enhances thyroid cancer progression, increasing KRAS signalling and thus proliferation (Levy *et al.*, 2010). In addition, gal-3 has been found overexpressed in pancreatic cancer, where it interacts with KRAS-GTP, influencing its active status and it membrane localization. Alterations in gal-3 level and consequent variation in RAS downstream signalling cascades strongly modulate the cancer phenotype. Downregulation of gal-3 decreases growth, invasiveness, anchorage independent growth and tumour growth in an *in vivo* orthotopic model, whereas gal-3 upregulation stimulates growth proliferation (Song *et al.*, 2012). V125A mutation, which reduces KRAS-GTP global amount and nanoclusters amount, decreases breast cancer cell proliferation and anchorage independent growth, while making them more sensitive to apoptosis (Shalom-Feuerstein *et al.*, 2008). On the other hand, in the same cell line, KRAS inhibitors can revert the transformation phenotype associated with gal-3 overexpression (Shalom-Feuerstein *et al.*, 2005). A colon cancer cell line was found to express gal-3 at high levels and this expression correlates with migration ability of cancer cells. In parallel, this migration ability seems to be mediated by RAF and ERK RAS effectors, since gal-3 downregulation simultaneously causes downregulation of RAF and p-ERK and decreases migratory phenotype. To further highlight the relationship between gal-3 and RAS pathway in colon cancer, tissue samples show a correlation between high levels of gal-3, RAF and ERK (Wu *et al.*, 2013). Finally, gal-3 seems to be the adaptor for the interaction between KRAS and $\alpha_v\beta_3$ integrin. The interaction between KRAS and this type of integrin is specific and causes tumour aggressiveness, indicated by anchorage independence and self-renewal, and tumour resistance to RTK inhibitors such as erlotinib. However, KRAS does not present any binding site for cytoplasmic β_3, implying that a mediator protein is necessary. Gal-3, as seen, can interact with KRAS and with $\alpha_v\beta_3$ (Markowska *et al.*, 2010), being the perfect candidate for this role. Indeed, knock-down of gal-3 in a pancreatic cancer cell line impedes

the formation of KRAS/β_3 interaction and consequently suppresses the erlotinib resistance and self-renewal driven by that interaction (Seguin *et al.*, 2014). This further shows the fundamental role of gal-3 in mediating KRAS function and its ability to ablate KRAS-mediated pro-survival signals, when downregulated.

Table 4: Main effects caused by gal-3/KRAS interaction in various cancer cell lines.

cancer type	gal-3/KRAS interaction	refs
breast cancer	• Gal-3 overexpression causes the switch from HRAS to KRAS usage, favouring an anti-apoptotic response	(Shalom-Feuerstein *et al.*, 2005)
	• Gal-3 stabilizes KRAS-GTP nanoclusters • V125A mutation causes loss of gal-3/KRAS functional interaction and consequent decrease in tumour severity	(Shalom-Feuerstein *et al.*, 2008)
thyroid cancer	• High expression of Gal-3 correlates with high level of KRAS-GTP • FTS inhibits gal-3/KRAS interaction and plasma membrane co-localization	(Levy *et al.*, 2011)
colon cancer	• Gal-3 promotes cancer migration through RAS/Raf/ERK pathway • Gal-3 high expression level correlates with high expression of Raf and ERK in cancer tissue samples	(Wu *et al.*, 2013)
pancreatic cancer	• Gal-3 mediated KRAS plasma membrane localization • Alterations in gal-3 levels cause variations in RAS activity and downstream pathway regulation, with silencing of gal-3 resulting in a less severe cancer phenotype	(Song *et al.*, 2012)
	• Gal-3 mediates the interaction between KRAS and $\alpha_v\beta_3$, favouring tumour stemness and erlotinib resistance	(Seguin *et al.*, 2014)

In conclusion, it has been discovered that an increase expression or availability of cytoplasmic gal-3 could confer to the cells the same tumorigenic properties, such as uncontrolled growth and unregulated apoptosis that they acquire upon mutation of *KRAS* oncogene. Moreover, several data indicate that the inhibition of gal-3 seems to be sufficient to downregulate KRAS activity and consequent pro-survival signalling pathways. These facts

are particularly important to better understand the interaction between these two proteins and try to find compounds able to decrease it, in light of therapeutic implications.

5. TUMOUR SUPPRESSOR $p16^{INK4a}$

$p16^{INK4a}$ (inhibitor of CDK4, known as well as CDKN2A – CDK inhibitor 2A -, or MTS1 – multiple tumour suppressor 1) is a well-established tumour suppressor protein, belonging to the INK4 family of CKIs (CDK inhibitors), which includes as well $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$. The proteins of the INK4 family of CDK inhibitors all present a similar structure of several ankyrin repeats (Li *et al.*, 2011) and are differentially expressed based on tissue and cell lineage, making their functions non redundant (Sherr and Roberts, 1999; Canepa *et al.*, 2007). Some of the proteins belonging to the INK4 family, including $p16^{INK4a}$, are encoded by genes in the *INK4/ARF* tumour suppressor *locus* on chromosome 9p21.3. This *locus* encodes for $p16^{INK4a}$ and its transcriptional variant, $p14^{ARF}$ in human and $p19^{ARF}$ in mouse, and for a third protein, $p15^{INK4b}$ (Hannon and Beach, 1994; Quelle *et al.*, 1995).

$p16^{INK4a}$ acts as inhibitor of CDK4 and 6, competing with cyclins-D (D1, D2 and D3) for their binding (**Fig. 18**) (Ruas and Peters, 1998). The formation of the complex made of CDK and cyclin allows cell cycle progression and, in the specific case of these complexes, the transition of the G1/S restriction point and the beginning of DNA replication. CDK-cyclin complexes phosphorylate the Rb protein. The hyperphosphorylation of Rb relieves it from its inhibitory role on E2F transcription-factor family, leading to the transcription of genes necessary for S phase (Reed, 1997; Sherr and Roberts, 1999; Coleman *et al.*, 2004; Bertoli *et al.*, 2013). By binding to CDK4 and CDK6, $p16^{INK4a}$ inhibits the proper formation of the cyclin-CDK complexes and therefore the entry in S phase, having therefore a role in stopping cell cycle progression (Hall *et al.*, 1995; Lukas *et al.*, 1995; Parry *et al.*, 1995; Reymond and Brent, 1995; Guan *et al.*, 1996). In addition, $p16^{INK4a}$ can disrupt the complexes formed by CDK4 and 6 and the members of the other class of CKIs, the Cip/Kip family. In doing so, $p16^{INK4a}$ frees inhibitors that can act on other CDKs, such as CDK2, further increasing Rb hypophosphorylated state (Sherr and Roberts, 1999). $p16^{INK4a}$ acts on cell cycle progression also through CDK-independent

mechanisms. For example, p16^{INK4a} can interact with the transcription factor TFIIH, which phosphorylates through its CDK7 subunit the C-terminal domain of RNA polymerase II, promoting cell cycle-associated transcription. The binding of p16^{INK4a} with TFIIH inhibits RNA polymerase phosphorylation, consequently inhibiting cell cycle progression (Serizawa, 1998; Nishiwaki *et al.*, 2000).

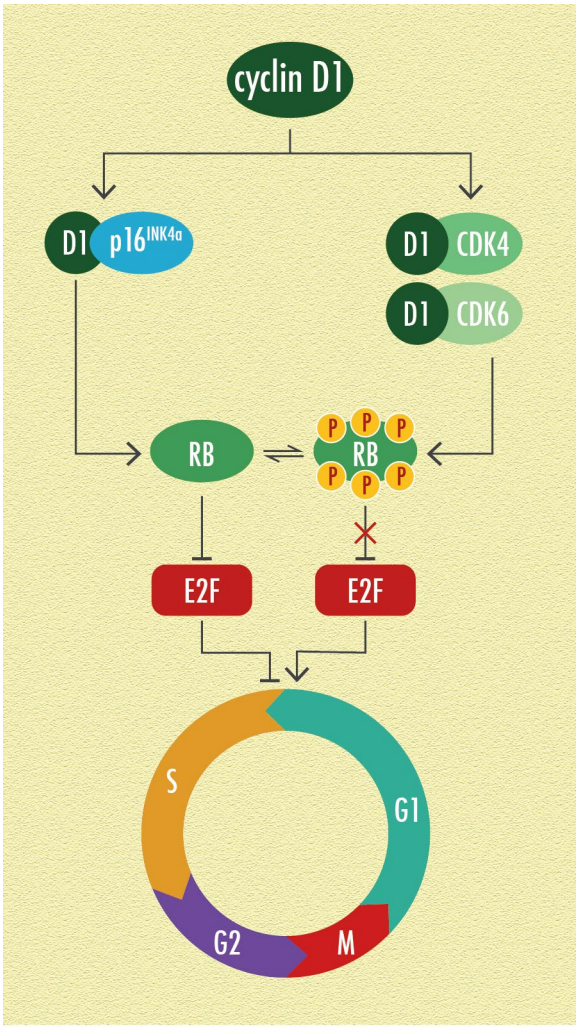


Figure 18: *Schematic representation of p16^{INK4a} role in cell cycle regulation*. p16^{INK4a} is an inhibitor specific for cyclin D1. When p16^{INK4a} binds to the cyclin, the complex formation between cyclin and CDK is blocked, Rb proteins remain in a hypophosphorylated state, impeding the activation of E2F transcription factors, and the cell cycle is stopped at G1-S phase boundary.

p16^{INK4a} has been implicated in replicative senescence (LaPak and Burd, 2014), since its expression and/or protein amount accumulates with increasing numbers of population doublings (Alcorta *et al.*, 1996; Hara *et al.*, 1996; Loughran *et al.*, 1996; Reznikoff *et al.*, 1996; Wong and Riabowol, 1996; Palmero *et al.*, 1997; Zindy *et al.*, 1997) and in senescent cells or cells subjected to stress stimuli (Ksiazek *et al.*, 2006; Canepa *et al.*, 2007; Quereda *et al.*, 2007; Wu *et al.*, 2007; Fordyce *et al.*, 2010). p16^{INK4a} seems to be shared in mechanisms

causing senescence and cell cycle arrest in non-senescent cells (Schmitt *et al.*, 2002). As further confirmation, introduction of human or mouse chromosome containing $p16^{INK4a}$ could impose senescence on an immortal cell (Klinger, 1982; Harris, 1988; Vojta and Barrett, 1995; England *et al.*, 1996; Smith and Pereira-Smith, 1996).

Not surprisingly, mutations of $p16^{INK4a}$ that lead to its functional inactivation are among the most frequently found aberrations in cancer, being present in almost 50% of all human cancer (Hirama and Koeffler, 1995; Ruas *et al.*, 1999; Esteller *et al.*, 2001; Ortega *et al.*, 2002; Gonzalez and Serrano, 2006; Li *et al.*, 2011; Romagosa *et al.*, 2011; LaPak and Burd, 2014). Often the all *INK4/ARF locus* is deleted in cancer, as in the examples of melanoma, pancreatic adenocarcinoma, glioblastoma, leukaemia and bladder cancer (Canepa *et al.*, 2007). As mentioned above, this *locus* comprises three different tumour suppressor genes, so it is evident why its deletion can cause dramatic outcomes. It has been shown that overexpression of the entire *locus* confers tumour resistance (Matheu *et al.*, 2004), whereas knockout studies of the three genes alone and in conjunction show that mice are more sensitive to the onset on cancer in the absence of one of the genes, and this sensitivity is enhanced when more than one gene belonging to the *INK4/ARF locus* is lacking (Sharpless *et al.*, 2004). $p16^{INK4a}$ is also subjected to point mutations or small deletions, which cause somatic loss in tumours (Ruas and Peters, 1998; Forbes *et al.*, 2006), and to epigenetic modifications, specifically promoter methylation, which silence gene translation (Ruas *et al.*, 1999; Canepa *et al.*, 2007). Interestingly, even if the loss of functionality of $p16^{INK4a}$ is very common, it seems to be specifically correlated only to certain types of cancer. In particularly, it has been found at very high frequency (80 to 100%) in mesothelioma (Geradts *et al.*, 1995) and head and neck squamous-cell cancer (Reed *et al.*, 1996), at intermedium frequency (40 to 80%) in non-small-cell lung cancer (Kinoshita *et al.*, 1996; Kratzke *et al.*, 1996; Sakaguchi *et al.*, 1996; Betticher *et al.*, 1997), glioma (Fueyo *et al.*, 1996; Nakamura *et al.*, 1996) and thymoma (Hirabayashi *et al.*, 1997), and at frequency lower than 40% in ovarian cancer (Dong *et al.*, 1997; Fujita *et al.*, 1997; Kanuma *et al.*, 1997) and acute lymphoblastic leukaemia (Volm *et al.*, 1997). In colon and prostate cancer almost no mutations in $p16^{INK4a}$ have been found (Geradts *et al.*, 1995; Konishi *et al.*, 1996). However, $p16^{INK4a}$ protein has been often found at

low level or inactivated in CRC (Tominaga *et al.*, 1997). Since point mutations and deletions occur at a very low frequency (Cairns *et al.*, 1995; Herman *et al.*, 1995; Ohhara *et al.*, 1996), the most common mechanism for $p16^{INK4a}$ inactivation is thought to be promoter methylation (Herman *et al.*, 1995; Merlo *et al.*, 1995). This hypothesis is supported by the fact that $p16^{INK4a}$ promoter is frequently methylated in primary cancer tissues and is almost never methylated in normal mucosa (Hsieh *et al.*, 1998; Guan *et al.*, 1999; Mitomi *et al.*, 2010), and that promoter methylation and consequent $p16^{INK4a}$ inactivation most probably favours tumour progression (Kim *et al.*, 2005; Ishiguro *et al.*, 2006). Even if there are evidences supporting that $p16^{INK4a}$ inactivation has an important impact on CRC formation, $p16^{INK4a}$ role in this type of cancer is still debatable. Indeed there are also studies reporting a normal $p16^{INK4a}$ protein expression in the majority (64-85%) of CRC tissue and a low expression in normal mucosa (Dai *et al.*, 2000; Palmqvist *et al.*, 2000), in addition to above mentioned studies showing low frequency of $p16^{INK4a}$ aberrations (Geradts *et al.*, 1995) and even methylation (Norrie *et al.*, 2003; Shima *et al.*, 2011) and no apparent $p16^{INK4a}$ inactivation correlated with tumour expansion (Ohhara *et al.*, 1996). In light of these conflicting information, a further investigation on the role of $p16^{INK4a}$ in the formation and progression of CRC seems even more important.

5.1 $p16^{INK4a}$ PATHWAYS OVERLAPPING WITH RAS PROTEINS AND GALECTINS

Considering their respective roles, it is clear that the functions of RAS proteins and $p16^{INK4a}$ are antagonistic. $p16^{INK4a}$ is a strong inhibitor of CDK4 and 6 and cell cycle progression, whereas it has been shown that RAS proteins are mediators of cell cycle progression through the activation of cyclins. RAS proteins promote the transcription of cyclin D1 through the RAF/ERK/MEK pathway and PI3K activation; they increase its stability by inhibiting the kinases that cause its ubiquitination and consequent degradation; through PI3K activation, they induce cyclin D1 mRNA translation and they also facilitate the assembly with CDK4 and CDK6. RAS pathway further helps cyclin D1 activity by reducing the level of

CDK-cyclins complexes inhibitors such as $p27^{Kip1}$ (Mittnacht *et al.*, 1997; Peeper *et al.*, 1997; Pruitt and Der, 2001; Coleman *et al.*, 2004). However, little is known about a possible direct relation between RAS proteins, in particular KRAS, and $p16^{INK4a}$.

In the middle 90´s Serrano *et al.* published several works highlighting an indirect relationship between HRAS and $p16^{INK4a}$. They showed that the introduction of a plasmid encoding $p16^{INK4a}$ in cells transformed with $HRAS^{G12V}$ and c-Myc decreased the tumorigenicity of the cells (Serrano *et al.*, 1995). On the other way around, when *HRAS* was transfected into $p16^{INK4a-/-}$ fibroblasts, the tumorigenicity of the cells greatly increased, while no effect was observed when using $p16^{INK4a+/+}$ or $p16^{INK4a+/-}$ cells (Serrano *et al.*, 1996). Moreover, they showed that cells lacking functional $p16^{INK4a}$ do not undergo senescence (Serrano *et al.*, 1996) and that RAS-caused cell cycle arrest, phenotypically indistinguishable from senescence, is mediated by $p16^{INK4a}$. Indeed cells capable to block cell cycle progression through RAS over-activity are growing continuously only when $p16^{INK4a}$ is inactivated (Serrano *et al.*, 1997). Oncogenic $HRAS^{G12V}$ was shown to increase $p16^{INK4a}$ expression in late papilloma and the balanced activity of RAS pathway and $p16^{INK4a}$ induced senescence (Yamakoshi *et al.*, 2009). A reciprocal regulation of the two proteins was discovered in the case of KRAS. In primary pancreatic duct epithelial cells it was noticed that KRASwt was able to increase $p16^{INK4a}$ expression, inducing senescence and preventing cancer formation, while KRASG12D repressed almost completely $p16^{INK4a}$ expression (Lee and Bar-Sagi, 2010). In addition, $p16^{INK4a}$ transcription could be regulated by RAS-activated transcription factors. In particular, activated RAF/ERK cascade phosphorylates and activates c-JUN, a component of AP-1, which in turn modulates $p16^{INK4a}$ expression (Passegue and Wagner, 2000). On the other side, also $p16^{INK4a}$ has been shown to affect oncogenic $KRAS^{G12V}$. Introduction of active $p16^{INK4a}$ in cells in which $p16^{INK4a}$ was not working restored its function and lowered selectively KRAS protein level by decreasing protein stability (Rabien *et al.*, 2012). Moreover, the simultaneous expression at normal level of $p16^{INK4a}$ and $KRAS^{G12V}$ caused the cell to become resistant to anoikis and highly clonogenic, while both these features did not occur in cells expressing $p16^{INK4a}$ only, showing that the suppression of oncogenic KRAS is necessary for $p16^{INK4a}$ to exert its anti-proliferative function (Rabien *et al.*, 2012).

Interestingly, a relationship between $p16^{INK4a}$ and gal-3 seems to exist as well. Upon $p16^{INK4a}$ expression, a clear decreased in gal-3 level was observed both *in vitro* and *in vivo*. In particular, $p16^{INK4a}$ diminished the expression of gal-3 and its consequent cell-surface presentation. The mechanism by which $p16^{INK4a}$ decreases gal-3 seems to be mediated by mRNA reduction, due more to post-transcriptional processes than to diminished *de novo* synthesis (Sanchez-Ruderisch *et al.*, 2010). On the other hand, overexpression or forced expression of $p16^{INK4a}$ causes enhanced gal-1 mRNA *de novo* synthesis and overexpression of gal-1 protein levels. In detail, most of the gal-1 newly produced seems to be re-located on the cell surface (Andre *et al.*, 2007). In addition to changing the expression levels of gal-3 and gal-1 in opposite directions, $p16^{INK4a}$ strongly influences the glycomic profile of pancreatic cancer cells (Andre *et al.*, 2007; Amano *et al.*, 2012). $p16^{INK4a}$ provokes variations in the expression of β1,4-galactosyltransferases and consequent downregulation of α2,3-sialylation of O-glycans and α2,6-sialylation of N-glycans (Andre *et al.*, 2007). This is consisted with the decrease of two enzymes of the sialic acid biosynthesis pathway observed in cells overexpressing $p16^{INK4a}$ (Amano *et al.*, 2012). Moreover, $p16^{INK4a}$ overexpression triggers an increase in the cell-surface presentation of fibronectin receptor (Plath *et al.*, 2000; Andre *et al.*, 2007). Considering the previously described roles of galectins and plasma membrane glycans in cell anchorage, it is conceivable to expect that this changes in glycomic profile and galectins expression induced by $p16^{INK4a}$ might cause different sensitivity to anoikis, the specific cell death induced by loss of attachment. Indeed gal-1 expression correlates with enhanced induction of anoikis (Andre *et al.*, 2007), whereas gal-3 significantly decreases the level of anoikis (Sanchez-Ruderisch *et al.*, 2010). Therefore $p16^{INK4a}$ can control anoikis induction by regulating the level of specific glycans on the plasma membrane necessary for cell attachment and the level of galectins necessary to mediate this attachment. In this model, $p16^{INK4a}$ acts as tumour suppressor by promoting anoikis, downregulating gal-3 and upregulating gal-1 (Andre *et al.*, 2007; Sanchez-Ruderisch *et al.*, 2010). Gal-3 actively competes with gal-1 for binding sites, decreasing gal-1 pro-anoikis effects, especially by diminishing caspase-8 activation (Sanchez-Ruderisch *et al.*, 2010).

In conclusion, it has been observed that $p16^{INK4a}$ indirectly competes with both KRAS

and gal-3 on different processes, such as cell cycle progression and anoikis, and often this competition correlates with reciprocal changes in protein and/or mRNA level.

6. YEAST AS A MODEL TO STUDY HUMAN PROTEINS

The study of model organisms, among which a special position is reserved to *S. cerevisiae*, has always been fundamental for biological discoveries and even now, with more advances techniques that allow the investigation of more complicated organisms such as human itself, it continues to be extremely useful (Fields and Johnston, 2005; Mager and Winderickx, 2005; Botstein and Fink, 2011). The reasons for that are several. Almost thirty years ago it was already clear that yeast special role as model organism was due to "the facility with which the relation between gene structure and protein function can be established" (Botstein and Fink, 1988), and from then several other reasons have risen, together with new technologies that utilize yeast. Importantly, in 1996 the complete genome of *S. cerevisiae* was the first eukaryotic genome to be sequenced and published (Goffeau *et al.*, 1996) and continuous updates were made (Engel *et al.*, 2014). In addition, an almost complete set of deletions of every open reading frame is available in yeast (Winzeler *et al.*, 1999; Giaever *et al.*, 2002), together with a collection of GFP-fused chimera proteins that helps localize endogenous yeast proteins (Ghaemmaghami *et al.*, 2003; Huh *et al.*, 2003) and an extensive database that gives detailed informations about every yeast gene, the Saccharomyces Genome Database (http://www.yeastgenome.org/). Moreover, in yeast there is a well-established and relatively simple methods to introduce gene mutations, which allows the discovery of the biochemical function of the analysed gene and the outcomes of the failure of that gene (Botstein and Fink, 2011). The availability of all the above-mentioned resources made possible to uncover the role of almost 85% of the 5800 protein-coding genes of *S. cerevisiae*, with important repercussion on the biology of other organisms, including human. When comparing yeast and other organism genomes, it is clear that both amino acid sequences and protein functions are conserved. Approximately 17% of yeast genes are

members of orthologous gene families associated with human diseases (Heinicke *et al.*, 2007) and, conversely, 30% of known genes involved in human disease have yeast orthologues, which can substitute for yeast gene function (Foury, 1997). For obvious reasons, including the simpler system that this unicellular organism provides, the easier manipulation and even the cheapest maintenance in laboratories, in addition to the above-mentioned resources, it has always been more practical to identify a protein function starting from yeast and later confirming it in higher eukaryotes that the other way around.

In addition to an important role in identifying genes and proteins functions, yeast has been chosen to test and validate new emerging technologies, mostly because the availability of prior data capable of confirming the newly acquired information (Botstein and Fink, 2011). Thereby, yeast was the first model organism in which methods that detects the mapping of the binding sites of transcription factors by chromatin precipitation followed by DNA microarray- ChIP-chip- (Iyer *et al.*, 2001; Lieb *et al.*, 2001) or by directing sequencing of the bound DNA- Chip-seq- (Robertson *et al.*, 2007) were tested, as well as methods that allow genome-wide assessment of translation rates or mRNA stability (Wang *et al.*, 2002b; Ingolia *et al.*, 2009). Yeast has also been the base for genome-wide experiments that integrate functional data using bioinformatics methods, creating putative integration networks (Myers *et al.*, 2005; Hibbs *et al.*, 2007). Also the experiments based on synthetic lethality have been extended to genome-scale with the synthetic gene array (SGA) analysis. Synthetic lethality is the phenomenon by which the deletion of two different genes causes yeast lethality, when none of the two gene deletions being lethal on its own, indicating that they cooperate in the same signalling pathway and/or have similar biological function. *S. cerevisiae*, due to the ease in which deletions are made, is an elective model organism for this type of studies (Novick *et al.*, 1989). SGA analysis allows the simultaneous study of an enormous number of genes and their respective interactions (Tong *et al.*, 2004; Costanzo *et al.*, 2010). Protein-protein interactions have been studied using yeast also with the two-hybrid method (Fields and Song, 1989), which also has been expanded to genome-scale analysis (Uetz *et al.*, 2000; Ito *et al.*, 2001; Fields, 2009). In addition, in yeast it is easier to perform co-immunoprecipitation of protein complexes, in which proteins can be tagged to facilitate the subsequent precipitation

(Krogan *et al.*, 2006).

It is clear from what written above that yeast provides a very useful model organism for the study of several aspect of biology. In particular, the fact that many of the proteins functions discovered in yeast can be then translated in higher eukaryotes is relevant in the study of proteins involved in medical disorders. At a first sight, it might sound that yeast proteins have little to do with processes implied in human diseases, however, some of this processes can be even better analysed in the simpler environment of yeast cells. Some examples can be the misfolding aggregations of proteins implicated in neurodegenerative disorders such as Parkinson's disease, Huntington's disease and Alzheimer's disease (Fields and Johnston, 2005; Mager and Winderickx, 2005; Zabrocki *et al.*, 2005), or the mechanisms underlying aging (Fields and Johnston, 2005). Of simpler understanding is the usage of *S. cerevisiae* in the study of the pathogenic yeast *C. albicans*, since both organisms are unicellular fungi.

In addition to study yeast protein functions and then translate them into higher eukaryotes, a different approach can be taken to directly understand the role of human proteins, the creation of humanized yeast, by expressing heterologous proteins in yeast (Laurent *et al.*, 2016) (**Fig. 19**). This method is particularly helpful in the discovery of human protein functions, especially when associated with a disease, in a "neutral" environment, which can facilitate the isolation of the protein function. It is also useful to determine the outcome of protein mutations (Botstein and Fink, 2011). The expression of human proteins in yeast can also be instrumental for the discovery of chemical or protein inhibitors in high-throughput screenings. These assays are based on the fact that the heterologous expressed human proteins might cause growth defects in yeast, which can be suppressed by chemical compound or by the expression of a second human protein (Mager and Winderickx, 2005).

Yeasts have also been humanized with genes that do not have a functional counterpart in yeast. This approach has been particularly useful in the study of neurological disorders such as Huntington's disease and Parkinson's disease (Outeiro and Lindquist, 2003; Willingham *et al.*, 2003; Outeiro and Muchowski, 2004; Zabrocki *et al.*, 2005).

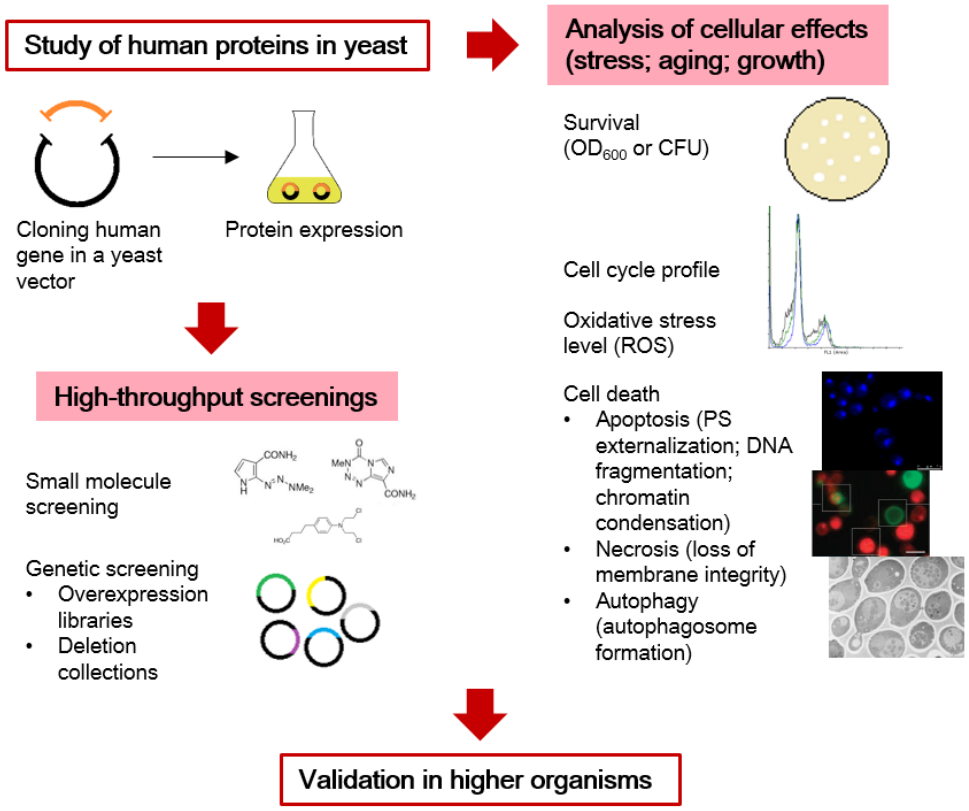


Figure 19: *Schematic representation of various methodologies in which humanized yeasts are used*. Yeast is transformed with expression vectors harbouring a human gene and then used in a variety of applications. It can be used in (i) high-throughput studies to screen small molecules that function as inhibitors or activators of the human protein expressed in yeast, (ii) to test other human molecules that influence the human protein behaviour equally expressed in yeast, or (iii) to test the influence on the cloned human protein-derived phenotypes of yeast genes deletions. Humanized yeasts can also be used in small-scale experiments, to test the effect of the expressed human protein on various yeast features, such as viability, cell cycle, and cell death (adapted from Pereira *et al.*, 2012; autophagic vacuole from Takeshige *et al.*, 1992).

6.1 Focus on drug screening and cancer

Many of the above mentioned resources available in yeast have been exploited for the testing of chemical agents, including pharmaceutical compounds, toxic agent and chemotherapeutics (Barberis *et al.*, 2005), and for uncovering pathways relevant for transformants cells adaptation and cancer progression. For example, the collections of yeast deletion mutants have been used to identify genes involved in UV sensitivity, whose human orthologues may be associated with cancer development (Birrell *et al.*, 2001), and have also been screened for drug effects, for example to test sensitivity and/or resistance to the common anticancer drug bleomycin (Aouida *et al.*, 2004). Another useful test performed using the deletion collections is the so-called "fitness-profiling" approach, in which a deletion strain is grown competitively with the wild type strain in the presence of specific chemical compounds. In this way it is possible to determine the fitness of the mutant strain and to confirm known targets of the drug, as well as identify new ones (Lum *et al.*, 2004; Mager and Winderickx, 2005). A slightly different approach is the one of the haplo-insufficiency screening. In this case diploid yeasts are used, in which only one out of two gene copies is deleted. The deletion does not cause a phenotypic effect by itself, but it may create one when the yeast is grown in the presence of a specific drug or toxin (Giaever *et al.*, 1999). With this method novel targets of known inhibitors have been identified, as well as targets of new drugs (Giaever *et al.*, 1999; Giaever *et al.*, 2002; Baetz *et al.*, 2004). A way to discover new target gene of a specific compound is a derivation of the synthetic lethality assay described earlier. In this particular case, instead of combining two gene deletions, one deletion is combined with a drug known to act on a different gene target. The yeast resulting unviable after the drug treatment most certainly carries a mutation in a gene involved in the same pathway and/or biological process of the gene targeted by the drug used (Mager and Winderickx, 2005). Importantly, all the described methodologies can be performed on a genome-wide scale, enabling the fast discovery of numerous data, which can be further confirmed in more detailed experiments (Mager and Winderickx, 2005; Botstein and Fink, 2011). In particular, a specific instrument exists for high-throughput analysis of yeast genes

involved in drug resistance and/or sensitivity, the molecularly barcoded yeast open reading frame (MoBY-ORF) collection. The MoBY-ORF contains approximately 5000 yeast genes cloned in expression plasmids flanked by upstream and downstream barcodes that allow their fast identification, making necessary the usage of a smaller amount of drug (Laurent *et al.*, 2016). Of course all the techniques described above that use yeast deletion mutant collections can be similarly performed using gene overexpression, equally easy to achieve in yeast. Also in this case it is possible to identify genes involved in pathway relevant for human diseases (Laurent *et al.*, 2016).

A further step into drug screening is the usage of humanized yeast. The heterologous expression of human proteins in yeast, even if lacking a functional orthologous in yeast, was described earlier as a way to better understand the role and function of the protein analysed. But of course this methodology can be employed in drug screening. If the insertion and expression of human cDNA in yeast is capable to cause a distinct and measurable phenotype, then the yeast can be screened for drug or protein that can revert that phenotype, finding in this way inhibitors of specific protein and cellular processes (Sekigawa *et al.*, 2010; Laurent *et al.*, 2016). One example in this way is the discovery of inhibitors of poly (ADP-ribose) polymerase (PARP) family of proteins, involved in breast and ovarian cancer (Telli, 2011). Yeast has no PARP homolog, but it shows decreased viability when human *PARP1* or *PARP2* are expressed, that was found to be rescue by two of the 16000 compounds tested (Perkins *et al.*, 2001). Even if this approach to discover new drug target and to test novel drug in yeast may seem highly cost and time effective, it must be taken into account that not all human genes are capable to trigger a phenotype in yeast, and among those which do cause variation, not all provoke phenotypes easy to monitor and test. Besides expressing human genes with no relation to yeast, it is also possible to mutate yeast proteins with human orthologues to make them more similar to their human counterparts, by changing specific sequence (Miller and Kumar, 2001), or directly expressed the human homologous in yeast. This last approach was actually one of the first attempts to humanize yeast and it was initially used to identify human proteins that could functionally replace yeast deletion. Now it is often used to study the human protein in a different context, to better identify the outcomes

of protein mutations and to isolate inhibitors of the protein (Mager and Winderickx, 2005; Laurent *et al.*, 2016).

Importantly, many of the above mentioned methods have been applied in the study of proteins important in cancer pathogenesis (2007; Pereira *et al.*, 2012) and in the discovery and testing of anticancer agents (Simon and Bedalov, 2004; Menacho-Marquez and Murguia, 2007; Matuo *et al.*, 2012; Guaragnella *et al.*, 2014). In particular, several cellular processes important in cancer onset and progression, such as apoptosis and cell growth, have been analysed in yeast, taking into consideration the main proteins involved. *S. cerevisiae* has been used in the study of proteins involved in apoptosis, such as the caspase family proteins and the Bcl-2 family proteins. In the case of caspase-3 and -7, yeast has been useful in identifying their binding partners through the two-hybrid system (Kamada *et al.*, 1998; Araya *et al.*, 2002). Moreover, the heterologous expression of caspases in yeast greatly helped the uncovering of their activation mode, especially in the case of those caspases that exist in a pre-active state (Kang *et al.*, 1999). Importantly, high expression level of caspases in yeast cause severe growth defects and this phenotype facilitates the identification, also in high-throughput scale, of caspase natural inhibitors, such as IAPs and p35 (Kang *et al.*, 1999; Wright *et al.*, 2000), and chemical compounds inhibitors and activators (Hayashi *et al.*, 2009). Similarly to the caspases, also members of the Bcl-2 family have been studied in yeast. As mentioned earlier, Bcl-2 family members have fundamental roles in apoptosis in mammals, covering both pro- and anti-apoptotic functions. Yeast has been extremely useful to identify Bcl-2 members interaction and their regulators. Important for these studies is the fact that a pro-apoptotic member, Bax, is capable to induce death in yeast. Screening for its inhibitors led to the discovery of Bax inhibitor 1, HMGB1, bifunctional apoptosis regulator and Calnexin orthologue Cnx1 (Torgler *et al.*, 1997; Xu and Reed, 1998; Zhang *et al.*, 2000b; Zhang *et al.*, 2000a; Brezniceanu *et al.*, 2003), and other inhibitors belonging to the same Bcl-2 family, such as Bcl-2 and Bcl-X_L (Sato *et al.*, 1994). In addition, yeast has been used to better analyse the post-translational modifications necessary for proper mitochondria targeting and regulation of cyt *c* release (Arokium *et al.*, 2007). Considering the role of apoptosis in cancer progression, the screening for new drugs targeting caspases and Bcl-2

family members, and thereby apoptosis, may be useful to isolate new cancer therapeutic. The research on fundamental proteins for cancer using yeast has been particularly focused on p53, one of the most important among these proteins. p53 is mutated in approximately 50% of human cancer and, when not mutated, often other proteins involved in its pathways are non-functional (Cheok *et al.*, 2011). There are no orthologues of p53 in yeast, but this protein can maintain most of its activity as transcription factor also in *S. cerevisiae* (Fields and Jang, 1990; Scharer and Iggo, 1992; Yousef *et al.*, 2008). Besides the conserved function, p53 can cause a mild decrease in growth of yeast (Nigro *et al.*, 1992; Mokdad-Gargouri *et al.*, 2001). All these factors have been extensively exploited to better analyse the function and regulation of p53. For example, p53 regulation by redox level and thioredoxin reductase were first discovered in yeast and then confirmed in mammals (Pearson and Merrill, 1998; Hu *et al.*, 2001). Also the importance of p53 mutations for pathogenesis has been analysed in yeast, facilitated by the amenability of yeast high-throughput assay. All the mutations of p53 representing amino acids substitutions were expressed in yeast and tested for various aspects, including the ability to activate proteins involved in cell cycle and apoptosis (Di Como and Prives, 1998; Robert *et al.*, 2000; Kato *et al.*, 2003). The status of p53 in tumour samples was also evaluated using yeast, through the functional analysis of separate alleles in yeast (Ishioka *et al.*, 1993; Fronza *et al.*, 2000).

6.2 Focus on RAS pathway and galectins

In the group of the above-mentioned pathways relevant for cancer progression that have been extensively studied in yeast, an important position is covered by RAS pathway. As described above, yeast and human RAS proteins share a high degree of homology (DeFeo-Jones *et al.*, 1983; Dhar *et al.*, 1984; Powers *et al.*, 1984). However, they do not activate the same downstream pathways, even if the resulting effects, as described above in detail, are often very similar, with cell cycle progression and consequent growth and cell death inhibition (**Fig. 20**). Interestingly, in the different yeast *Schizosaccharomyces pombe*, RAS

protein activates a kinase signalling cascade very similar to the one found in mammals (Masuda *et al.*, 1995).

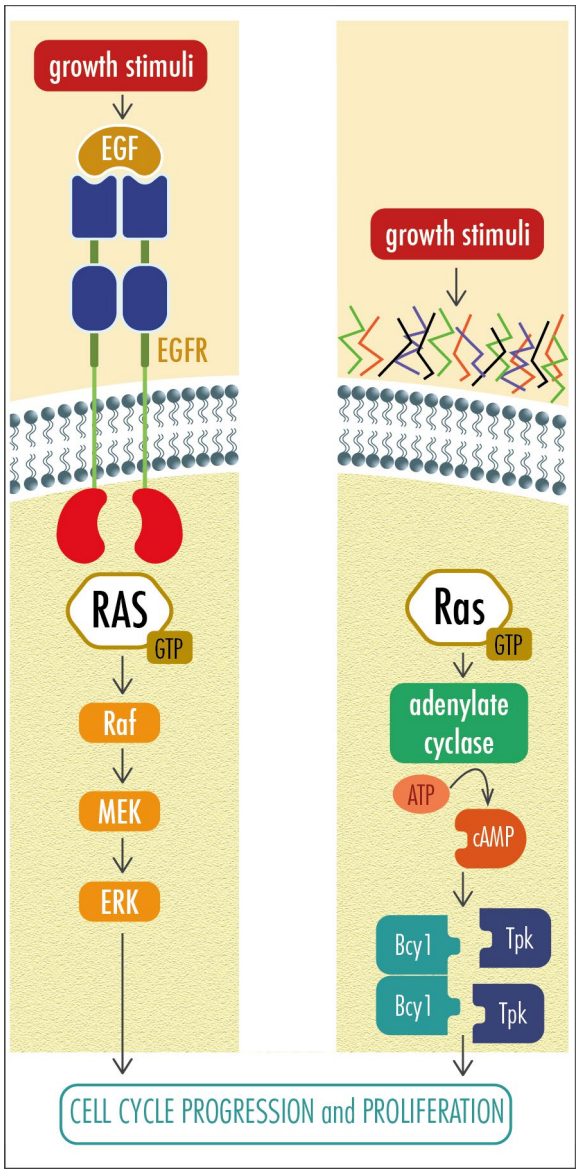


Figure 20: *Comparison between human and yeast RAS pathways*. Both human and yeast RAS pathway are activated upon growth stimuli and ultimately lead to cell cycle progression and cell proliferation. However, the downstream proteins activated by human and yeast RAS are different. Human RAS activates various downstream targets, among which one of the most well-studied is RAF/MEK/ERK cascade, whereas yeast Ras proteins activate adenylate cyclase, which produce cAMP. This second messenger activates PKA, sequestering the inhibitory subunit Bcy1 and releasing the catalytic subunit Tpk.

The study of the yeast Ras pathway brought during the years great insight in the understanding of the mammalian RAS pathway. As examples, yeast was very useful to understand that Ras proteins, both mammalian and yeast, need a post-translational processing in order to translocate to the plasma membrane (Clark *et al.*, 1985). Moreover, it should be noticed that the first Ras effector, adenylate cyclase, and the first GEF protein, Cdc25, were identified in yeast (Toda *et al.*, 1985; Broek *et al.*, 1987; Robinson *et al.*, 1987). These discoveries, especially the one of Cdc25, greatly helped the uncovering of similar

proteins in other organisms based on homology (Bowtell *et al.*, 1992; Shou *et al.*, 1992; Wei *et al.*, 1992). In addition to GEF proteins, yeast has been useful to deepen the understanding of the other class of Ras regulators, GAP. Indeed, the functions of *NF1* gene, whose mutations are responsible for neurofibromatosis type 1, were clarified thanks to the similarity with *IRA* genes of *S. cerevisiae*, especially found in the region appointed to the GTPase activity (Xu *et al.*, 1990). Importantly, expression of *NF1* in yeast can suppress the phenotypes caused by *IRA* genes deletions, such as heat shock sensitivity (Gibbs *et al.*, 1988), showing that mammalian and yeast GAPs are interchangeable and highlighting the similarity between yeast and mammalian Ras proteins. As further confirmation, also mammalian RAS proteins can complement for yeast deficient Ras and *vice versa*. In the middle '80s, several studies showed that human activated HRAS could suppress the lethality of simultaneous *RAS1* and *RAS2* deletion in yeast, because of its equal capacity to activate yeast adenylate cyclase (Broek *et al.*, 1985; Clark *et al.*, 1985; DeFeo-Jones *et al.*, 1985; Kataoka *et al.*, 1985; Toda *et al.*, 1986). Conversely, it was discovered that a yeast-mammalian hybrid gene expressed in mouse cells had the ability to induce morphological changes extremely similar to those induced by mammalian oncogenic HRAS (DeFeo-Jones *et al.*, 1985). In addition, a mutant variant of yeast Ras protein that resembles oncogenic HRAS was also identified, Ras^{val19}, which was capable to differentially activate adenylate cyclase (Broek *et al.*, 1985; Toda *et al.*, 1986). The functional complementation of *RAS2* deletion in yeast by human *HRAS* was later showed also for different *Δras2* induced phenotypes, such as temperature-sensitive growth and temperature-dependent depolarization of the actin cytoskeleton (Ho and Bretscher, 2001).

More recently, the similarities between human and yeast Ras proteins have been exploited for the study of autophagy in a humanized yeast model (Alves *et al.*, 2015). Autophagy is a highly conserved metabolic process that the cells use to recycle their components to maintain cellular homeostasis (Yang and Klionsky, 2010). This process is involved in cancer on a double front. On one side, autophagy can weaken the cells by degrading their components, but on the other side, the recycling of peptides makes the cells less sensitive to nutrient depletion and therefore death. Autophagy has been implicated in the resistance of tumours to

chemotherapy (Chen and Karantza, 2011). Importantly, autophagy is conserved also in yeast and RAS proteins have been involved in autophagy regulation (Furuta *et al.*, 2004; Elgendy *et al.*, 2011; Guo *et al.*, 2011; Kim *et al.*, 2011a; 2011b; Lock *et al.*, 2011). The humanized yeast in this case helped determine the role of mutated and wild type KRAS in the autophagic process. Yeast lacking *RAS2* was transformed with either *KRASwt* or mutant (*KRAS*^{G13D}, *KRAS*^{G12D}, *KRAS*^{G12V}) and the level of autophagy was measured using the endogenous yeast autophagic pathway (Alves *et al.*, 2015). This experiment confirms that human RAS proteins are functional in yeast and that they can activate yeast proteins involved in processes similar to the ones activated in human. Yeast is therefore an excellent model to study human RAS proteins and downstream pathway.

Contrarily to RAS proteins, galectins orthologues do not exist in yeast (Kasai and Hirabayashi, 1996; Dodd and Drickamer, 2001). However, as seen before in the case of other proteins, one for all p53, this must not be discouraging. On the contrary, it appears clear from the previous descriptions that yeast can provide a useful model to better understand function, localization and binding partners of human proteins without yeast orthologues, acting as a blank slate. Even if galectins have never been transformed in yeast to study their physiological function, a previous study reports yeast successful transformation with galectins, later exposed on the outer leaflet of the cell (Ryckaert *et al.*, 2008). This study employed yeast in a yet undescribed function, considering this introduction. The purpose of galectins expression and following surface translocation was to mimic the glycan-binding proteins distribution on a physiological and living surface, which resembles protein clusters and membrane microdomains in a much better way compared to a synthetic surface (Ryckaert *et al.*, 2008).

In conclusion, yeast can provide a useful model in the study of human RAS proteins because of the functional conservation of Ras proteins roles. In particular, yeast can be used to analyse cancer-related processes in which RAS proteins are involved, with a specific focus on KRAS interaction with gal-3. The fact that gal-3 does not have an orthologue in yeast should be seen as an advantage, since this model could provide a “clean” environment in which gal-3 function and relationship with KRAS might be studied without the interference of redundant pathways.

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Aims and Thesis Outline

The use of humanized yeast to better understand and study the role of human proteins has been well established. Usually the discoveries made in *S. cerevisiae* regarding human proteins are subsequently translated and confirmed in higher eukaryotes. Among the proteins studied in yeast, in this work a special focus has been directed to the RAS protein family, namely KRAS, which has been extensively characterized both in yeast and human cells. An important partner/regulator of the most studied human RAS protein, KRAS, is galectin-3, a carbohydrate binding protein belonging to the galectin family. Gal-3 has been involved in a variety of different cellular processes, from cell-cell adhesion to cell cycle progression and apoptosis evasion. The relationship between KRAS and gal-3 is particularly relevant for cell growth and survival, and has a pivotal role in the progression of different cancer types.

The work presented in this thesis was in the scope of a wider network, the Marie Curie Project GLYCOPHARM, whose general objective was the "*development of innovative therapeutic agents and strategies, new diagnostic/prognostic tests and new methodologies, e.g., for drug screening. GLYCOPHARM focuses on a family of endogenous lectins, the galectins, which play key roles in many clinically relevant processes, including cancer and the immune and inflammatory processes*" (www.glycopharm.eu).

The general aim of this thesis was to deepen the knowledge and understand the functions of galectins, in particular gal-3. The work performed examines both the role of galectins in the extracellular environment, executed through their CRDs, and the intracellular roles of gal-3, mainly occurring through protein-protein interaction. The specific aims of the thesis were to i) study of the behaviour and effects of extracellular galectins and to ii) analyse intracellular gal-3 and its interaction with RAS proteins.

For the sake of simplicity, the thesis was divided in 4 chapters according to the specific aims established. The study of the behaviour and effects of extracellular galectins is described in chapters 1 and 2, and the analysis of intracellular gal-3 and its interaction with RAS proteins is described in chapters 3 and 4:

- Chapter 1: MOLECULAR RESPONSE OF YEAST TO HUMAN GALECTINS.

  The effects of the presence of exogenous galectins (gal-3, gal-4, gal-7 and gal-1) on the yeast *S. cerevisiae* and *C. albicans* were evaluated.

- Chapter 2: MICROARRAY-BASED STUDY ON THE BINDING INTERACTION BETWEEN GALECTINS AND YEAST CELL SURFACE

  The binding ability of various galectins, the same used in assessing the effects of exogenous galectins on yeasts, to the surface of *S. cerevisiae* and *C. albicans* was determined.

- Chapter 3: CONSTRUCTION OF A *S. CEREVISIAE*-BASED PLATFORM EXPRESSING HUMAN GALECTIN-3 AND KRAS

  A *S. cerevisiae*-based platform expressing the human protein KRAS and gal-3, both alone and in combination was built. To operationalize the platform, the behaviour of these proteins expressed in yeast was investigated, so that specific yeast phenotypes were identified, exploitable for further screening KRAS and gal-3 interacting drugs and proteins.

- Chapter 4: UNCOVER THE ROLE OF KRAS/GALECTIN-3/$p16^{INK4a}$ AXIS REGULATION IN COLORECTAL CANCER.

  KRAS/gal-3 interaction and its role in in colorectal cancer survival was unveiled. Special attention was given to the mutual interplay between KRAS, gal-3 and the tumour suppressor protein $p16^{INK4a}$.

To achieve these goals, two different yeasts species were used, *S. cerevisiae* and *C. albicans.* Moreover, two *S. cerevisiae* genetic backgrounds were necessary (BY4741 and W303-1A) to assess the significance of the results, since yeast genetic background often impacts on cell responses/phenotyping. The human proteins gal-3 and KRAS were cloned in yeast through specific expression vectors. *S. cerevisiae* wt strains and mutants deleted for either *RAS1* or *RAS2* genes were used as recipients. For the experiments done in human cells, mostly the CRC-derived cell line SW480 was used. Immunofluorescence was performed in the immortalized normal epithelial colon derived cell line NCM460 transformed with $FlagKRAS^{wt}$.

The work intended first and foremost to build a yeast-based platform expressing human gal-3. For that, it was mandatory to unveil the responses of yeasts to human gal-3 expressed intracellularly, and compare those with the effect of the same galectin when added extracellularly, as well as with the effects of other galectins representing the different structural families. Moreover, the yeast-based platform was built bearing in mind the aim of expressing gal-3 simultaneously to KRAS. For that purpose, KRAS was also expressed in yeast and phenotyped. Simultaneously, human cells were assayed to verify the role of gal-3/KRAS/$p16^{INK4a}$ interaction, using as model CRC cells. The platform, once finished, will enable screening for clinically relevant gal-3 and/or KRAS antagonistic drugs or proteins.

Chapter 1

Molecular Responses of Yeasts to Human Galectins

Abstract

Galectins are high affinity β-galactosides-binding lectins that share a conserved carbohydrate-binding domain. In humans 15 galectins were described, differing in specificity, protein structure and ability to multimerize. The most studied is galectin-3, which is ubiquitously expressed, both inside and outside the cell and varies in role depending on the subcellular localization, the cell type and the proliferation state. Extracellular gal-3 mediates the recognition of specific carbohydrate patterns that are important for cell adhesion and morphology and for self-molecules discrimination during the immune response. Moreover, gal-3 has a role in the recognition of various pathogens, including *Candida albicans*.

This study analyses the biological responses of the yeast *Saccharomyces cerevisiae* to the contact with human gal-3. For comparison, gal-1, gal-4 and gal-7 were used. Also the non-galectin lectin Concanavalin A (ConA) was used as control. The assays were done in parallel in a strain of *Candida albicans*. Parameters associated with viability/cell cycle progression, as well as apoptotic/necrotic cell death were assessed.

Every galectin caused a pattern of effects that varied in type and intensity, depending on the yeast specie. This suggests that each galectin may interact with a different ligand, putatively located on the yeast cell surface, able to transduce the signal into the cell and stimulate a different response pathway. The highly mannosylated proteins located on the outer cell wall are probable candidates. Gal-3 showed to affect *S. cerevisiae* more than the other galectins, decreasing cell viability and increasing cell size and reactive oxygen species (ROS) production, but not causing an apoptotic-like death. The effects of gal-3 on *S. cerevisiae* appeared to be mostly mediated by the carbohydrate recognition domain (CRD) of the galectin, since the yeast phenotype was maintained when using truncated gal-3 (without the CRD). The same was not observed using *C. albicans*. The further use of *S. cerevisiae Δras2* mutants suggested that the effects observed depended on the RAS/cAMP/PKA pathway, which is known to be responsible for cell cycle progression, cell size and proliferation.

Introduction

The galectin family of lectins, or S-type lectins, is characterized by a conserved peptide sequence element of about 130 amino acids in the carbohydrate recognition domain (CRD) that confers them high affinity and specificity for β-galactosides N- or O-linked to proteins (Barondes *et al.*, 1994). Until now, 15 mammalian galectins have been discovered. They are subdivided into three groups according to their structure. The *proto-type* group includes gal-1, gal-2, gal-5, gal-7, gal-10, gal-11, gal-13, gal-14 and gal-15 and is characterized by a unique CRD with the ability to act as monomer or in complexes (Hirabayashi and Kasai, 1993; Römer and Elling, 2011). The *tandem-repeat* type includes gal-4, gal-6, gal-8, gal-9 and gal-12, and is defined by the presence of two distinct CRDs connected by a linker polypeptide (Hirabayashi and Kasai, 1993; Römer and Elling, 2011). The *chimera* type comprises a single member, gal-3, whose peculiarity is to include two distinct domains: a CRD at the C-terminal and a non-carbohydrate-binding arm at the N-terminal, atypically long (Hirabayashi and Kasai, 1993; Rabinovich, 1999) and rich in proline and glycine (Hsu *et al.*, 1992; Agrwal *et al.*, 1993). One of the main characteristic of all galectins, regardless their subtype, is the ability of cross-linking. *Tandem-repeat* type galectins can cross-link their targets thanks to the presence of two CRDs, while *proto-type* galectins and *chimera* type gal-3 form multivalent complexes. In particular, gal-3 can form multivalent chimeras through its N-terminal domain. These complexes increase the avidity of the galectins binding to their ligands, which compensates the affinity, lower compared to protein-protein interactions (Brewer, 2002; Compagno *et al.*, 2014).

Galectins are not membrane-bound like other lectins. They are present in the soluble state (Ho and Springer, 1982) in different localizations, both inside and outside the cells (Hughes, 1999). Accordingly, they have multiple biological roles that depend on their localization. Intracellularly, they are involved in signalling, regulation of RNA splicing and apoptosis. When localized outside the cells, they act on cell-cell adhesion and they are important player in immunity defense. They can serve as pathogen recognition receptors (PRRs), which specifically bind to pathogen-associated molecular patterns (PAMPs); they can

bind to self glycans, stopping or attenuating the immune response, or they can work as damage-associate molecular patterns (DAMPs), specific molecules released from the cell upon non-programmed cell death, which regulate innate immunity by activating immune cells (Perillo *et al.*, 1998; Cooper and Barondes, 1999; Liu *et al.*, 2002; Yang and Liu, 2003; Boscher *et al.*, 2011; Compagno *et al.*, 2014).

Gal-3 is the only *chimera* type member and the most studied among the galectins identified in humans. The N-terminal domain is essential for the biological activity of gal-3 because it promotes oligomerization and consequent multimers formation (Massa *et al.*, 1993) and it is involved in gal-3 secretion outside the cell (Menon and Hughes, 1999). Gal-3 has multiple ligands, which differ in both structure and function. The preference of gal-3 for a given ligand depends primarily on sugar-binding affinity and on the lectin phosphorylation status, but also on the coordinate expression of gal-3 and the ligand (Sato and Hughes, 1992; Hirabayashi and Kasai, 1993). The transcription of gal-3 encoding gene, *LGALS3* (Raimond *et al.*, 1997) is multifactorially modulated, namely according to the proliferation state of the cell (Moutsatsos *et al.*, 1987), the presence of infectious agents (Elliott *et al.*, 1991) or tumour onset and progression (Hebert and Monsigny, 1994). The biological roles of gal-3 are defined by the localization of the galectin and the cell type where it is expressed (Dumic *et al.*, 2006). Gal-3 is ubiquitously localized, but it is found in particular in epithelial cells and myeloid and amoeboid cells. Importantly, gal-3 is highly expressed in a variety of tumours and its expression and localization within the cancer cell vary from the healthy equivalent (Cay, 2012). Gal-3 is synthetized in the cytoplasm, but it can localize also in the nucleus, at the cell membrane and in the extracellular matrix. In the nucleus, gal-3 is associated with the ribonucleoprotein complexes (Laing and Wang, 1988), being involved in the spliceosome assembly and in pre-mRNA splicing (Dagher *et al.*, 1995). Cytoplasmic gal-3 is involved in various intracellular events, such as the regulation of apoptosis, through the interaction with key proteins like Bcl-2 (Yang *et al.*, 1996; Fukumori *et al.*, 2006), CD95 (Fukumori *et al.*, 2004), Alix (Liu *et al.*, 2002), Annexin VII (Yu *et al.*, 2002) and Nucling (Liu *et al.*, 2004). Additionally, gal-3 is involved in the regulation of cell proliferation and differentiation, influencing cancer progression, through the interaction with the oncoprotein KRAS (Shalom-Feuerstein *et al.*,

2005; Ashery *et al.*, 2006; Shalom-Feuerstein *et al.*, 2008; Abankwa *et al.*, 2010; Bhagatji *et al.*, 2010; Levy *et al.*, 2010; Levy *et al.*, 2011; Song *et al.*, 2012).

Once secreted in the extracellular space through the leaderless (or alternative) secretory pathway, gal-3 is mainly involved in innate immunity. Gal-3 binds primarily to self-glycans, *i.e.* glycans present on the surface of the cells of the organism that produces the galectin (Mandrell *et al.*, 1994). When gal-3 cross-links various glycans on the same cell, it forms two- and three-dimensional complexes called lattices (Lee and Lee, 2000; Sacchettini *et al.*, 2001), which cover large areas of the cell surface due to a high steric dimension. Lattices noticeably reduce the lateral mobility of the other proteins present on the cell surface, including the receptor responsible to activate the pathway of the innate immunity, raising the threshold for ligand-dependent receptor clustering, this way impeding or reducing signal transduction (Sacchettini *et al.*, 2001). Gal-3 has also the ability to bind to non-self glycans, *i.e.* glycans present on the surface of pathogens or other microorganisms. In this case, the response to gal-3 varies. Gal-3 can actively reduce cell proliferation or even kill the pathogen, as in the case of its direct bacteriostatic activity towards *Streptococcus pneumonia* (Farnworth *et al.*, 2008). When gal-3 binds to the O-antigen of *Helicobacter pylori* or to the LacdiNAc glycans of *Schistosoma mansoni*, it enhances the recruitment of the immunity cells (Lim *et al.*, 2003; Huff *et al.*, 2004; van den Berg *et al.*, 2004). In opposition, gal-3 defensive activity can be subverted by the microorganisms, which may take advantage of the galectin cross-linking ability to adhere and invade the host cells. Examples of this behaviour are the parasite *Trypanosoma cruzi* (Vray *et al.*, 2004), the virus *Herpex simplex* (Woodward *et al.*, 2013) and the bacterium *Neisseria meningitidis* (Quattroni *et al.*, 2012).

It has been reported that gal-3 can bind to glycans on the wall of the human commensal and pathogenic yeast *Candida albicans*. This yeast, as well as other yeast species from the same genus, like *C. parapsilosis, C. glabrata* or *C. dublinensis*, are very common human pathogens. Although most of these species are commensal, their outburst affects mucocutaneous surfaces in the body and induces high levels of morbidity and mortality in hospitalised patients (Guinea, 2014; Netea *et al.*, 2015). Therefore it is highly relevant to study the mechanisms associated with the immune control of these microorganisms

proliferation. Gal-3 was reported to recognize specifically β-1,2-linked mannopyranose units on the cell wall of *C. albicans* (Fradin *et al.*, 2000; Jouault *et al.*, 2006; Kohatsu *et al.*, 2006). This interaction appeared very specific since it was not observed using gal-1, other *Candida* species, or the common model organism *Saccharomyces cerevisiae* (Kohatsu *et al.*, 2006). The absence of β-1,2-linked mannopyranose from *S. cerevisiae* cell wall was considered the cause for the absence of gal-3 recognition/ligation (Jouault *et al.*, 2006; Kohatsu *et al.*, 2006). Since gal-3 was found to specifically recognize *C. albicans* and not *S. cerevisiae*, it was hypothesized to be the protein responsible for discrimination between pathogenic and non-pathogenic yeasts and for the onset of an appropriate immune response (Jouault *et al.*, 2006; Kohatsu *et al.*, 2006).

The binding of gal-3 to *C. albicans* appears to have a role in this pathogen recognition and clearance. This was inferred from the increased gal-3 expression along the maturation process of monocyte into macrophages (Jouault *et al.*, 2006; Reales-Calderon *et al.*, 2012) and from the morphological changes associated with yeast death caused by gal-3 in *C. albicans* cells (Kohatsu *et al.*, 2006). Various other evidences reinforce the relationship between gal-3 and the inhibition of *C. albicans* infection. In human gingival epithelial cell line Ca9-22 and in human gingival fibroblasts, gal-3 secretion was found to be upregulated upon *C. albicans* infection (Tamai and Kiyoura, 2014). This increased secretion was verified also in neutrophils and caused an enhanced phagocytosis of the pathogens (Linden *et al.*, 2013a). Gal-3 seems to be necessary for an appropriate TNF-α production through association with dectin-1, a C-type lectin that is the major receptor on macrophages for fungal β-1,3-glucan (Esteban *et al.*, 2011). The association of gal-3 with dectin-1 triggers pro-inflammatory response of macrophages against pathogenic fungi, and this depends on the recognition of exposed yeast cell wall β-glucans (Esteban *et al.*, 2011). In addition, gal-3 is crucial for fungal clearance (Linden *et al.*, 2013b). The results obtained using cell cultures model were corroborated in gal-3 knocked-out mouse model, which showed increased mortality upon infection with *C. albicans* and an enlarged area of the brain affected by infection (Linden *et al.*, 2013b). Taken together, these evidences reveal a clear biological role for gal-3 in the recognition and clearance of *C. albicans.* The same was demonstrated for other equally pathogenic Candida

species (Linden *et al.*, 2013a). Still, the mechanisms underlying are mostly unknown.

S. cerevisiae is the most well-known eukaryotic organism. It is phylogenetically afar and biologically different from *C. albicans* in many ways. Importantly, *S. cerevisiae* differs from *C. albicans* in the ability to filament into true hyphae forming extremely invasive mycelia. Indeed *S. cerevisiae* can form pseudohyphae, but it does not possess a mechanism for yeast/hyphae dimorphic transition. This difference is one of the reason why *S. cerevisiae* is considered a non-pathogenic yeast, although it has been increasingly reported to cause systemic infections in immunocompromised patients (de Llanos *et al.*, 2011).

The organization of the cell wall of *S. cerevisiae* and *C. albicans* is very similar (Aguilar-Uscanga and Francois, 2003; Ruiz-Herrera *et al.*, 2006; Free, 2013). In both yeast species, the side closer to the plasma membrane is composed mainly by β-1,3-glucans and, to a lesser extent, by β-1,6-glucans, and it includes as well small amount of chitin (Klis *et al.*, 2006; Free, 2013). The layer faced outside is richer in highly mannosylated proteins, mainly GPI-anchored to the glucans network (Gemmill and Trimble, 1999; Klis *et al.*, 2006). Even though the basal structure of the wall is very similar between the two yeast species, the specific composition in polysaccharides and proteins vary extensively. In particular, differences in the side branching of the glucans have been shown (Ruiz-Herrera *et al.*, 2006), as well as a great variety in glycoprotein glycan structures (Fukazawa *et al.*, 1995; Gemmill and Trimble, 1999). For example, all pathogenic *Candida albicans* produce poly-β-1,2-linked mannose chains that bind specifically the organism to the macrophages (Li and Cutler, 1993; Gemmill and Trimble, 1999), but these structures may differ among different Candida species and even among different serotypes belonging to the same species (Gemmill and Trimble, 1999). This type of mannosylated structures have not been found in *S. cerevisiae* (Suzuki, 1997; Gemmill and Trimble, 1999; Fradin *et al.*, 2000; Jouault *et al.*, 2006).

The present work aims at identifying the response of yeasts to the presence of extracellular galectins. Therefore, living yeast cultures were exposed to purified galectin suspensions. The effects of gal-3 were assessed in comparison with galectins from the *proto-type* and *tandem-repeat* groups as well as a non-galectin lectin, ConA. Two very different model yeasts were used, *S. cerevisiae* and *C. albicans*. Basic aspects of the yeast cell biology, such as viability,

cell morphology, cell cycle, ROS level and DNA status, were tested. Results showed that one of the *S. cerevisiae* strains used in this work was sensitive to gal-3, showing decreased viability, increased ROS level and altered cell size and only moderate stress symptoms in *C. albicans*, *i.e.* a small percentage of cells with membrane breakages. Gal-4 and gal-7 produced different effects, not altering cell viability but increasing ROS and membrane rupture levels. Gal-1 caused no detectable effect. These results suggest that the effects caused by the galectins do not relate directly on their structure, since the two proto-type galectins gal-1 and gal-7 behaved very differently. The effect of gal-3 was found to be largely mediated by its CRD, since most of the effects caused by full-length gal-3 were similarly caused, or even enhanced, by gal-3 lacking the N-terminal domain. In addition, gal-3 action on *S .cerevisiae* cells seems to depend of the functionality of the Ras/cAMP/PKA pathway.

In conclusion, this work shows that gal-3 is not exclusively specific for *C. albicans* and that is not the only galectin able to affect yeast behaviour. Our discoveries highlight the differences that may be encountered using different yeast genetic background and instil doubt that gal-3 may actually be responsible for discriminating between pathogenic and non-pathogenic yeasts.

Materials and methods

Strains, growth conditions and treatment with lectins

Microbial strains, maintenance and growth conditions. The yeast strains used in this study were *Saccharomyces cerevisiae* W303-1A wild type (MATa leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100) and *Δras2* (isogenic to W303-1A but ras2::KanX), BY4741 wild type (MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0) and *Candida albicans* BPW17 (ura3:: imm434/ ura3:: imm434iro1/ iro1:: imm434 his1:: hisG/ his1:: hisG arg4/ arg4). Yeasts were maintained at 4°C on solid YPD (1% yeast extract, 1% peptone, 2% glucose and 2% agar) and refreshed on the same medium for 72 h (*S. cerevisiae*) and 48 h (*C. albicans*) before every assay. These were used to inoculate YPD batch cultures (liquid:air ratio of 1:2.5 and 200 rpm orbital shaking) at $O.D._{600}$ 0.04 (*S. cerevisiae*) and 0.02 (*C. albicans*). These cultures were allowed to multiply up to $O.D._{600}$ 1. *S. cerevisiae* was cultivated at all times at 30°C and *C. albicans* at 37°C.

Yeasts exposure to galectins. $O.D._{600}$ 1 yeast cultures were diluted 1:2 in fresh YPD, distributed in 140 µl aliquots, supplemented with 40 µl of galectin-x or ConA suspended in PBS (phosphate buffered saline) to a final concentration of 60 µM, and incubated for 8 h in the same conditions as above. Controls were made using the equivalent volume of buffer without any lectin. In the case of flow cytometry assays, a control was made to set the analysis parameters of dying cells, which consisted of yeast culture incubated with buffer alone and heated for 5 min at 85°C at the end of the 8 h.

Assessment of yeast viability

Viability was assessed by colony forming units (CFU) assay, using the cells incubated with lectins. A sample was taken at T_0 and T_{8h} consisting of a 10 µl culture aliquot, which was then serially diluted in sterile dH_2O 1:10 four times. From the last dilution, 8 drops of 40 µl were taken and plated on solid YPD and incubated for 48 h. Viability was quantified as the ratio between the mean number of colonies at T_{8h} and at T_0. Every assay was repeated 4 to 6 times to ensure statistical significance.

Fluorescence microscopy

A Leica Microsystems DM-5000B epifluorescence microscope with DIC (differential interference contrast) filter setting and a 100x/1.0 oil-immersion objective was used.

Measure of cell size. Cell size analysis was performed using the ImageJ software (http://imagej.nih.gov/ij/), which estimates the area of each cell in μm^2. To ensure statistical significance, 300 to 500 cells of each strain/treatment in each of 4-5 independent experiments were measured.

Assessment of DNA alterations. Staining yeast DNA with DAPI (4',6-diamidino-2-phenylindole) was performed using 20 µl of cells from T_{8h} lectin-treated cells. These were spun down, fixed in 70% ethanol for 10 min and washed 1x in PBS. DAPI was added to a final concentration of 50 ng/µl. The cells were incubated in the dark for 15 min, washed 2x in dH_2O and then observed using fluorescence microscopy with a 100x/1.0 oil-immersion objective and appropriate filter settings.

Mitochondrial membrane integrity. Staining yeast mitochondria membrane with $DiOC_6$ (3,3'-dihexyloxacarbocyanine iodide) was performed in 20 µl of T_{8h} lectin-treated cells. The culture was spun down and washed twice in $DiOC_6$ buffer (10mM MES, 0,1mM $MgCl_2$, 2% glucose). $DiOC_6$ was added to the cell suspension to a final concentration of 50 nM, incubated for 15 min in the dark, washed twice in dH_2O and then observed using fluorescence microscopy with a 40x/1.0 objective and appropriate filter settings. In both staining assays, 3 independent experiments were analysed, each corresponding to 300-400 cells.

Flow cytometry analysis

All flow cytometry data were analysed using the freeware Flowing Software 2. At least 4 independent replicates of each strain/lectin treatment were analysed.

Quantification of ROS and assessment of plasma membrane integrity. An aliquot of 30 µl of T_{8h} culture was subjected to the protocol previously described in Tulha *et al.* (2012). The samples were analysed in an Epics® XL™ (Beckman Coulter) flow cytometer, at 370nm/420nm and 535nm/617nm excitation/emission, respectively.

Cell cycle analysis. This was performed as described in the literature (Fortuna *et al.*, 2001;

Haase and Reed, 2002), with some modifications. Briefly, 50 µl of cells at T_{8h} were harvested and fixed overnight in 70% ethanol at 4°C. The cells were washed 1x in 50 mM sodium citrate buffer pH 7.5, resuspended in 2 mg/ml RNAase A in Tris-EDTA pH 8.0 and incubated at 37°C overnight. The cells were incubated in a 5 mg/ml protease K solution (H_2O-HCl pH 7.5) for 45 min at 37°C and resuspended in 50 mM sodium citrate buffer pH 7.5. SYTOX®-Green (Life Technologies) probe was added to a final concentration of 10 µM and the cells were incubated in the dark overnight at 4°C. Finally, 600 µl of Triton X-100 (0.25% v/v in 50mM sodium citrate buffer, pH 7.5) was added. The final suspension was sonicated 2x 2 sec at 30 W, incubated on ice in between sonications. The samples were analysed in an Epics® XL™ (Beckman Coulter) flow cytometer at 497nm/520nm excitation/emission according to SYTOX®-Green manufacturer instructions.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software. To evaluate the statistical significance of results, One-way ANOVA analysis was used. In the case of the comparison between the effect of truncated gal-3 and full-length gal-3, the values were compared two by two using Student t-test. The difference was considered significant when p-value ≤ 0.05.

Results

Gal-3, but not gal-4 and gal-7, decreases S. cerevisiae *viability*

The effect of gal-3 on the growth ability of the model yeast *Saccharomyces cerevisiae* was assessed by CFU assays. Two different gal-3 concentrations, 60 and 100 µM, were tested at several exposure times, from 2 to 14 hours (not shown). The 8 h treatment was chosen as the minimum amount of time necessary to induce an effect using 60 µM galectin. This concentration/exposure time combination was identically applied to assess the putative effects of gal-1, gal-4 and gal-7, and of the control mannose-binding lectin ConA, on the survival of *S. cerevisiae* and the human commensal/pathogen yeast *C. albicans*. The selected galectins represent different structural subgroups: gal-3 represents the *chimera* group, gal-1 and gal-7 the *proto-type* group, and gal-4 the *tandem-repeat* group (Cummings and Liu, 2009). *C. albicans* was chosen for being phylogenetically and physiologically afar from *S. cerevisiae* and because previous studies showed that gal-3 affected *C. albicans* morphology and viability (Kohatsu *et al.*, 2006). Moreover, there is a strong correlation between gal-3 expression and function and candidiasis in human (Jawhara *et al.*, 2008; Reales-Calderon *et al.*, 2012; Linden *et al.*, 2013a; Linden *et al.*, 2013b; Tamai and Kiyoura, 2014).

Gal-3 (60 µM/8 h - hereon unmentioned) induced in *S. cerevisiae* a decrease in CFU of 40% compared to the control (**Fig. 1A**). Gal-1 induced a decrease in CFU similar to gal-3 (approximately 35%), while gal-4 and gal-7 did not affect the yeast viability at a statistical significant value (**Fig. 1A**). ConA caused 50% decrease in the number of colonies formed by *S. cerevisiae* (**Fig. 1A**). On the other hand, the effect on *C. albicans* viability was quite different. All galectins, as well as ConA, caused CFU fluctuations, apparently less pronounced than in *S. cerevisiae,* but statistically invariable (**Fig. 1B**). These results contrast with the previous findings from Kohatsu *et al.* (2006), who observed an effect of gal-3 on the viability of *C. albicans* but not of *S. cerevisiae*. These authors used strains of both species different from the ones used in the present work. In particular, they used the *S. cerevisiae* BY4741, which differs from W303-1A used in this work in several amino acids auxotrophy (http://www.yeastgenome.org/) and has frequently been reported to respond very differently from

W303-1A (Cohen and Engelberg, 2007; Petrezselyova *et al.*, 2010). CFU assays using gal-3 were therefore repeated using *S. cerevisiae* BY4741. Similarly to Kohatsu *et al.* (2006), no differences in growth were observed between the gal-3 treated *S. cerevisiae* BY4741 and the untreated control culture (not shown). Yeast response to gal-3 therefore appears to be strain-dependent.

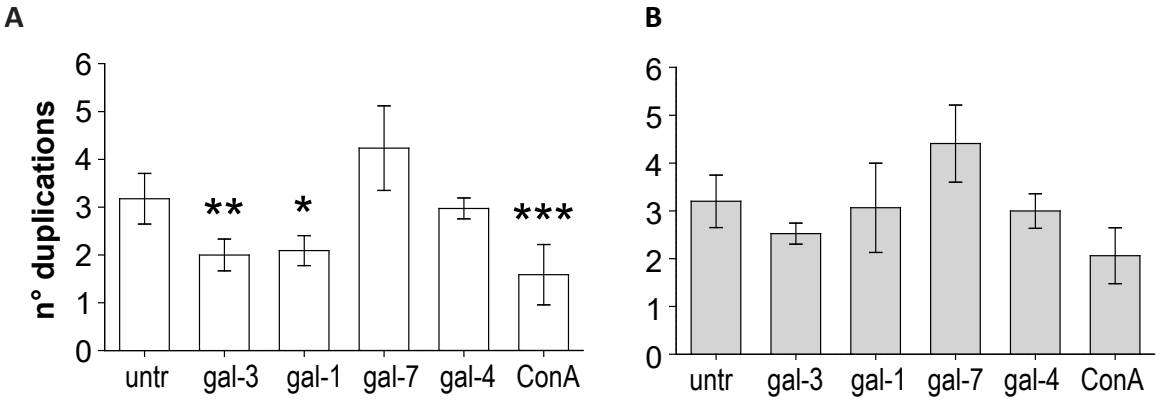


Figure 1: *How lectins affect yeasts multiplication ability.* Number of times the cultures of *S. cerevisiae* **(A)** and *C. albicans* **(B)** doubled in number of cells (equivalent to a full culture cell cycle) after 8 h incubation with a lectin. The number of duplications is calculated as a ratio of CFUs at T_{8h} and T_0. Values are an average of 3-4 independent assays. Statistically significant differences between the buffer-only (untreated) and the lectin treated samples are shown: * (p-value ≤ 0.05), ** (p-value ≤ 0.01) and *** (p-value ≤ 0.001).

Decreased yeast population viability may be the result of cellular metabolic and molecular imbalance due to specific cultivation conditions, but may also derive from the death of a part of the population. A death-inducing response is usually associated with an increased production and intracellular accumulation of ROS. The accumulation of ROS is one of the first event occurring after yeast is subjected to stress stimuli and it is necessary for the further induction of either programmed cell death (PCD) or cell death (necrosis). When PCD is triggered, other significant alterations are visible in the cell, such as chromatin condensation and DNA fragmentation, loss of mitochondria membrane potential and its subsequent permeabilization, and phosphatidylserine externalization. On the other hand, necrosis is a less organized and controlled processed, in which one of the main event is the rupture of plasma membrane and the following release of intracellular content. Therefore, plasma

membrane integrity is often used to discriminate between PCD and necrosis (Gourlay *et al.*, 2006; Frohlich *et al.*, 2007; Carmona-Gutierrez *et al.*, 2010; Wloch-Salamon and Bem, 2012; Giannattasio *et al.*, 2013).

The levels of ROS were measured by DHE (dihydroethidium) staining. Results showed a significant increase in the level of ROS following the incubation of *S. cerevisiae* cells with gal-3, gal-7 and gal-4, while no effect was detected using gal-1 or ConA (**Fig. 2A**). *C. albicans* cells presented generally a lower level of ROS than *S. cerevisiae* (**Fig. 2B**) and only gal-4 caused a significant increase compared to the buffer-only treated cells. In both yeasts, the ROS accumulation induced by the galectins was significantly lower than the one caused by a strong death inducer such as high temperature (85°C) for a short period of time (**Fig. 2**).

ROS-induced yeast death was characterized assessing the parameters above mentioned. Firstly, the percentage of cells that lost their membrane integrity and became permeable to propidium iodide (PI^+) was quantified in each yeast culture. Gal-3 did not induce a significant increase in the percentage of PI^+ cells of *S. cerevisiae* (**Fig. 2C**), in opposition to *C. albicans*, that presented a statistically significant increase (**Fig. 2D**). Gal-3 induced more ROS in *S. cerevisiae* than in *C. albicans* and, the other way round, more membrane disruption in *C. albicans* than in *S. cerevisiae* (**Fig. 2**). Gal-4 and gal-7, on the other hand, triggered the disruption of a significant percentage of yeast cells, concomitantly with the results from ROS production. They provoked similar results, though to a lesser extent, in *C. albicans* (**Fig. 2C, D**), while gal-1 and ConA did not induce any change in either yeast (**Fig. 2C, D**). Importantly, the observed increased number of cells with high ROS levels and disrupted membrane are significant but far from the values of the heat-shocked positive controls. In these cases the cells likely become irreversibly unviable, while the gal-4 or gal-7 treated cells might have recovered during the 48 h incubation on rich media needed to obtain results from CFU.

Secondly, DNA damage, *i.e.* chromatin condensation or DNA fragmentation, was assessed by DAPI staining followed by microscopic observation. Gal-3 induced almost 50% of the *S. cerevisiae* cells to display DNA alterations, while the other galectins, as well as ConA, induced a percentage of cells statistically invariant in relation to the control population (**Fig. 3A, B**). *C. albicans* in turn was statistically insensitive to the presence of all lectins (**Fig. 3C, D**).

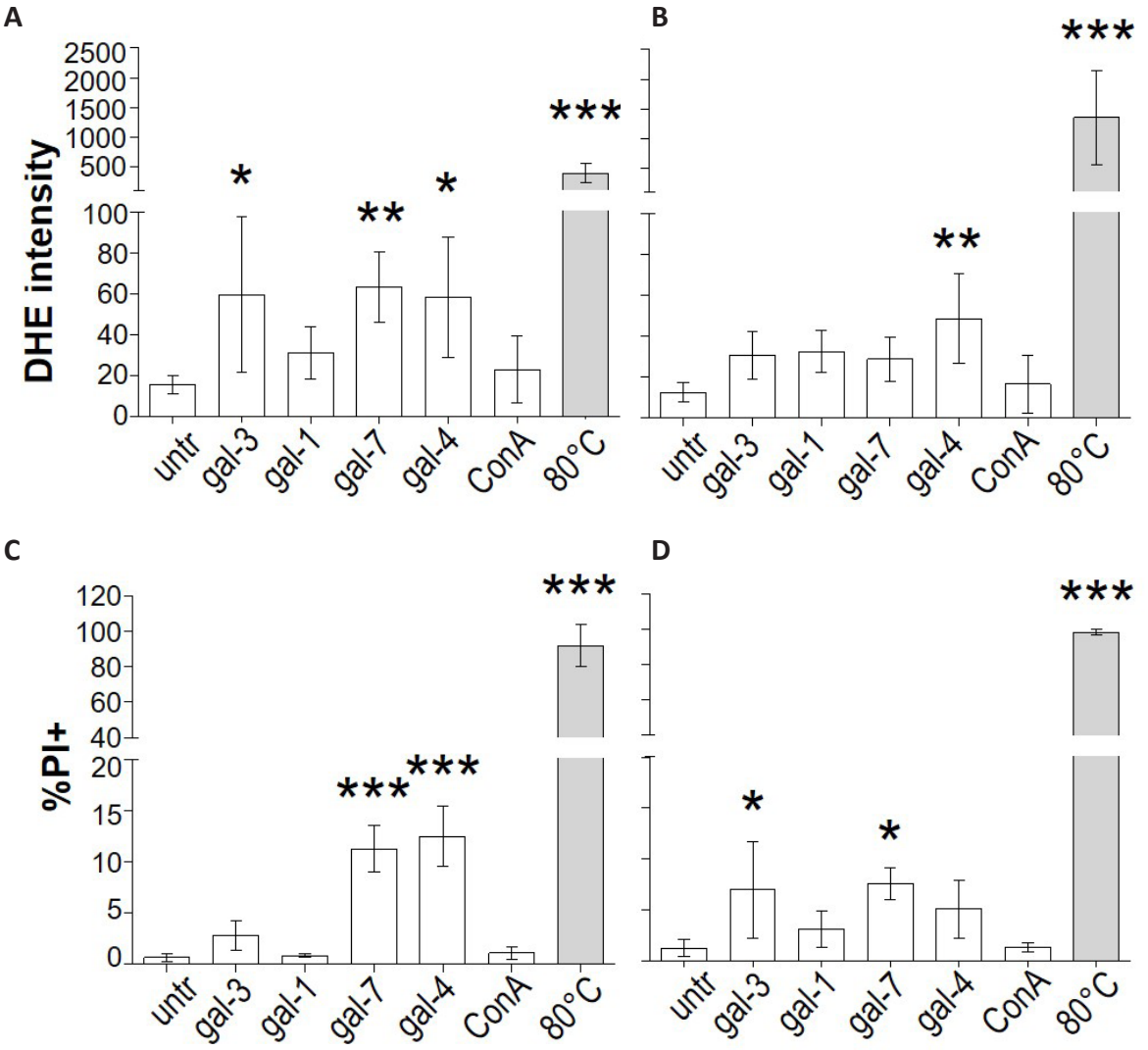


Figure 2: *Flow cytometry analysis of yeasts responses to lectins.* Level of ROS in *S. cerevisiae* **(A)** and *C. albicans* **(B)** in response to lectins. Values correspond to the average intensity of 20.000-30.000 cells stained with DHE (arbitrary unit) from 3-5 independent assays per strain/treatment. Percentage of *S. cerevisiae* **(C)** and *C. albicans* **(D)** cells that responded to lectins with plasma membrane breakage. Cells were identified using PI staining in an identical number of cells as for DHE staining. In both cases, a positive control consisting of heat-killed cells (85°C, 5min) is presented. Significant differences between the buffer-only (untreated) and the lectins treated samples are shown: * (p-value ≤ 0.05), ** (p-value ≤ 0.01), and *** (p-value ≤ 0.001).

Thirdly, the mitochondrial membrane depolarization was measured by $DiOC_6$ staining. Only gal-3-treated *S. cerevisiae* cultures were used in view of the lesser effects of gal-3 on *C. albicans*. No significant deviation from the buffer-only culture was observed in the percentage of cells that presented damaged mitochondria (**Fig. 3E, F**).

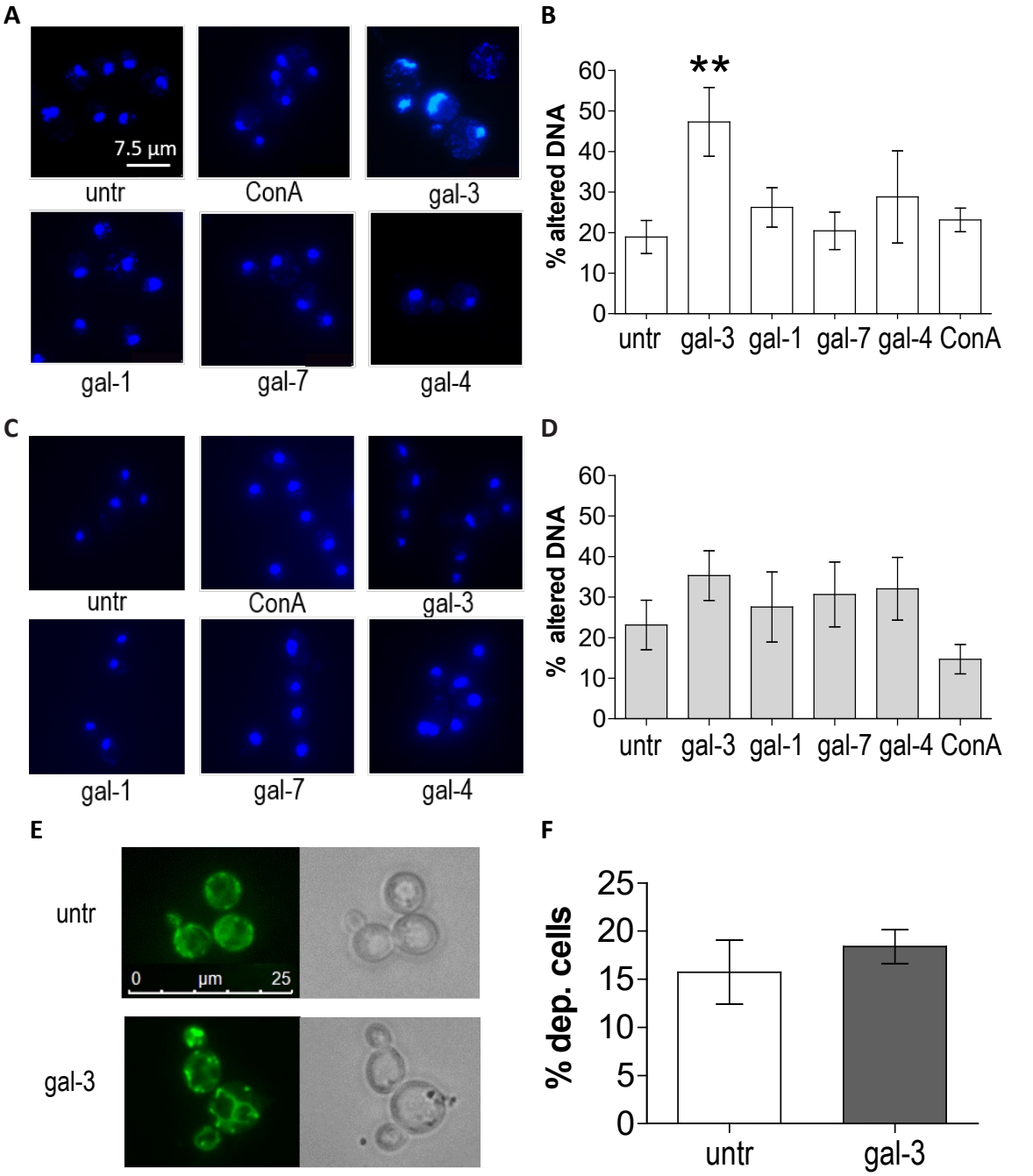


Figure 3: *Fluorescence microscopy of yeasts responses to lectins.* DNA fragmentation and chromatin condensation in response to lectin treatment were assessed in *S. cerevisiae* **(A, B)** and *C. albicans* **(C, D)**. Representative images of DAPI stained cells by fluorescence microscopy can be seen in **A** and **C**. The images of 300-400 cells were taken in each assay (100x/1.0 magnitude) and the DNA shape was evaluated manually. The average and standard deviation of the number of cells presenting DNA abnormalities from 3 independent assays were plotted (**B, D**). Significant differences between the buffer-only (untreated) and the lectin treated samples are shown: ** (p-value ≤ 0.01). Mitochondrial membrane depolarization in response to gal-3 treatment was determined in *S. cerevisiae* by $DiOC_6$ staining (**E, F**). The average and standard deviation of the number of cells presenting mitochondrial membrane depolarization from 3 independent assays were plotted.

Gal-3 seems therefore to have a very specific effect on *S. cerevisiae* compared to the other galectins. It increased the accumulation of ROS and the DNA alterations (chromatin condensation and DNA fragmentation), without altering the plasma membrane integrity, suggesting an increased stress level. The possible induction of PCD was discarded since no visible effect on mitochondria membrane was observed (Carmona-Gutierrez *et al.*, 2010; Giannattasio *et al.*, 2013). On the other hand, *S. cerevisiae* responded similarly to gal-4 and gal-7, both increasing ROS level and provoking membrane rupture, without affecting the DNA. These results are more compatible with the first stages of a necrotic death, in which an increased level of stressed cells is observed (Wloch-Salamon and Bem, 2012). Finally, gal-1 and ConA did not induce any significant effect.

All taken, each galectin caused a specific effect, and *S. cerevisiae* and *C. albicans* responded differently. In particular *C. albicans* resulted less affected: gal-3 only caused a slight increase in plasma membrane rupture (~5% of cells), while a mild increase in ROS levels was caused by gal-4 and in membrane breakage by gal-7.

All the lectins cause the appearance of a second population of cells, with different levels of ROS and membrane breakage

When *S. cerevisiae* and *C. albicans* were incubated with either galectin or ConA, the analysis of the population of cells in the flow cytometer revealed the appearance of two distinct populations of cells (Pi and Pii), one of which was absent from the controls (**Fig. 4A, C**). The two populations differed in shape and cell abundance depending on the lectin used, but maintained their characteristics in several independent experiments of each galectin/yeast combination, confirming that both shape and size of the populations were not results of chance. Concurrently, gal-4 and gal-7 caused identically shaped and sized Pii population in either yeast species (**Fig. 4**), while the population emerging in the presence of gal-3 was similar to these in shape, but much smaller (Pii ~2% of the all population compared to ~15% in the presence of gal-4 or gal-7). In all cases, the galectin-induced Pii population was composed by more heterogeneous cells than the Pi, as inferred from increased values of side and forward scattering. This means that cells within the Pii population vary greatly in

cell size and present different surface and internal complexity. On the other hand, gal-1 and ConA seem to induce the appearance of a similar Pii population in shape, with smaller and less complex cells. The abundance is however very different, with ConA Pii population being almost 80% of the total (Pi+Pii) and gal-1 no more than 5%.

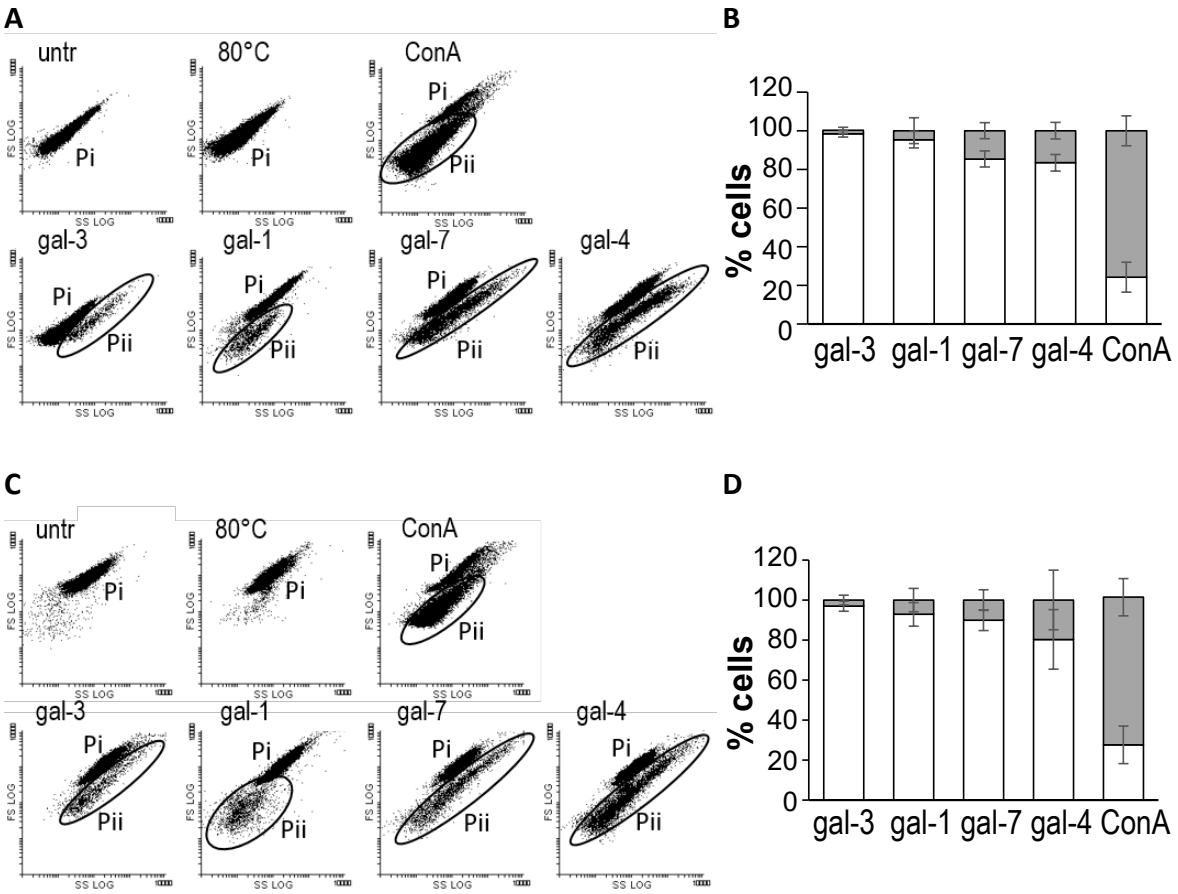
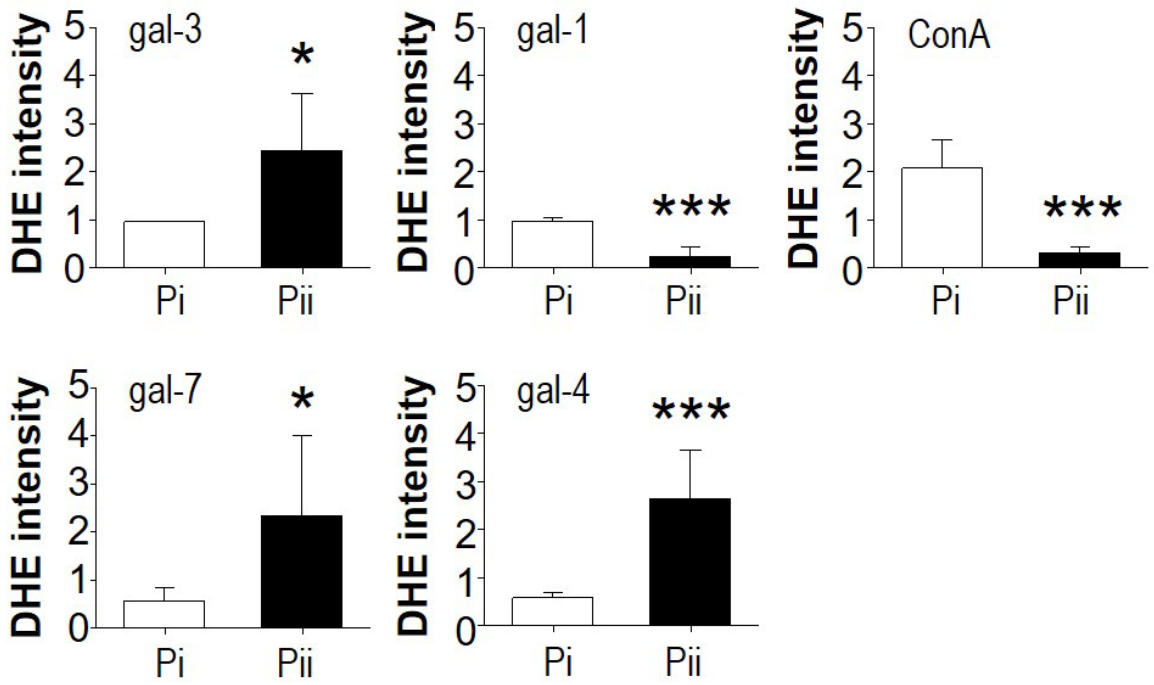


Figure 4: *Structure of yeast populations derived from exposure to lectins. S. cerevisiae* **(A, B)** and *C. albicans* **(C, D)** cell populations differently treated – buffer-only (untreated), lectins incubation, heat-killed samples (85°C, 5 min) – were analysed by flow cytometry. Images are representative of 8-10 independent assays. *Circles* indicate a second population of cells (Pii) with different morphological characteristics compared to the main one (Pi). The shape of Pii relates to the lectin in use, being similar for gal-3, gal-7 and gal-4, and for gal-1 and ConA. The abundance of cells in Pii varied, but maintained the relative size when compared with Pi. This was observed both in *S. cerevisiae* **(B)** and *C. albicans* **(D)**. Bars show the average of the percentage of number of cells that composed each population in 8 to 10 independent assays: (☐) Pi; (■) Pii; 100% was Pi+Pii.

Interestingly, when the two *S. cerevisiae* populations were analysed separately for both ROS level (**Fig. 5A**) and membrane breakage (**Fig. 5B**), it was possible to notice a very clear difference between them. Pii populations with similar shape, such as the ones induced by the treatment with gal-3, gal-4 and gal-7, presented similar outcome in regards of ROS level and membrane breakage. Indeed, when the cells were incubated with gal-3, gal-7 and gal-4, the Pii population presented 2.5 times more ROS than the Pi population (**Fig. 5A**), and the number of cells that presented membrane rupture grew to 40%, 50% and 60% respectively (**Fig. 5B**). Differently, the Pii populations resulting from either gal-1 or ConA treatment displayed the same or less ROS production and membrane breakage. Equivalent experiments in *C. albicans* (not shown) yielded qualitatively identical though quantitatively slightly different results. All taken, it appears that the amounts of lectin used are acting only on a percentage of the cells in each culture, and this is reflected by the appearance of the Pii population. This might therefore represent the actually affected cells.

A



B

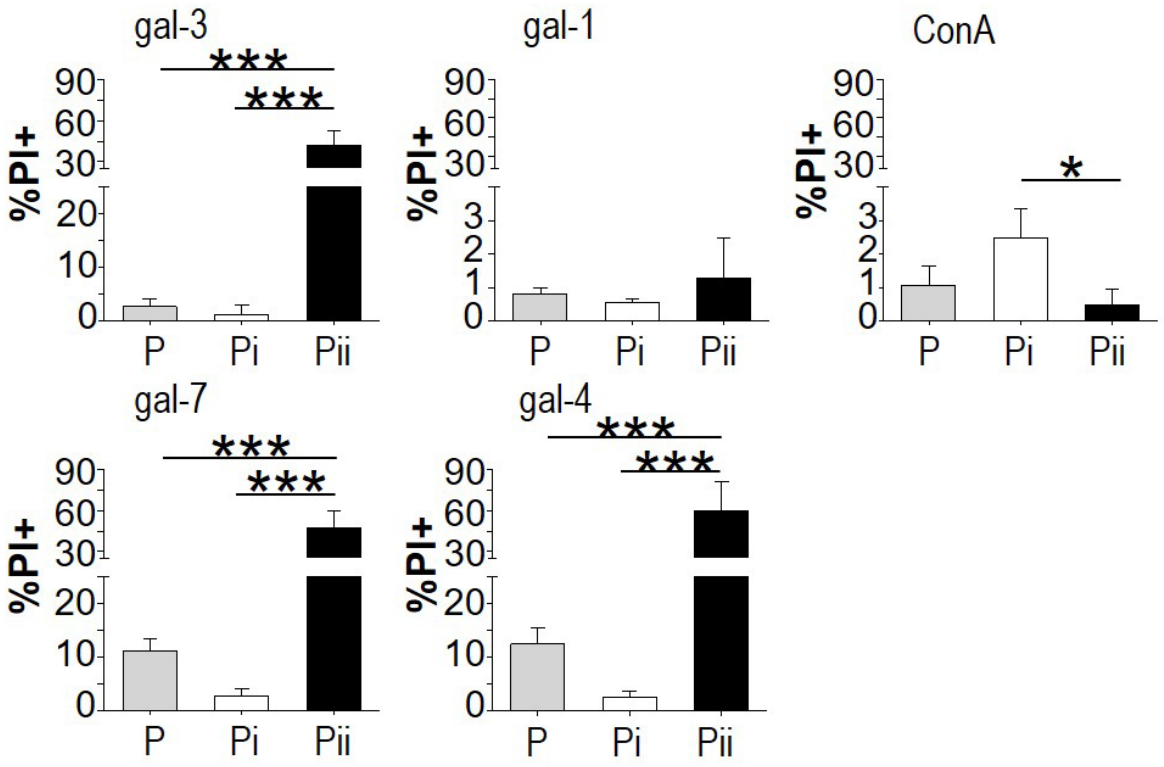


Figure 5: *Different populations are differently responding to lectins. S. cerevisiae* was chosen to analyse separately each of the two populations observed by flow cytometer, Pi and Pii. P represents the sum Pi+Pii (100% in Fig. 4). Results of ROS production **(A)** correspond to the average of the ratio of DHE intensity between either the two populations (Pi or Pii) and the total population (P), Pi/P or Pii/P. Results of plasma membrane rupture **(B)** correspond to the percentage of cells that presented PI staining in each population, P, Pi and Pii, average from 4 to 5 independent assays. Statistical significant differences are shown: * (p-value ≤ 0.05), ** (p-value ≤ 0.01), and *** (p-value ≤ 0.001).

Gal-3, but not gal-4 and gal-7, triggers an increase in cell size without altering the cell cycle in a Ras2 dependent manner

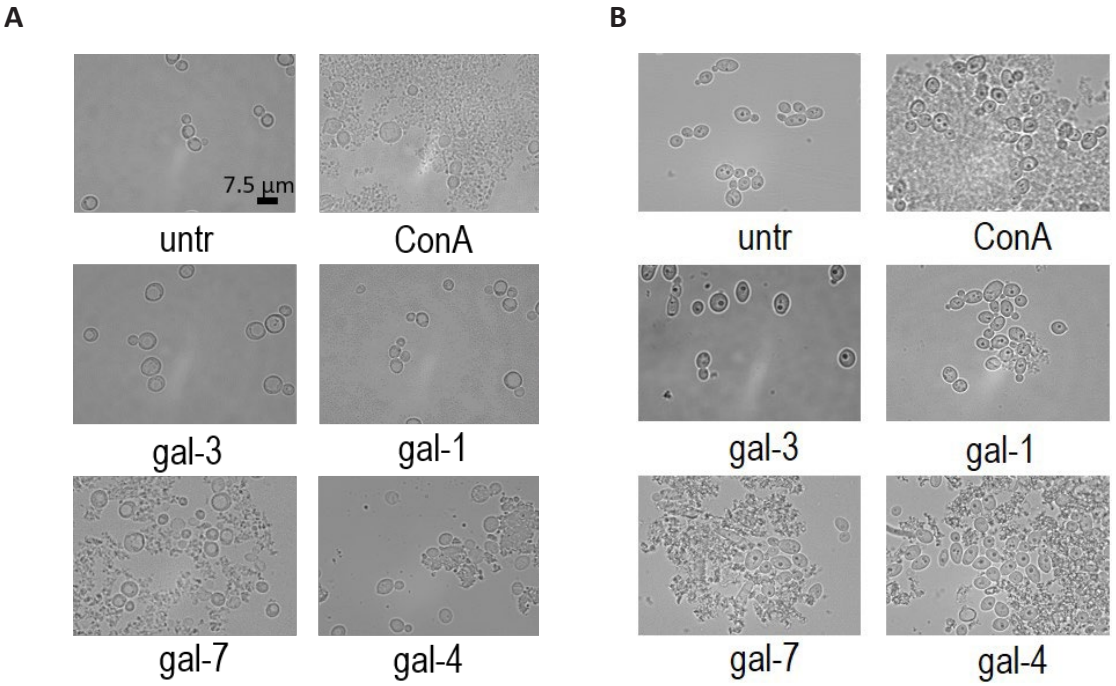
The results of CFU assays using *S. cerevisiae* W303-1A indicate that gal-3, gal-1 and ConA, but not gal-7 or gal-4, affected yeast viability (**Fig. 1A, B**). This should be accompanied by decreased values in the optical density (A_{600nm}) of the correspondent cultures, which did not occur (not shown). There are two other common possible causes for biased $O.D._{600}$ readings in relation to colony formation ability: increased cell size or the formation of cell aggregates. Therefore, the formation of multicellular aggregates was verified, and the size of the cells was measured. Moreover, since the change in yeast cell size may derive from G1 cell cycle

arrest (Busti *et al.*, 2010; Turner *et al.*, 2012; Soifer and Barkai, 2014), the sub-populations of cells in each of the main phases of the cell cycle were quantified.

S. cerevisiae cultures treated with galectins and ConA were checked for aggregation. ConA induced the formation of massive conglomerates of yeast cells (**Fig. 6A**). The same phenotype was observed in cultures incubated with gal-7 and to a much lesser extent gal-4, but not gal-3 or gal-1 (**Fig. 6A**). *C. albicans* behaved essentially as *S. cerevisiae* (**Fig. 6B**), except that the cells incubated with gal-4 presented larger aggregates. All these aggregates were not mechanically destroyed using vigorous pipetting or vortexing, or physically using sonication, suggesting that cells are connected by strong chemical bonds, as predicted for lectins like ConA (Stratford, 1992a, 1992b; Nenoff *et al.*, 2000; Touhami *et al.*, 2003a; 2003b).

Cell size was assessed determining the average area of a large number of cells. Gal-3 induced an increase of approximately 25% in the size of *S. cerevisiae* compared with the buffer-only treated cells (**Fig. 6C**). The other galectins also induced a cell size increase, though less pronounced (approximately 10-15%) (**Fig. 6C**). Interestingly, also ConA provoked an increase of approximately 20% in the cell size (**Fig. 6C**). On the other hand, the effect of gal-3 on *C. albicans* was opposite, causing the area to decrease of approximately 10% (**Fig. 6D**), as previously observed using a different genetic background (Kohatsu *et al.*, 2006). The other galectins, as well as ConA, did not alter significantly the size of *C. albicans* cells (**Fig. 6D**).

It is well known that in *S. cerevisiae* cell size and cell cycle progression are tightly interconnected. In particular, the increase in size may be a consequence of cell cycle arrest in G1 phase, before DNA replication occurs (Busti *et al.*, 2010; Turner *et al.*, 2012; Soifer and Barkai, 2014). The distribution of *S. cerevisiae* yeast cultures throughout the three phases of the cell cycle was quantified. No differences between the buffer-only and the galectin or ConA-treated cells were observed (**Fig. 6E**), suggesting that the above described galectin-induced size increase is not a consequence of G1 arrest.


A
B
7.5 µm
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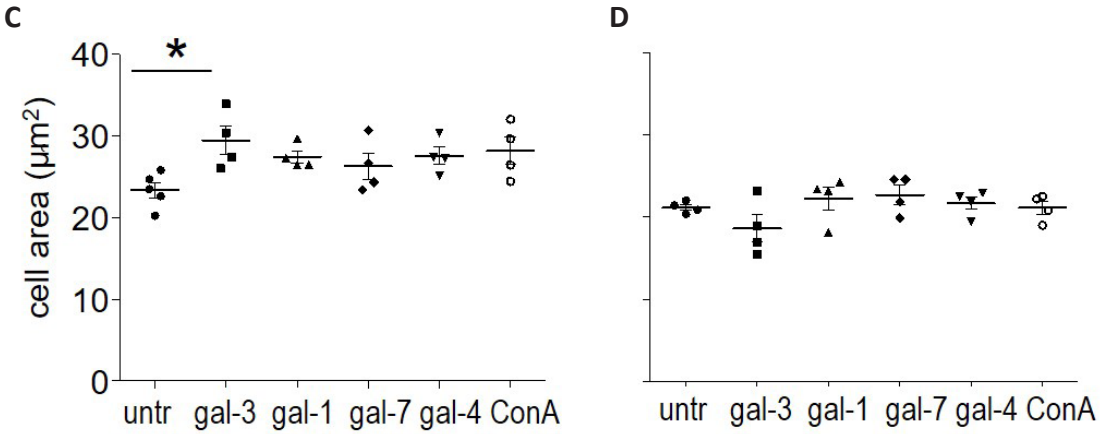
C
D
cell area (µm²)
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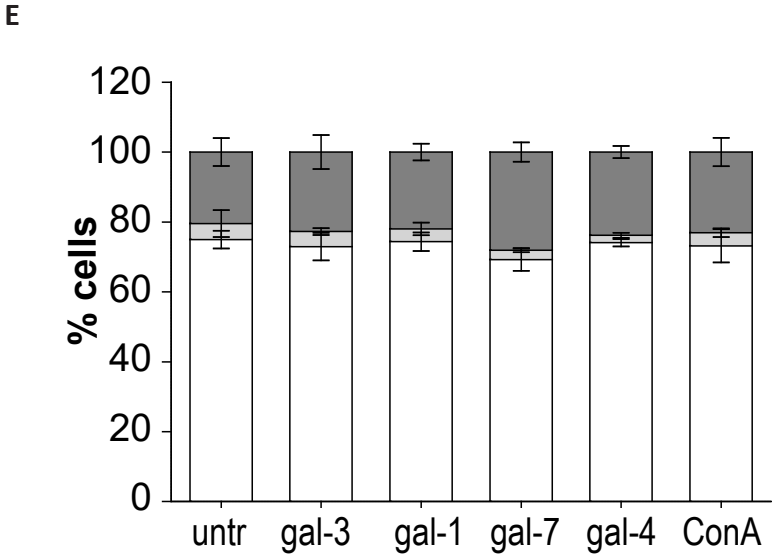
gal-4
ConA


E
% cells
120
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ConA

Figure 6: *How lectins affect yeasts shape and size.* Representative images of the cells of *S. cerevisiae* **(A)** and *C. albicans* **(B)** after 8 h incubation with a lectin. Control consists of identical incubation in buffer w/o lectin. All the images were taken using the same amplification (100x/1.0). Cell size/area (μm^2) of *S. cerevisiae* **(C)** and *C. albicans* **(D)** cells. Values are an average of 4 independent assays. In each assay, the area of 300 to 400 cells was measured. Statistically significant differences between the buffer-only (untreated) and the lectin treated samples are shown: * (p-value ≤ 0.05). Distribution of *S. cerevisiae* cells throughout the phases of cell cycle- (□) G1, (■) G2 and (■) S- established by flow cytometry analysis using SYTOX®-Green probe **(E)**. Each value represents the average of 4-6 independent assays.

The increased cell size might also be the consequence of nutrient signalling RAS/cAMP pathway hyperactivation (Mitsuzawa, 1994; Enserink, 2014). In *S. cerevisiae*, Ras/cAMP/PKA is one of the pathways involved in mediating the response to carbon source availability (Tatchell *et al.*, 1985; Tamanoi, 2011), with implications in, namely, cell size (Baroni *et al.*, 1989) and the induction of PCD (Gourlay and Ayscough, 2006; Gourlay *et al.*, 2006).

Gal-3 provoked in *S. cerevisiae* cells a phenotype that has traits in common with apoptotic cells, displaying increased ROS level and DNA alteration and maintaining plasma membrane integrity (Frohlich *et al.,* 2007; Carmona-Gutierrez *et al.,* 2010; Giannattasio *et al.,* 2013; Wloch-Salamon and Bem, 2013). Moreover, other effects provoked by gal-3 on *S. cerevisiae* are compatible with cells undergoing nutrient depletion: the cells decreased the number of duplication and at the same time they increased their cell size. These characteristics are typical of altered glucose signalling through the Ras/cAMP/PKA signalling pathway (Baroni *et al.*, 1989; Busti *et al.*, 2010; Turner *et al.*, 2012).

In order to determine whether RAS/cAMP/PKA pathway might be involved in the response of *S. cerevisiae* to gal-3, a *RAS2*-deleted mutant strain was used to repeat all the experiments previously done using the wild type strain. In all assays, results showed no difference between the gal-3 and the buffer-only treated samples (**Fig. 7A-H**). These results concur with the possibility that the Ras/cAMP/PKA pathway is involved in the yeast response to gal-3 and, in particular, that Ras2 protein may be necessary to trigger the stress response observed in the *wt* strain.

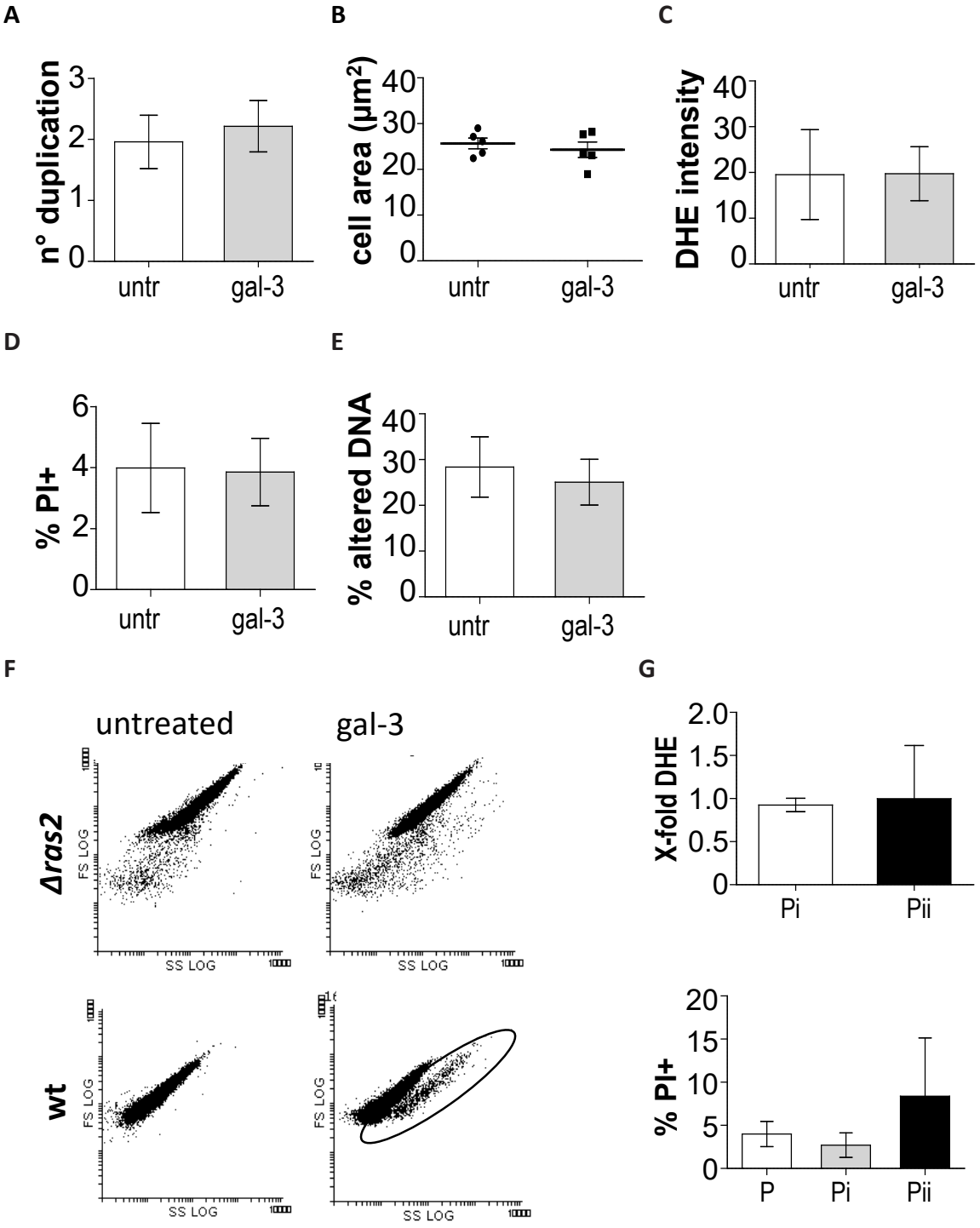

Figure 7*: Gal-3 does not induce any of the previously detected effects in* S. cerevisiae *lacking Ras2 protein*. The viability (CFUs) as in Fig.1 **(A)**, the cell size as in Fig. 2 **(B)**, the ROS production and plasma membrane fragmentation as in Fig. 3 **(C, D)**, and the DNA modification as in Fig. 4 **(E)** were assayed. The structure of the second population formed by gal-3 in the mutant *Δras2* (above), in comparison with the wt strain (below) **(F)**. The ROS level **(G)** and the percentage of membrane rupture **(H)** were analysed separately for the second population as in Fig. 5. Number of independent assays and number of measurements as before ensured statistical validity.

The CRD domain is largely responsible for the effects provoked by the full gal-3 protein

Gal-3 is the only galectin presenting a unique long N-terminal arm opposite to the CRD (Dumic *et al.*, 2006). In order to determine whether the gal-3 CRD or the N-terminal long arm domain were separately determining the effects observed on yeasts, all the above assays were repeated using a truncated form of gal-3 (gal-3^{tr}) consisting only of the CRD (Kopitz *et al.*, 2014). It is predictable that an effect that is dependent on the CRD specificity might still occur, in opposition to an effect that depends on the absent arm, namely through the formation of gal-3 multimers (Ahmad *et al.*, 2004; Halimi *et al.*, 2014).

Gal-3^{tr} affected *S. cerevisiae* differently compared to the full protein: 15% reduction in CFU (**Fig. 8A**), 15% cell size increase (**Fig. 8B**) and 35% cells with DNA alterations (**Fig. 8C**). The values obtained with the entire protein were respectively 40%, 30% and 50%. These results suggest that the truncated gal-3, while causing on *S. cerevisiae* basically the same effects as the full protein, is less effective on the outcome specific for gal-3, such as cell size and DNA alteration. In opposition, the lack of the N-terminal domain provoked a 1.6-fold increase of cells with membrane breakage (**Fig. 8D**), and a 3-fold increase in the level of ROS (**Fig. 8E**), resulting in a phenotype more similar to the one caused by gal-7 and gal-4. All taken, it is possible to postulate that the CRD is sufficient to cause the specific effect of gal-3 on *S. cerevisiae*, but the intensity of the outcome is reduced. This may indicate that the N-terminal domain, possibly through multimers formation, is necessary to signal efficiently to the cells. On the other hand, the truncation of the protein leads to the formation of a galectin more similar in structure to gal-4 and gal-7, and this reflects on the effects on *S. cerevisiae*: increased membrane breakage and ROS level.

C. albicans, on the other hand, was almost indifferent to the truncation of the N-terminal arm of gal-3. The similarity between the effects of full and gal-3^{tr} is evident (**Fig. 8F-H**). In 3/5 tests the results obtained were identical: the CFUs were reduced in ~20%, the size reduction accounted approximately for 10% and the DNA alterations were invariant (**Fig. 8F-H**). The number of cells presenting membrane breakage slightly decreased from ~7% to ~3% (**Fig. 8I**). The only prominent difference was a very significant increase in ROS production (**Fig. 8J**).

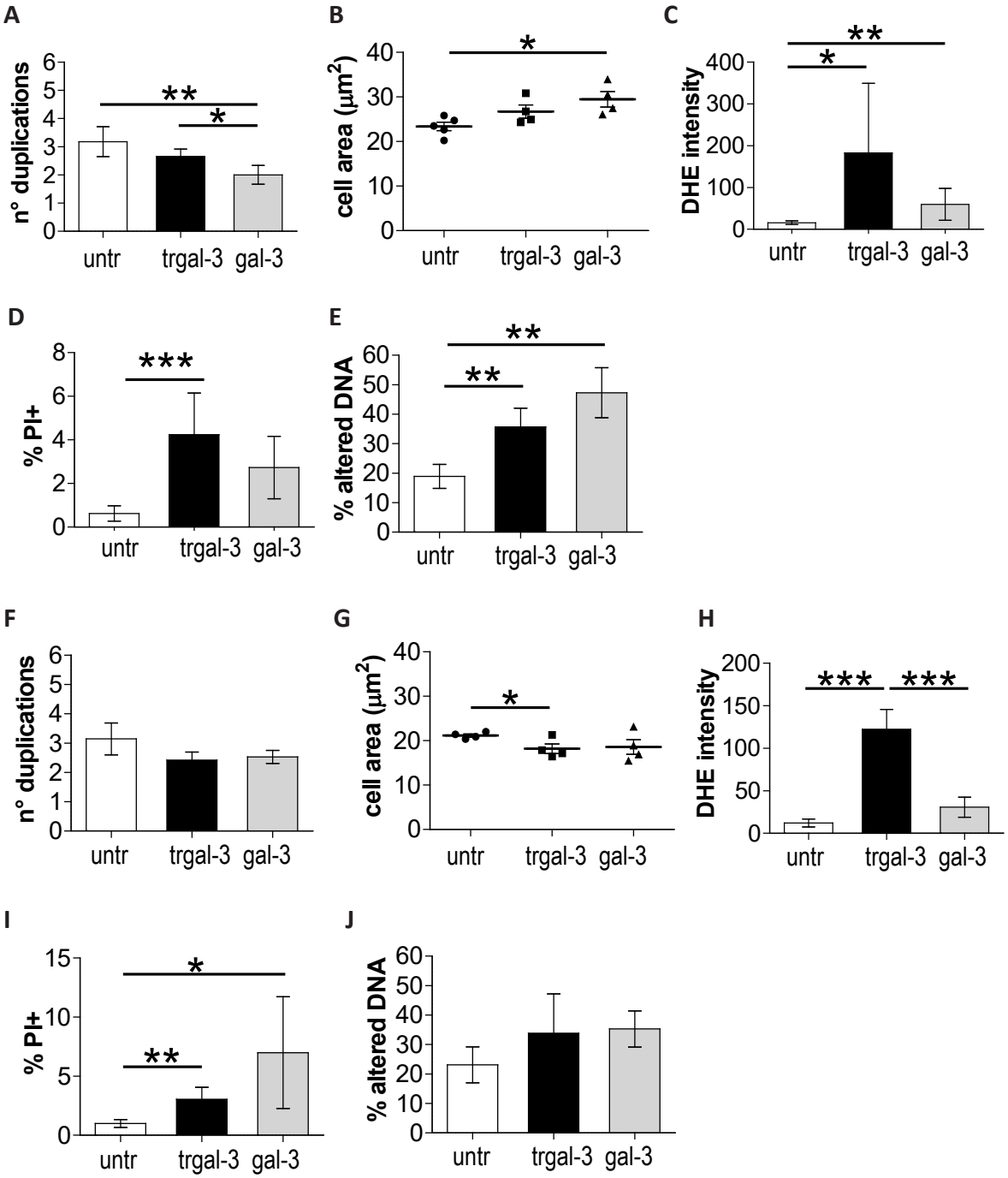

Figure 8: *The CRD of galectin-3 protein is sufficient but not as efficient to induce the same level of responses in yeasts caused by the entire proteins.* The truncated gal-3 was tested in comparison to the full-length protein and a buffer-only (untreated) control under the same experimental conditions using *S. cerevisiae* **(A-E)** and *C. albicans* **(F-J)**. The viability (CFUs) were determined as in Fig. 1 **(A, F)**, the cell size as in Fig. 6 **(B, G)**, the ROS production and plasma membrane fragmentation were observed as in Fig. 2 **(C, H** and **D, I),** and the DNA modifications were verified as in Fig. 3 **(E, J)**. The numbers of independent assays and measurements ensured statistical validity. Statistical significant differences are shown: * (p-value ≤ 0.05), ** (p-value ≤ 0.01), and *** (p-value ≤ 0.001).

Discussion

Galectins, in particular gal-3, are relevant for host-pathogen interaction, participating in the recognition, entrance and clearance of bacteria (Mey *et al.*, 1996; Sato *et al.*, 2002; Fowler *et al.*, 2006; Nieminen *et al.*, 2008), viruses (Mandrell *et al.*, 1994; Fogel *et al.*, 1999; Hsu *et al.*, 1999) and parasites (Pelletier and Sato, 2002; van den Berg *et al.*, 2004; Vray *et al.*, 2004; Bernardes *et al.*, 2006). Moreover, gal-3 has been shown to specifically recognize *C. albicans* outer cell wall glycoconjugates, triggering the clearance of the microorganism *in vitro* (Jouault *et al.*, 2006; Kohatsu *et al.*, 2006; Linden *et al.*, 2013a) and *in vivo* (Jawhara *et al.*, 2008; Linden *et al.*, 2013b), in a dectin-1-dependent manner (Esteban *et al.*, 2011). However, the ability of gal-3 to bind specifically β-1,2-mannopyranosides belonging to *C. albicans* cell wall has so far only been proven *in vitro* (Fradin *et al.*, 2000). *S. cerevisiae*, on the other hand, was reported as not responding to gal-3 (Jouault *et al.*, 2006; Kohatsu *et al.*, 2006). Concurrently, it was hypothesized that macrophages discrimination between non-pathogenic yeasts like *S. cerevisiae* and pathogenic yeasts like *C. albicans* might be mediated by gal-3 (Jouault *et al.*, 2006; Kohatsu *et al.*, 2006).

The present study examines for the first time the effect of exogenously added galectins on the basic features of yeast life. The model yeasts *S. cerevisiae* and *C. albicans* were used, each representing very different genera and biology. Well-known laboratory strains of each of these species were chosen. Yeasts were exposed, under physiologically controlled conditions, to galectins representing different structural groups: gal-3, the only member of the *chimera* group, gal-1 and gal-7 from the *proto-type* group, and gal-4 for the *tandem-repeat* group. ConA, a non-galectin lectin known for its ability to bind to both *S. cerevisiae* (Montijn *et al.*, 1994; Arvindekar and Patil, 2002; Coulibaly and Youan, 2014) and *C. albicans* (Sandin, 1987; Warolin *et al.*, 2005) was used as control. The galectin-exposed yeasts were assessed for culture growth and viability, death-associated processes, cell cycle progression, budding pattern, cell shape and size. Moreover, the level of ROS accumulated intracellularly was quantified, as a means to verify the degree of stress generated by the presence of the galectins. These are well-established protocols, but they require large amounts of yeast cells,

which could not be used due to the quantities of purified galectins necessary. Therefore, all the assays were miniaturized and tested prior to using cells exposed to galectins. Moreover, while the gal-3 concentration was the one Kohatsu *et al.* (2006) used, the ideal time of exposure necessary to detect a measurable response from the cells was determined. For the sake of comparison, the chosen conditions were also applied to study the cells exposed to the other galectins as well as ConA.

Results showed that gal-3 decreased *S. cerevisiae* growth ability, but it did not affect growth rate (μ_g) during culture exponential growth on glucose. It also provoked a considerable increase in cell size, without changes in cell shape, or irregularity in haploid apical budding pattern. The regulation of cell size of *S. cerevisiae* has not been completely understood so far. It is established that yeast regulates cell size primarily controlling the cell cycle in response to nutrients availability and growth rate (Busti *et al.*, 2010; Turner *et al.*, 2012). Yeast cell volume increases regularly up to the verge of G1-S progression (Di Talia *et al.*, 2009), until it reaches the proper size, sensed by the proteins content, to enter in S phase. The time needed for the cell to reach that size is therefore dependent on its initial size, longer for smaller cells and *vice versa* (Di Talia *et al.*, 2007; Alberghina *et al.*, 2009; Alberghina *et al.*, 2012). Moreover, *S. cerevisiae* naturally displays a large variation in cell volume, which is due mainly to the co-existence in a culture of mothers of different ages, many of which attached to buds in several states of development (Alberghina *et al.*, 2009). These volume variations do not have implications in the culture μ_g during exponential phase, or mass doubling time. Accordingly, cultures of yeast mutants affected in cell size (higher or smaller) grow at a μ_g undistinguishable from the correspondent wild type (Jorgensen *et al.*, 2002). Identically, the μ_g of gal-3 exposed cells, as well as the cell cycle progression through G1, were unperturbed. Cell size is controlled mainly by nutrient availability. The leading nutrient receptors able to sense external nutrients activate the downstream pathways of PKA (protein kinase A) and TOR (target of rapamycin) (Zaman *et al.*, 2008; Busti *et al.*, 2010; Turner *et al.*, 2012). In addition, in yeast cell, some TOR and PKA independent signalling pathways are present that contribute to the sensing of nutrient availability. These are the cases of acetyl-coA concentration (Cai *et al.*, 2011; Cai and Tu, 2011) and the accumulation of storage carbohydrates (Futcher, 2006).

The yeast cultures used in the present study were grown exponentially on glucose, conditions that favour large cells (mothers as well as buds). The facts that (1) the cells exposed to gal-3 were larger, and (2) the length of G1 was not perturbed suggest that gal-3 induces a rupture in the molecular control of size. As said, the knowledge on the key operators of this control is not deep (Turner *et al.*, 2012). The progression through G1 in relation to size control is achieved through the inactivation of Whi5, a negative regulator of SBF transcription factor (Turner *et al.*, 2012). Whi5 inactivation is obtained through a molecular complex that harbours the key cyclin Cln3, whose transcription is nutrient dependent through acetyl-coA-mediated histone acetylation. Acetyl-CoA may derive from glucose fermentation or from acetate respiration (Shi and Tu, 2013). In view of the results above, it can be hypothesised that gal-3 might interfere in Cln3/ Whi5 regulation, promoting the regular timing of G1/S transition in spite of the oversized cells.

Since *S. cerevisiae* viability was affected by gal-3, the parameters that characterize the underlying death process were analysed. Different processes can lead to yeast cell death, among which the better characterized is PCD (Gourlay *et al.*, 2006; Carmona-Gutierrez *et al.*, 2010; Wloch-Salamon and Bem, 2012). To characterize gal-3-induced death, firstly intracellular ROS levels and plasma membrane breakage were assessed, then chromatin condensation, DNA fragmentation and mitochondria depolarization were evaluated. Cells of *S. cerevisiae* exposed to gal-3 displayed increased ROS levels, no plasma membrane rupture, condensed chromatin and fragmented DNA, but not mitochondria depolarization, ruling out PCD. These results suggest that *S. cerevisiae*, when exposed to gal-3, undergoes a non-apoptotic stress-related process. In opposition, gal-3 had a much less incisive effect on *C. albicans*, causing neither reduced viability nor DNA alteration nor ROS accumulation, although app. 7% cells presented plasma membrane breakage. Cell size apparently decreased slightly (-12%).

These results apparently contradict previous findings (Jouault *et al.*, 2006; Kohatsu *et al.*, 2006). Two key differences distinguish these works from the present one: genetic background and culture metabolic status. The present work used *S. cerevisiae* strain W303-1A, known for being more favourable for phenotypic assessment, but less resistant to stress than other genetic backgrounds (Petrezselyova *et al.*, 2010; Kokina *et al.*, 2014). In agreement, when

the assays were repeated with *S. cerevisiae* BY4741 used by Kohatsu *et al.* (2006) (Jouault *et al.* (2006) used BY4741 mother strain SC288) the same gal-3 amounts and exposure times did not induce a response. Importantly, unlike in (Kohatsu *et al.*, 2006), cells were exposed to galectins when they were young and in exponentially growing culture, *i.e.* highly homogenous cells under catabolite repression. The same is true for *C. albicans* assessment. Thereby, very different strains and culture conditions were used. These discrepancies clearly show that gal-3-induced responses in yeast are strain- and metabolic state-dependent, concurring with what discussed above regarding cell size control, and therefore not generalizable. Since it is hypothesized that the effect of gal-3 on yeast cells directly depends on al-3 binding to yeast surface, the variations of results for different strains and metabolic conditions was actually expected. Indeed there is a large variability in cell wall composition according to physiological status or environmental cues, such as temperature, pH, oxygen levels and growth medium (Aguilar-Uscanga and Francois, 2003; Klis *et al.*, 2006; Free, 2013). Differences in yeast cell wall molecular composition and mechanical resistance exist also between exponentially growing cells and cells that reached stationary phase (Smits *et al.*, 1999; Klis *et al.*, 2006). Cells in stationary phase have been proven to have thicker cell wall and be therefore stronger and more resistant to stress compared to exponentially growing cells (Smith *et al.*, 2000). Moreover, after post-diauxic shift cells are less permeable and more resistant to glucanase (De Nobel *et al.*, 1990). These changes are accompanied by variations in the wall proteins profile (Lagorce *et al.*, 2003). Similar changes were observed in cells subjected to stress, such as poor carbon source, or hyperosmotic or heat stress (Boorsma *et al.*, 2004).

Gal-4 and gal-7 (but not gal-1) caused increased ROS production and plasma membrane breakage, but not a reduction of viability. This was observed in both *S. cerevisiae* and *C. albicans*, although more severely in *S. cerevisiae*. Noticeably, although the observed increase of ROS production and membrane rupture was significant, it was not at the level of the heat-shocked cells (positive control). Altogether, the responses to the different galectins do not reflect their structural differences. Gal-7 and gal-1 belong to the *proto-type* group and gal-4 to the *tandem-repeat* group (Hirabayashi and Kasai, 1993), but gal-7 and gal-4 induced similar responses and gal-1 had no detectable effect. Of course, it cannot be ruled out the

possibility that the amounts of galectin and the exposure times used, which were optimized for gal-3, might not be the ideal ones for assessing the effects of other galectins.

The structure of gal-3 raised the question of whether the effects observed using this galectin would derive from the CRD recognizing and binding a specific ligand, and what role might play the N-terminal arm. The results obtained using a truncated form of gal-3 without the N-terminal arm suggest that the CRD is sufficient to promote all the effects caused by the full protein, though their intensity varied. ROS production and membrane breakage were enhanced, reaching levels very similar to the ones induced by gal-4 and gal-7, galectins that do not possess an N-terminal arm. On the other hand, truncated gal-3 induced a less evident decrease in viability, increase in cell size and DNA damage. These results suggest that, although the CRD is enough to trigger these responses, the N-terminal domain is important for full efficiency. N-terminal is known to promote multimerization of the protein, which in human is necessary to increase the efficiency of binding, increasing avidity to supply for low affinity (Brewer, 2002; Compagno *et al.*, 2014).

ConA is a lectin that binds yeast outer cell wall α-D-mannosyl residues (Lis and Sharon, 1973). It promotes the agglutination of many microorganisms including *C. albicans* (Cassone *et al.*, 1978; Sandin, 1987) and *S. cerevisiae* (Lis and Sharon, 1973; Flemming *et al.*, 1985). It was therefore expected to promote, as observed, the formation of large conglomerates of *S. cerevisiae* and *C. albicans*. These were indestructible by mechanical or physical action. The apparent reduction of *S. cerevisiae* viability upon incubation with ConA may actually derive from a bias in CFU caused by the extensive clumping. No other response to ConA was detected. Conglomeration was also observed in response to the presence of gal-4 and gal-7, though to a lesser extent than to ConA. This poses a question regarding binding specificity of the assayed galectins. The cell walls of *S. cerevisiae* and *C. albicans* do not display galactose residues (Klis *et al.*, 2006; Free, 2013). Yet, the phenotypic responses possibly require recognition and binding of the galectins to some component of the yeasts outer cell wall and signalling therein, while conglomeration demands for steady bridging between two cells after binding to components of the outer cell wall. Gal-3 was shown to bind to isolated β-1,2-oligomannosides derived from *C. albicans* VW32 (Fradin *et al.*, 2000). However, *S. cerevisiae*

does not produce that type of glycans (Fradin *et al.*, 2000; Jouault *et al.*, 2006). Therefore, we hypothesize that gal-3, and the other galectins, probably ligate as well to other components of the yeast outer layers of the cell wall.

In human cells, gal-3 is involved in the regulation of cell proliferation and differentiation, interacting with the oncoprotein KRAS (Elad-Sfadia *et al.*, 2004; Shalom-Feuerstein *et al.*, 2005; Shalom-Feuerstein *et al.*, 2008; Levy *et al.*, 2010; Levy *et al.*, 2011; Song *et al.*, 2012). In yeast, the Ras/cAMP/PKA pathway is very important for physiology and multiplication. Yeasts have two highly homologous and partially redundant Ras proteins, Ras1 and Ras2. These are palmitoylated and attached to the inner leaflet of the plasma membrane, mediating intracellular signalling progression upon glucose sensing (Thevelein and de Winde, 1999; Busti *et al.*, 2010; Tamanoi, 2011). When nutrients are suitable, Ras proteins are active and activate adenylate cyclase. This produces cAMP from ATP, which acts as a messenger to signal the good state of the environment, activating mainly PKA (protein kinase A). The activation of PKA leads to many outcomes. First, it regulates cyclin synthesis allowing the cell cycle to progress (Thevelein, 1992; Hubler *et al.*, 1993; Mizunuma *et al.*, 2013) and it controls the nutritional reserve of the cells, regulating the enzymes involved in lipid and carbon metabolism and the level of storage of carbohydrates (Ortiz *et al.*, 1983; Francois *et al.*, 1984; Francois and Hers, 1988). Moreover, it has a role in the defence against ROS and other stress stimuli, negatively regulating genes controlled by promoters harbouring STRE (stress response elements) (Lee *et al.*, 1999; Longo, 2003). One of the main outcomes of the Ras pathway activation is culture growth in response to nutrients availability, but Ras/cAMP/PKA pathway is also involved in apoptosis (Heeren *et al.*, 2004; Gourlay and Ayscough, 2006; Frohlich *et al.*, 2007; Almeida *et al.*, 2008) and aging (Hlavata *et al.*, 2003; Burtner *et al.*, 2009; Santos *et al.*, 2012), mainly allowing the accumulation of ROS inside the cell. Moreover, Ras proteins have a significant role in the regulation of cell size. As stated above, the two main pathways able to regulate cell volume include PKA and TOR (Zaman *et al.*, 2008; Busti *et al.*, 2010; Turner *et al.*, 2012). Ras proteins are well known upstream regulators of both PKA and TOR (Zaman *et al.*, 2008; Busti *et al.*, 2010). As confirmation of their direct involvement in cell size regulation, the cells harbouring the overactive variant of *RAS2*, $RAS2^{val19}$, were proven

to be bigger than the ones expressing the wt Ras2 protein (Baroni *et al.*, 1989; Baroni *et al.*, 1994). Taking into account all these factors, an involvement of this pathway in the response to gal-3 was hypothesized.

Results using the mutant deleted for *RAS2*, therefore not producing the more expressed of the two Ras proteins, clearly showed that the Ras pathway is necessary to mediate the effects of gal-3 on *S. cerevisiae*. All of the previously found gal-3-generated phenotypes were absent from the *Δras2* mutant. One possible explanation is that gal-3 might perturb Ras/cAMP/PKA pathway, erroneously signalling a false abundance of nutrient. Indeed the cells start to enlarge- gal-3 increased cell size-, but when the nutrient is not found, the cells undergoes stress stimuli, - the ROS level increases and DNA is damaged -, culminating in viability loss. A similar outcome of Ras/cAMP/PKA hyper-activation was noticed by Gourlay and Ayscough (2006). Another hypothesis is that gal-3 causes an abnormal activity of PKA and TOR driven by Ras proteins and this might cause an anticipated ageing effect (Hlavata *et al.*, 2003; Burtner *et al.*, 2009; Santos *et al.*, 2012), leading as well to an increased ROS production and a decreased viability.

Lectins induced in *S. cerevisiae* and *C. albicans* the appearance of two populations of cells (Pi and Pii) with different shapes that include cells with different complexity and size. The untreated sample, on the contrary, only showed one population of cells (Pi). Pii generated by gal-3, gal-4 and gal-7 had the same shape, and cells belonging to it showed similar ROS production and degree of plasma membrane rupture, though gal-3-induced Pii contained fewer cells. Both the ROS level and the membrane breakage were much higher in Pii compared to Pi. On the other hand, gal-1 and ConA generated a Pii shaped differently, with cells that showed less complexity compared to the ones in Pii triggered by gal-3,-4 and-7. Gal-1- and ConA-induced Pii did not show different level of ROS or plasma membrane breakage compared to the main population (Pi), contrarily to what happened for the other galectins. These results were identical in both yeast species and extremely well conserved in multiple replicas. The increased response to the galectins (-3,-4 and-7) and the reproducibility of the results led to hypothesise that the Pii population actually represents the cells in touch with the galectins. The fact that gal-4 and gal-7 generated a more numerous Pii population than

gal-3 could derive from the fact that these galectins have two CRD, which also propitiates the agglutination of the yeasts, as observed. Following this reasoning, gal-3, having only one CRD, should have half the chances of establishing a productive binding. This means that all the results in which the yeast sample exposed to a galectin was taken as a whole, actually are diluted by a part of the sample that was not in effective contact with the galectin. Assays like DAPI staining or CFU quantification do not allow the visualization of two populations, but ROS production and plasma membrane rupture assays using each population separately confirmed this suspicion. Importantly, in the *RAS2* deletion mutant, Pii is absent, reinforcing the ideas of Ras2 being necessary to mediate gal-3 effects and Pii being the population actually affected by galectins exposure. Therefore, and bearing in mind that more time of exposure did not increase the effect of gal-3, it can be stated that the observed responses to the galectins should be considerably stronger if it were possible to increase the ratio of [galectin] / [yeast cells].

In summary, we proved for the first time that galectin-3 has a significant effect on *S. cerevisiae*, leading to stress-related symptoms that do not match with an apoptotic profile. The effects on *S. cerevisiae* depend on the strains and the physiological status of the cells. This may prove that gal-3 cannot be the only protein involved in the process of discriminating between pathogenic (*C. albicans*) and non-pathogenic yeast. The responses of the yeasts to gal-3 are mainly mediated by the CRD of the protein, since when the truncated form (no N-terminal domain) was used, all the phenotypes were maintained, although their intensity varied. Therefore, the CRD is the minimum requirement to elicit an effect, but alone it does not reach full efficiency. We also showed that gal-3 action on *S. cerevisiae* requires a completely functional Ras/cAMP/PKA pathway, since all the gal-3 induced phenotypes were absent from *Δras2* mutant. Finally, we demonstrated that the effects are galectin specific, with the exception of gal-4 and gal-7, which provoked very similar phenotypes, and that the similarities do not relate to the galectins subgroup.

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Chapter 2

Microarray-based Study on the Binding of Galectins to Yeast Cell Surface

Abstract

Galectins are carbohydrate binding proteins belonging to the lectin superfamily. Their carbohydrate recognition domain (CRD) presents high affinity for β-galactosides, but some galectins have been proved to be able to bind different types of glycans, such as mannosides. There are several members in the galectin family and each has a specific affinity for differentially long and branched β-galactosides. Galectins are subdivided into three subgroups according to their structure and number of CRDs, the *proto-type* group, the *tandem-repeat* group and the *chimera* type group. Galectins have an important role in the recognition of glycans pattern on self and non-self cells, including various pathogenic microorganisms, from viruses to bacteria, to parasites. Gal-3, the only *chimera* type galectins, has been reported to be able to bind to β-1,2-linked oligomannosides on the cell wall of *C. albicans*. In this work, we analysed the binding of various galectins, representing each galectin subgroup, to the cells of two different yeast species, the non-pathogenic *S. cerevisiae* and the pathogenic *C. albicans*, using the microarray technology. We used both whole yeast cells and fractions derived from sub-cellular compartments and yeast extracellular matrix. We printed on the microarrays two different *S. cerevisiae* genetic background, and both wt and two *RAS* deletion mutants. We proved that all the galectins tested except gal-1 could successfully bind to the yeast sub-cellular fractions, with a higher efficiency to *S. cerevisiae* than to *C. albicans*. We showed that the galectin binding using whole cells as ligands was slightly less efficient than using sub-cellular concentrated fractions, but still capable to present significant binding, especially in the case of gal-7. Finally, we demonstrated that binding to *S. cerevisiae* was stronger than binding to *C. albicans* with both whole cells and sub-cellular fractions and with all the galectins tested, except gal-1, which did not present any binding to none of the two yeasts. In addition, binding to *RAS2* mutant was higher than to the wild type in most of the cases. Importantly, the genetic background influenced the binding ability of the galectins. In conclusion, we proved that microarrays might be an efficient and fast methodology to determine galectin binding to yeast surface and, with deeper studies, to characterize galectins binding profile and yeast surface components, as well as factors that may be important in discriminate between pathogenic and non-pathogenic yeast recognition.

Introduction

Galectins belong to the lectin superfamily of glycan binding proteins. They are identified based on the presence of approximately 130 conserved amino acids that form the carbohydrate recognition domain (CRD) (Cooper and Barondes, 1999; Compagno *et al.*, 2014), which has a specific tertiary structure of two antiparallel β sheets forming a sandwich-like structure (Rini and Lobsanov, 1999) and presents a high affinity for β-galactosides (Barondes *et al.*, 1994). The specificity of the binding depends on the member of the galectin family, but a generalization can be made. Usually the interaction with the monosaccharide galactose is weaker compared to the disaccharide lactose and longer oligosaccharides (Leffler and Barondes, 1986; Brewer, 2002). Until now, 15 mammalian galectins have been discovered and they are subdivided into three groups based on their structure and CRDs number. The *proto-type* galectins are characterized by a unique CRD and by the ability to act as monomer or homodimer (gal-1,-2,-5,-7,-10,-11,-13,-14,-15); *tandem-repeat* type galectins are defined by the presence of two distinct CRDs, not necessarily with the same binding properties, connected by a linker polypeptide (gal-4,-6,-8,-9,-12); *chimera* type is composed by a CRD at the C-terminal and a non-carbohydrate-binding domain and the N-terminal (gal-3). Different galectins do not have a redundant function in the cells, since they bind glycans with different specific affinities and each galectin is expressed in a tissue-specific or developmentally regulated fashion (Colnot *et al.*, 1996; Compagno *et al.*, 2014).

Galectins can be found both inside and outside the cells and they have specific functions in both cases (Yang *et al.*, 2008). Some galectins, such as gal-1,-3,-4, are found both intra- and extracellularly, while some others have a preferential localization, like gal-7, which is mainly intracellular (Liu *et al.*, 2002; Yang and Liu, 2003). Extracellular galectin functions depend on their ability to oligomerize and cross-link their ligands. Indeed all galectins, regardless their subtype, are able to cross-link. *Tandem repeat* types use their two CRDs, while *proto-type* galectins and the *chimer*a type galectin-3 form multivalent complexes (Boscher *et al.*, 2011). When galectins cross-link their ligands, they form supramolecular complexes called *lattices* and they promote the redistribution or the segregation of the bound ligands at the cellular

membrane, increasing the avidity and half-life of galectin-glycans interactions (Yang *et al.*, 2008; Rabinovich and Croci, 2012; Compagno *et al.*, 2014). Usually, most of the extracellular functions of galectins relate on their ability to bind to the glycans of glycoconjugates from the extracellular matrix, the plasma membrane, or the cell wall, while their intracellular functions mainly depend on protein-protein and protein-nucleic acids interaction (Rabinovich and Croci, 2012).

Extracellular galectins can bind to various glycans and exert several different functions, including cell adhesion, interaction with transmembrane receptors that influence cell growth, apoptosis, cell differentiation and immunity (Rabinovich and Toscano, 2009; Rabinovich and Croci, 2012; Compagno *et al.*, 2014). In particular, the role of galectins in immunity is dual and the particular function depends on the member of the galectin family and its binding specificity. Galectins can recognize both self-glycans and foreign glycans, helping the cell to distinguish pathogens from their own cells, in order to set up a proper defense (Rabinovich and Toscano, 2009; Sato *et al.*, 2009). Importantly, cells from different origins are able to synthetize different glycoconjugates, making the glycomic profile of a cell a specific and unique characteristic. In particular, mammalian cells have an array of glycoconjugates distinct from the one of pathogenic microorganisms, such as viruses, bacteria, fungi and parasites (Reuter and Gabius, 1999; Gabius *et al.*, 2002), making glycans on the cell surface highly specific for a particular class of cells or organisms, so that they serve as a basis for recognition by host proteins (Buzas *et al.*, 2006). In the specific case of pathogen recognition, galectins can either block or promote the infection. Galectins can block invasion either directly killing the microorganism or stimulating the activation of the cells of the immune system (Buzas *et al.*, 2006; Sato *et al.*, 2009; Vasta, 2009; Baum *et al.*, 2014). For example, gal-1 promotes the entry and attachment of HIV-1 (human immunodeficiency virus type 1) (St-Pierre *et al.*, 2011; Sato *et al.*, 2012), Nipah viruses (Levroney *et al.*, 2005) and human T cell leukaemia virus type 1 (HTLV-1) (Gauthier *et al.*, 2008) by cross-binding glycans on both the pathogen cell wall and human cells, while gal-4 impedes *Bordetella pertussis* and *Helicobacter pylori* to attach to glycolipids receptor occupying them (Ideo *et al.*, 2005; Danielsen and Hansen, 2006). Galectins can bind to a high variety of glycans present on the surface of several different

pathogens, from viruses, such as the above-mentioned HIV-1 (St-Pierre *et al.*, 2011; Sato *et al.*, 2012), to bacteria, such as *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*, to parasites, such as *Leishmania major*, to fungi, such as *Candida albicans* (Sato *et al.*, 2009).

The interaction between *C. albicans* and one of the galectin proteins, gal-3, has been analysed. Gal-3 has been found to bind to β-1,2-linked mannopyranose units present on the cell wall of *C. albicans* (Fradin *et al.*, 2000). Interestingly, these glycans are a specific feature of *C. albicans*, absent from *Saccharomyces cerevisiae* or in other species of *Candida.* Indeed it is reported that gal-3 can only bind to *C. albicans* and not to *S. cerevisiae* or *C. granulomata* (Kohatsu *et al.*, 2006). The interaction between gal-3 and this type of glycan was found unusual, since gal-3 binds specifically to β-galactosides. However, it is not unique among galectins. Gal-10 has indeed been found to have a higher affinity for mannose-related sugars than to β-galactosides (Yang *et al.*, 2008; Cummings and Liu, 2009). It was verified that gal-3 was actually bound to *C. albicans* cell wall in living yeast cells in a carbohydrate-dependent manner (Jouault *et al.*, 2006) and had a direct fungicidal effect on *C. albicans*. Indeed, yeast cells treated with gal-3 diminished their viability and simultaneously decreased the cell size and enhanced the cell granularity (Kohatsu *et al.*, 2006). Notably, the importance of gal-3 role in the invasion of *C. albicans* is underlined by the fact that tissues affected by systemic candidiasis show a broader expression of gal-3 (Kohatsu *et al.*, 2006) and that the absence of gal-3 increases mortality upon infection with *C. albicans* (Linden *et al.*, 2013a). In a proteomic analysis, gal-3 expression has been found to increase in macrophages after treatment with *C. albicans* (Reales-Calderon *et al.*, 2012). The observation that the secretion of gal-3 increased upon *C. albicans* invasion was validated also in other models. For example, gal-3 secretion is upregulated in human yeast infected gingival epithelial cell line Ca9-22 and gingival fibroblasts (Tamai and Kiyoura, 2014). Also neutrophils increase their secretion of gal-3 upon treatment with *C. albicans* and this causes an enhanced phagocytosis of the pathogens (Linden *et al.*, 2013b). Even if an interaction between gal-3 and *C. albicans* seems to be well-established and relevant for *C. albicans* pathogenic process and host cell defense, little is known about the interaction of *C. albicans* with the other members of the galectin family. Considering the unicity of gal-3 structure, being the only *chimera* type galectin, it

would be useful to understand if the putative specific binding to *C. albicans* is due to gal-3 ability to form supramolecular lattices or to the binding affinity of its CRD. For this purpose, binding to *C. albicans* cells and components of its surface should be tested also for other galectins, possibly representing different galectins subgroups.

Importantly, gal-3 has been proposed to be responsible for distinguishing between pathogenic – *C. albicans* – and non-pathogenic – *S. cerevisiae* – yeasts, based on its ability to bind solely to *C. albicans* (Kohatsu *et al.*, 2006). However, authors report using two serotypes of *C. albicans* and one strain of *S. cerevisiae*. The strains of *C. albicans* originated from a credited culture collection. Laboratory strains are well known for loosing infectiousness. Therefore, other more virulent *C. albicans* strains (preferably clinical isolates), as well as other more common species in human yeast infections, like *C. glabrata*, *C. parapsilosis, C. krusei* or *C. dublinensis* (Vazquez-Gonzalez *et al.*, 2013) should have been used. Additionally, also *S. cerevisiae* strains strongly diverge genetically. This is extremely important when considering the possible contact of a patient with a wild yeast strain of any origin, including the ones causing nosocomial infections (Pillai *et al.*, 2014). Finally, it cannot be forgotten that most yeasts causing serious infections in humans (including *S. cerevisiae*) are commensal, and that the transition to the infectious aggressive state demands yeast to undergo profound molecular remodelling. Specific culture conditions are needed to trigger these changes (like for example cultivating the yeast in human blood serum), which were not used by Kohatsu *et al.*, (2006). Results of these authors are this way far too scarce and innapropriate to support such a daring hypothesis as gal-3 being responsible *in vivo* for discriminating between the pathogeneic and non-pathogenic state or ability of yeasts. Therefore, the binding of galectins to *S. cerevisiae* should be verified, both to confirm its putative absence and to better understand gal-3 specificity towards *C. albicans*.

The differential binding of gal-3 to *C. albicans* and *S. cerevisiae* seems to be due to different glycans expressed on the cell wall, the outer layer of a yeast cell. Specifically, all pathogenic *C. albicans* strains produce poly-β-1,2-linked mannose chains that bind specifically the organism to the macrophages (Li and Cutler, 1993; Gemmill and Trimble, 1999), but these structures differ among different Candida species and even among different serotypes belonging to

the same species (Gemmill and Trimble, 1999). This type of mannosylated structures were not found in *S. cerevisiae* (Suzuki, 1997; Gemmill and Trimble, 1999; Fradin *et al.*, 2000). Regardless to the specificity in composition, the organization of the cell wall of *S. cerevisiae* and *C. albicans* is very similar (Aguilar-Uscanga and Francois, 2003; Ruiz-Herrera *et al.*, 2006; Free, 2013). In both yeast species, the side closer to the plasma membrane is composed mainly by β-1,3-glucans and, to a lesser extent, by β-1,6-glucans, and it includes as well small amount of chitin (Klis *et al.*, 2006; Free, 2013). The layer faced outside is richer in highly mannosylated proteins, mainly GPI-anchored to the glucans network (Gemmill and Trimble, 1999; Klis *et al.*, 2006). As mentioned, even though the basal structure of the wall is very similar between the two yeast species, the specific composition in polysaccharides and proteins varies extensively. In particular, differences in the side branching of the glucans have been shown (Ruiz-Herrera *et al.*, 2006), as well as a great variety in glycoprotein glycan structures (Fukazawa *et al.*, 1995; Gemmill and Trimble, 1999). Besides presenting differences between yeast species, yeast wall composition can also change upon different environmental cues, such as temperature, pH, oxygen levels and growth medium (Aguilar-Uscanga and Francois, 2003; Klis *et al.*, 2006), and in different growth phases (Smits *et al.*, 1999). Cells in stationary phase have been proven to have thicker cell wall, to be less permeable and to be more resistant to glucanase compared to exponentially growing cells (De Nobel *et al.*, 1990; Smith *et al.*, 2000). These changes are accompanied by variations in the wall proteins profile (Lagorce *et al.*, 2003). Similar changes were observed in cells subjected to stress, such as poor carbon source, or hyperosmotic and heat stress (Boorsma *et al.*, 2004).

In addition to bind glycoconjugates on the cell wall, galectin can also bind to glycoprotein in the extracellular matrix (ECM), such as laminin (Zhou and Cummings, 1990; Sato and Hughes, 1992), fibronectin (Sato and Hughes, 1992; Ozeki *et al.*, 1995), and tenascin (Probstmeier *et al.*, 1995). These interactions, similarly to the one occurring on the cell wall, activate important signalling cascades that have a pivotal role in cell adhesion and motility. Variations in adhesion and motility mainly depend on reorganization of actin cytoskeleton and formation of adhesion plaques (Levy *et al.*, 2003; Goetz *et al.*, 2008). The ECM in multicellular organisms is fundamental for cells stability and organization, as well as for

cell-cell communication and adhesion (Nelson and Cox, 2008). It is composed of diverse molecules, including glycosaminoglycans and proteoglycans (Esko *et al.*, 2009). Similarly to the glycans present on the cell surface, also the glycans present in the ECM vary a lot (Schaefer and Schaefer, 2010), making galectins once again the perfect transducer of the ECM glycans signals. Importantly, also unicellular organisms such as bacteria and fungi can create a substrate resembling the mammalian ECM, when grown in communities such as stalks, mats/biofilms and colonies (Sutherland, 2001; Nobile and Mitchell, 2007; Lal *et al.*, 2010). Also the ECM of the yeast *S. cerevisiae* and *C. albicans* is formed by various components, including proteins and glycans (Chandra *et al.*, 2001; Kuthan *et al.*, 2003; Al-Fattani and Douglas, 2006; Vachova *et al.*, 2011). Recently, a specific method was developed to isolate and extract the ECM of *S. cerevisiae* and *C. albicans*, and to identify the components of the correspondent sugar and protein fractions (Faria-Oliveira *et al.*, 2014). The glycans composing the ECM of *S. cerevisiae* are mostly oligosaccharides of mannose, constituting 44% of total sugar content, glucose, accounting for the 52%, and, to a lesser extent, galactose (4%). The presence of uronic acid was also detected (Faria-Oliveira *et al.*, 2015). Possibly, many of the above mentioned sugars are attached to proteins, forming glycoproteins and proteoglycans (Sutherland, 2001; Vachova *et al.*, 2011). Giving the prevalence of glycans in the composition of yeast ECM, including a small percentage of galactose, it is conceivable that galectins could bind to components of yeast ECM. Moreover, ECM has been identified as involved in *C. albicans* drug resistance (Chandra *et al.*, 2001; Al-Fattani and Douglas, 2006) and gal-3 has been involved in identifying specifically pathogenic fungi. Therefore, it could be informative to analyse the possible binding of galectins to yeast ECM, both *S. cerevisiae* and *C. albicans*.

One method to analyse quickly the binding of galectins to yeast cells and yeast cell components is through the use of microarrays. This methodology, since its first application more than 20 years ago (Ekins, 1989), has been used for a variety of different applications. DNA microarrays were the first to be optimized and they have been used for DNA sequencing (Pease *et al.*, 1994), analysis of gene expression profiling (Schena *et al.*, 1995; Morley *et al.*, 2004) and diagnosis (Mikhailovich *et al.*, 2008; Donatin and Drancourt, 2012), among other applications (Szameit *et al.*, 2009; Fan *et al.*, 2010; Horvath *et al.*, 2011). Now, new types of

microarrays have been developed, such as proteome microarrays (Zhu *et al.*, 2001; Chen *et al.*, 2008), antibody microarrays (Haab *et al.*, 2001; Rho and Lampe, 2013), reverse-phase protein arrays (Paweletz *et al.*, 2001; Sheehan *et al.*, 2005) and lectin microarrays (Tao *et al.*, 2008; Hirabayashi *et al.*, 2013). Various groups simultaneously developed microarrays for lectins in 2005 (Angeloni *et al.*, 2005; Kuno *et al.*, 2005; Pilobello *et al.*, 2005; Zheng *et al.*, 2005). These are based on the immobilization of lectins on a glass substrate, possibly presenting a wide array of glycans specificity. The advantages of this methodology are the direct usage of crude samples containing glycoproteins, without the need of glycans isolation, and the high number of samples that can be analysed to obtain their glycomic profiling (Hirabayashi *et al.*, 2013). A natural extension of the lectins microarrays are the glycans microarrays. Indeed, if in the case of lectins microarrays lectins are immobilized and probed with glycans, in the case of glycans microarrays, the carbohydrates are the ones to be printed on the microarrays and then tested with different lectins and/or antibodies (Liu *et al.*, 2007; Park and Shin, 2007; Blixt *et al.*, 2008; Karamanska *et al.*, 2008; Narla and Sun, 2012; Park *et al.*, 2013; Narla *et al.*, 2015). Glycans microarrays allows the detection of binding specificity of disease-associated antibodies (Galanina *et al.*, 2003; Wang *et al.*, 2008) and lectins, as well as the carbohydrates recognized by endogenous lectins (Blixt *et al.*, 2004; Palma *et al.*, 2006; Graham *et al.*, 2012). In addition, they have been particularly helpful in the analysis of the glycans recognized by pathogenic microorganism such as viruses and bacteria, which therefore can have pivotal roles in the onset of immune response, or pathogen-host cell adhesion and invasion (Blumenschein *et al.*, 2007; Childs *et al.*, 2009; Flannery *et al.*, 2015). The field of microarrays technology is constantly growing and one of the last developments was the direct printing of fixed bacterial cells to the microarray, to later test their glycans profile through the binding of various lectins (Campanero-Rhodes *et al.*, 2015). This approach avoids the isolation of glycans from the organism surfaces, making the recognition of binding specificity even faster.

In this work, the binding ability of whole cells of *S. cerevisiae* and *C. albicans* to various galectins using the microarray technology was tested for the first time. Additionally, yeast cells were fractioned and the yeast cell components isolated. Fractions consisting of cytoplasmic

contents, plasma membrane, cell wall and ECM, were used to confirm the galectins binding capacity. Four different galectins were used, representing each subgroup, gal-3 for the *chimera* type, gal-1 and gal-7 for the *proto-type* and gal-4 for the *tandem-repeat* type. In addition, different *S. cerevisiae* genetic backgrounds were used: the frequently used laboratory strains BY4741 and W303-1A. This strategy aimed at showing that different genetic backgrounds also have differences at the level of surface glycans. Finally, also the *RAS* mutant strains of both genetic backgrounds were used, in view of the demonstrated mediation of yeast Ras proteins for exogenous (chapter 1, this thesis) and endogenous (chapter 3, this thesis) gal-3 activity on *S. cerevisiae*, and because of the involvement of Ras proteins in the modelling of yeast cell wall (Imazu and Sakurai, 2005).

For the first time a microarray methodology was shown to be efficient for testing the glycans profile of the surface of yeasts, both *C. albicans* and *S. cerevisiae*. Moreover, contrarily to previous reports (Kohatsu *et al.*, 2006), gal-3 bound to *S. cerevisiae* cells, actually with a higher intensity than to *C. albicans*. Binding analysis also displayed differences between the various galectins specific binding profile. Importantly, the variation of galectins binding not only confirm the involvement of the Ras pathway in the yeast cell response to galectins, as predicted from the results in the chapters 1 and 3, but also indicated that the pathway does that by altering the pattern of the cell wall implicating in binding ability.

MATERIALS AND METHODS

Preparation of samples with yeast whole cells

Whole cells used for microarrays printing are listed in **Table 1**. Printing was done using cells fixed in paraformaldehyde (PFA) or alive in suspension.

Fixation in paraformaldehyde. Cells were grown in 10 ml YPD (2% glucose, 1% peptone, 1% yeast extract) batch cultures, at 30°C and 200 rpm, to $O.D._{600}$ 1.5, and collected by gentle centrifugation, to avoid disruption. PFA 37% was added to the pellet to a final concentration of 1:10 and incubated 10 min at room temperature. This fixation procedure preserves the cells physiological status and morphology. Cells were washed 3 times with filtered PBS and resuspended in a final volume of 1 ml TBS (tris-buffered saline, 10 mM Tris/HCl, 0.15 M NaCl).

Alive cells suspension. Yeasts were grown on solid YPD at 30°C for 48 h. A portion of the yeast biomass was used to dissolve in TBS and the suspension was adjusted to $O.D._{600}$ of 10.

Considerable differences in cell size exist between the different yeast strains causing biases in $O.D._{600}$ readings. Therefore, the cell concentration in each sample, both PFA-fixed cells and cells in suspension, was confirmed and normalized by using a Neubauer chamber, in order to assess the precise correlation between $O.D._{600}$ and number of cells. Each sample was supplemented with 1 µl 5 mM SYTO® 13 dye (Life Technologies), and the samples were diluted with TBS to $O.D._{600}$ 6.0, 3.0 and 1.0.

The fluorescence spectrum of the last sample ($O.D._{600}$ 1) was obtained using a Horiba Jobin Yvon Fluoromax-4 spectrofluorometer (excitation 488 nm and emission 500-600 nm).

Table 1: Yeast strains used for the printing of the microarrays.

N°	yeast strain	genotype
	Saccharomyces cerevisiae	
1	BY4741 wt	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*
2	BY4741 *Δras1*	Isogenic to BY4741 but *ras1::KanMX4*
3	BY4741 *Δras2*	Isogenic to BY4741 but *ras2::KanMX4*
4	W303-1A wt	*MATa leu2Δ3 leu2Δ112 ura3Δ1 trp1Δ1 his3Δ11 his3Δ15 ade2Δ1 can1Δ100*
5	W303-1A *Δras1*	Isogenic to W303-1A but *ras1::HIS3*
6	W303-1A *Δras2*	Isogenic to W303-1A but *ras2::KanMX4*
	Candida albicans	
7	BPW17	*ura3::imm434/ura3::imm434 iro1/iro1::imm434 his1::hisG/ his1::hisG arg4/arg4*

Whole cells binding signal normalization

The binding signal of the galectins bound to the whole yeast cell samples was normalized, in order to obtain the fluorescence/cell ratio. The number of cells in each printed spot was calculated using the following rule: fluorescence at 488 nm (SYTO® 13 dye) x n° cells in the printed suspension / maximum fluorescence at 511 nm (measured by spectrofluorometer). The galectin binding fluorescence emitted from a single cell was then calculated dividing the total spot fluoresce at 635 nm by the number of cells in each spot.

Cellular fractions preparation

Chosen yeast strains number 4, 6 and 7 (**Table 1**) were used to obtain the sub-cellular fractions of cytoplasm, membranes and cell wall, as well as the soluble fraction of the extra cellular matrix. ECM was extracted, freeze-dried and suspended in TBS as in Faria-Oliveira *et al.* (2014), without further processing, therefore including all the proteinaceous and

saccharide components of the matrix. Wall, membrane and cytoplasm were obtained as previously described (Ferreira *et al.*, 2006; Ferreira and Lucas, 2008). The protein content of all samples was quantified using the Lowry method (Lowry *et al.*, 1951). For that purpose, an aliquot of the samples corresponding to 100 µg was mixed with Laemmli buffer (Laemmli, 1970) 2x in a final volume of 20 µl, heated for 10 minutes at 95°C and let cool down at room temperature. In order to verify the protein abundance in each sample, these were analyzed by SDS-PAGE, using a 5% stacking and a 10% running polyacrylamide gel and staining with Coomassie Blue (Meyer and Lamberts, 1965). Before printing, the protein concentration of the samples was adjusted to 10 mg/ml and 1 µl of Cy3 fluorophore (GE Healthcare) was added to each sample to monitor printing efficiency. Samples were diluted in TBS to final concentrations of 3 mg/ml, 1 mg/ml and 0.3 mg/ml.

Printing of microarrays

Microarrays were printed manually as described in Campanero-Rhodes *et al.* (2015). The four concentrations- $O.D._{600}$ 10, 6, 3, 1 – of whole cells PFA-fixed and in TBS suspension and the four concentrations – 10 mg/ml, 3 mg/ml, 1 mg/ml and 0.3 mg/ml – of yeast cell fractions were used for microarray printing. Each sample dilution was printed in triplicate. The control glycoproteins Ribonuclease A (RbA) and B (RbB), Fetuin (Fet), Asialofetuin (AFet), Fibrinogen (Fg) and Asialofibrinogen (AFg) were printed in four concentrations 10-fold lower than yeast cell fractions (0.03 mg/ml, 0.1 mg/ml, 0.3 mg/ml and 1mg/ml). Arrays were scanned for detection of SYTO® 13 dye in whole cells sample at 488 nm (blue laser) and of Cy3 dye in fractions and control proteins at 532 nm (green laser). A GenePix 200-AL scanner (Axon, Molecular Devices) was used. Fluorescence signal was quantified with GenePix Pro 6.0 software (Molecular Devices).

Microarrays binding and inhibition assay

Lectins used for overlay (binding) of microarrays were Concanavalin A (ConA) at 20 µg/ml as control and gal-3, gal-1, gal-4 and gal-7 at 20 µg/ml and 50 µg/ml. Inhibition was performed adding to the overlay buffer the inhibitors of ConA (mannose 100 mM), and galectins (lactose

75 mM together with AFet 1 mg/ml). Binding and inhibition of the lectins to the yeast microarrays was performed as in Campanero-Rhodes *et al.*, 2015, with modifications. The microarrays were overlaid for 1.5 h. Some of the galectins required an extra step for detection. After washing, the microarrays were first incubated for 45 minutes with an anti-His (Sigma-Aldrich) antibody together with biotinylated anti-mouse (Invitrogen) (gal-3 and gal-4) or with anti-gal-1 (Santa Cruz Biotechnology) together with biotinylated anti-rabbit (Invitrogen) (gal-1) for 45 minutes and AlexaFluor 647 (AF647)-labelled streptavidin (Invitrogen) at 1 µg/ml was added. The mixture was incubated for 35 minutes. Different buffers were used: ConA required TBS supplemented with 10 mM $CaCl_2$ and 2 mM $MgCl_2$, gal-1, gal-3 and gal-4 required PBS + 4mM β-mercaptoethanol and gal-7 potassium phosphate buffer (0.2 M KH_2PO_4, 0.2 M K_2HPO_4 at pH 7.0) supplemented with 2 mM DTT (1,4-dithiothreitol). Microarrays were scanned for AlexaFluor 647 detection (excitation at 635 nm, red laser). Fluorescence signal of each spot was quantified with GenePix Pro 6.0 software (Molecular Devices).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software. One-way ANOVA analysis was used. The difference between experimental data was considered significant whenever p-value ≤ 0.05.

Results

Designing of the yeast microarrays and their validation

Two publications, Jouault *et al.* (2006) and Kohatsu *et al.* (2006), reported that gal-3 does not bind to *S. cerevisiae*, but can bind to *C. albicans*. Quite the contrary, the present work showed (chapter 1) gal-3 inducing a stronger phenotypic effect on *S. cerevisiae* than on *C. albicans.* A contribution to sustain the present results would come from experimental evidence that gal-3 and the other galectins used are able to bind efficiently to yeast cells. Microarrays allow the simultaneous evaluation of the binding intensity of multiple ligands, so they were chosen to evaluate the binding ability of galectins to yeasts, in particular to *S. cerevisiae.* Bacteria cells were successfully used before (Campanero-Rhodes *et al.*, 2015), but yeasts were never tried. That was done for the first time in the present work.

Microarrays were printed with yeast whole cells treated in two different ways. Cells were either fixed in paraformaldehyde or kept in suspension in TBS. First, the printing of the yeast whole cells was tested, since as said above this was the first time in which yeast whole cells were used to print directly on microarrays. PFA fixation can maintain the cell *status quo,* since the cells are physiologically dead, but supposedly intact (Fox *et al.*, 1985). However, it was previously noticed that this fixation procedure may alter the binding ability of *C. albicans* (Mleczko *et al.*, 1989). Considering that PFA is routinely used to cause micro-fractures in the yeast cell wall (Williamson and Fennell, 1979; Inacio and da Luz Martins, 2013), it is expectable that this compound alters some of the characteristic of the yeast surface, which is critical for evaluating binding ability. It was therefore chosen to pair the PFA-fixed yeasts with yeast in TBS suspension. TBS is a mild buffer that does not affect the yeast cell surface characteristics. The method though presents some problems. The samples are easily contaminated with bacteria or fungi, and because the TBS does not have nutrients, cells quickly enter into starvation and begin to die. The assays must thus be performed in a very narrow window of time.

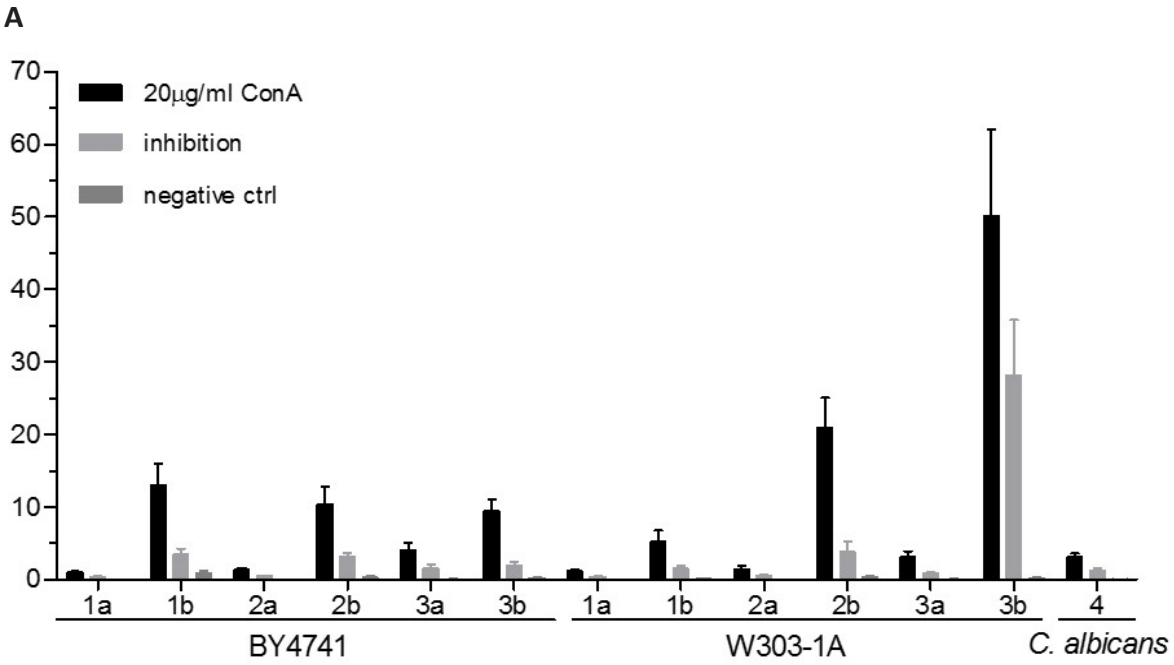
Several strains of *S. cerevisiae* and one of *C. albicans* (BPW17) were used to print onto microarrays. In the case of *S. cerevisiae*, both fixation methods were used, while in the case

of *C. albicans*, only the PFA-fixed yeast was available. This was due to restrictions imposed by the equipment and the laboratory (Agencia Estatal Consejo Superior de Investigaciones Científicas, Instituto de Química-Física Rocasolano, Madrid, Spain) to the manipulation of potentially pathogenic microbes. Two different genetic backgrounds of *S. cerevisiae* were used. In chapters 1 and 3 of this thesis different behaviour of the strains BY4741 and W303-1A in several conditions was often described. We reported that they respond differently to stress *stimuli* (chapter 3) and, importantly, to exogenous gal-3 in solution (chapter 1). Thus, it was particularly important to compare the cells of BY4741 and W303-1A in regard of their ability to bind different galectins. In addition to the wt strains of *S. cerevisiae*, also the *RAS* deletion mutants were used. During our study on the interaction between gal-3 and Ras proteins, it appeared clear that not only human Ras, but also yeast Ras proteins relate with gal-3. Indeed, yeast *RAS2* mutant did not present any significant alterations induced by exogenous gal-3 in solution (chapter 1), further evidencing this notion. BY4741 and W303-1A *Δras1* and *Δras2* were therefore printed as well on the microarray. Several controls were performed in order to confirm the binding capacity of the yeasts printed on the microarrays and the binding efficiency of the galectins used.

ConA (20 µg/ml) was used to validate if the cells were printed properly, and if the methods used to maintain them – PFA fixation or TBS suspension – were suitable to evidence different lectin binding efficiency and specificity. ConA is a plant lectin reported to be able to bind very efficiently to the glycoconjugates of the yeast cell wall of both *S. cerevisiae* and *C. albicans* (Lis and Sharon, 1973; Sandin, 1987; Arvindekar and Patil, 2002; Warolin *et al.*, 2005). The binding of all the lectins (galectins as well as ConA) was accompanied by two controls. One control consisted in using the lectin together with a recognized specific inhibitor. Mannose was used to inhibit ConA and lactose together with AFet was used to inhibit all the galectins. The inhibitor should saturate the carbohydrate-binding site of ConA impeding this lectin to bind to the cell wall carbohydrates. The second control consisted in using the binding mixture without lectin and should evidence unspecific signal background. Results showed that the microarrays were printed properly. Every cell printed presented a binding signal, which varied according to the cell type (**Fig. 1A**). Moreover, the binding signal was higher than the

inhibition in all samples, indicating that mannose inhibition effectively impeded the signal from ConA binding (**Fig. 1A**). Moreover, the signal was almost absent in the negative control (without ConA), proving that the results were specific for the binding of ConA and that most of the signal obtained came from the binding of the lectin to the glycans on the yeast surface (**Fig. 1A**). These results indicate that, when present, the signal truly corresponded to the binding of ConA to the yeast cell wall constituents. The intensity of the binding of ConA to PFA-fixed cells was 3- to 16-fold smaller than the one of cells in TBS suspension (**Fig. 1A**), showing that the fixation method can be crucial for the detection of binding and prone to erroneously suggest binding ability and specificity.

Besides using ConA as binding lectin in alternative to galectins, and preventing the binding with specific inhibitors, other controls were made. To test the binding ability of the galectins, specific highly glycosylated proteins- Fg, AFg, Fet and AFet- were printed on the microarray. Two scarcely glycosylated proteins – RbA and RbB- were used as negative control. The proteins were printed in four different concentrations (0.03 mg/ml, 0.1 mg/ml, 0.3 mg/ml, 1 mg/ml), to determine the correlation between the protein concentration and the binding intensity. These proteins arrays were incubated with ConA (**Fig. 1B**). As expected, a strong binding to the highly glycosylated proteins and a much weaker signal coming from RbA and RbB, almost absent for RbA, was observed (**Fig. 1B**). Moreover, results showed that in most of the protein/concentration combinations, the binding of ConA to the control proteins depended on the protein concentration, increasing proportionally to their increasing amounts. The binding specificity and the accuracy of the microarray construction were confirmed by the absence of signal in the negative control and the inhibition sample.


A
70
60
50
40
30
20
10
0
20μg/ml ConA
inhibition
negative ctrl
1a
1b
2a
2b
3a
3b
1a
1b
2a
2b
3a
3b
4
BY4741
W303-1A
C. albicans

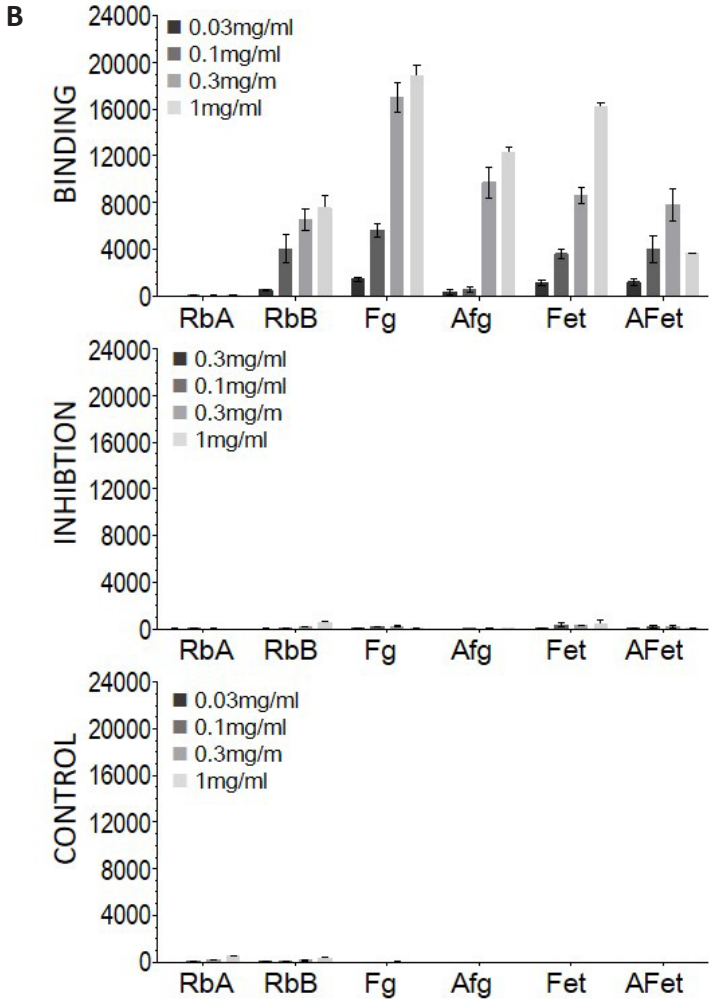

B
BINDING
INHIBTION
CONTROL
24000
20000
16000
12000
8000
4000
0
0.03mg/ml
0.1mg/ml
0.3mg/m
1mg/ml
RbA
RbB
Fg
Afg
Fet
AFet

Figure 1: *Concanavalin A binding to yeast whole cells and control proteins*. Normalized fluorescence intensity of the binding of ConA to different yeast cells was compared to the normalized fluorescence derived from the ConA inhibited by addition of mannose and to the negative control. Samples indicated as "a" are PFA-fixed whole cells, while samples named as "b" are cells in TBS suspension. Samples "1", "2" and "3" correspond respectively to the *S. cerevisiae* wt, *Δras1* and *Δras2* strains. Sample "4" is PFA-fixed *C. albicans* wt (**A**). The values of the normalized intensity are the average ±SD of 6 to 12 printed spots. The fluorescence derived from the binding of ConA to the control proteins RbA, RbB, Fg, AFg, Fet and AFet was determined (upper panel) and compared with the one derived from ConA inhibited by mannose addition (central panel) and the negative control (lower panel) (**B**). The intensity values are the average ±SD of the three spots printed with the same control protein concentration.

These control proteins (Fg, AFg, Fet and AFet; RbA and RbB) were subsequently used to test galectins. Following the work presented in chapter 1, four different galectins were used, gal-3, gal-4, gal-7 and gal-1. The choice was done bearing in mind each of the three galectins subgroups, *chimera, tandem-repeat* and *prototype*. Gal-3 is a *chimera* galectin, with one CRD and a long N-terminal domain, gal-4 is a *tandem-repeat* galectin, with two CRDs linked together, and gal-7 and gal-1 are *prototype* galectins, with only one CRD, but almost always dimerizing in solution (Hirabayashi and Kasai, 1993; Kasai and Hirabayashi, 1996; Römer and Elling, 2011). All the galectins were able to bind the control Fg, AFg, Fet and AFet proteins, even if with significantly different intensity (**Fig. 2**).

The highest binding signals (**Fig. 2A, B**, gal-3 and gal-4 to Fet) were comparable to, or even higher than the ones obtained using ConA. However, some differences between the behaviour of ConA and galectins were observed. There was not always a direct correlation between the protein concentration and the binding intensity. In the case of gal-3 (**Fig. 2A**) and gal-4 (**Fig. 2B**), the highest binding intensity was achieved with 0.3 mg/ml of AFet and not with the highest concentration (1 mg/ml). This could be due to signal saturation at the highest protein concentration, altering the actual perception of the signal intensity. Moreover, when using gal-7 (**Fig. 2C**) and gal-1 (**Fig. 2D**), the inhibition and the negative control signals were comparable or even higher than the binding signal. This means that the signal is unspecific, not originating from the binding of the lectins, but possibly from some reagent used for the detection of the signal. This is particularly so for gal-1 binding to Fg and AFg (**Fig. 2D**).

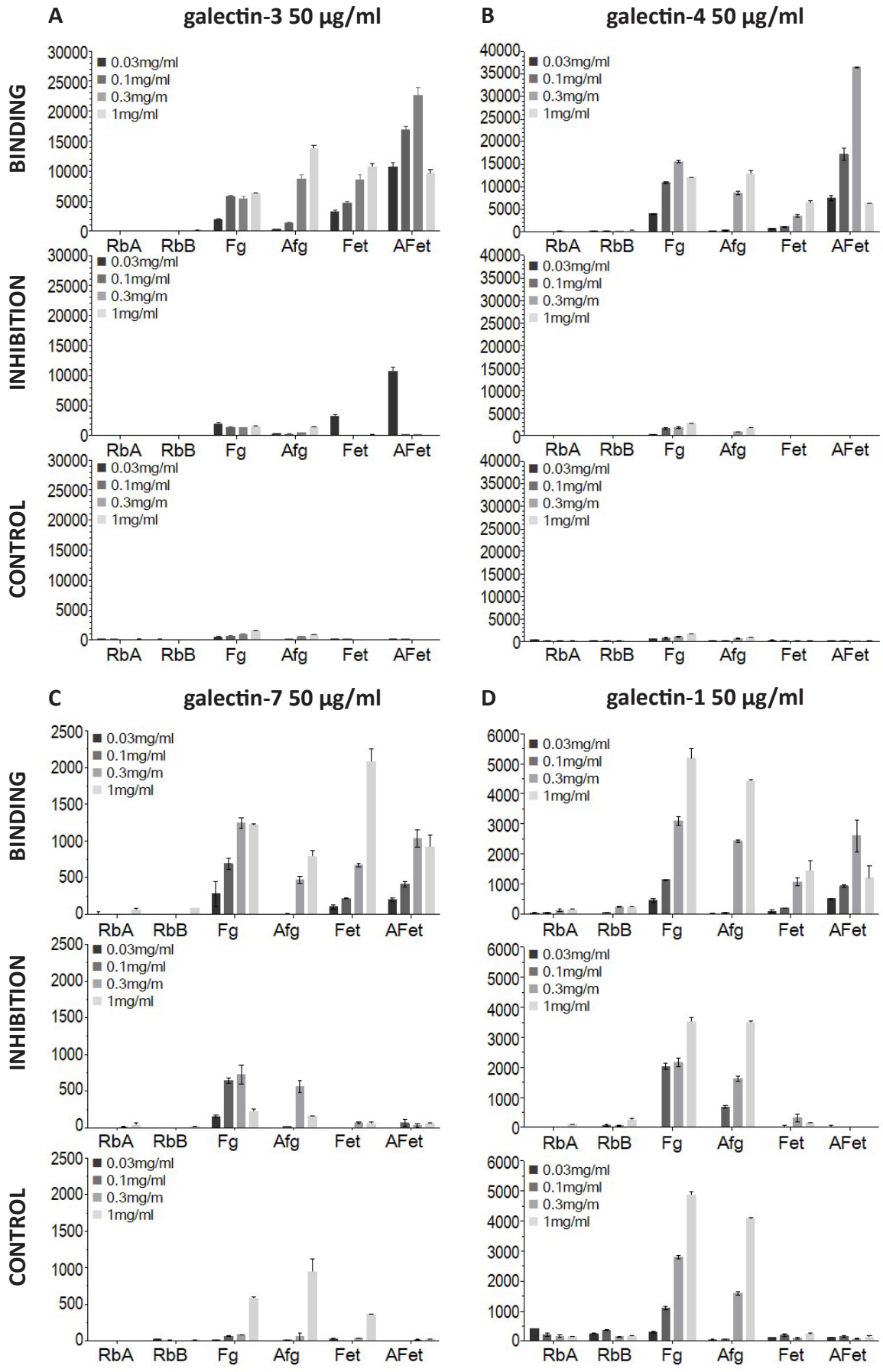

A
galectin-3 50 µg/ml
B
galectin-4 50 µg/ml
C
galectin-7 50 µg/ml
D
galectin-1 50 µg/ml
BINDING
INHIBITION
CONTROL
0.03mg/ml
0.1mg/ml
0.3mg/m
1mg/ml
RbA
RbB
Fg
Afg
Fet
AFet

Figure 2: *Galectins binding to the control proteins*. The fluorescence derived from the binding of gal-3 (**A**), gal-4 (**B**), gal-7 (**C**) and gal-1 (**D**) to the control proteins RbA, RbB, Fg, AFg, Fet and AFet was determined (upper panel) and compared with the one derived from the galectins inhibited by lactose and AFet addition (central panel) and with the negative control (lower panel). The intensity values are the average ±SD of the three spots printed with the same control protein concentration. Please note that the scale of the fluorescence intensity is maintained when binding, inhibition and negative control are compared, but it may change among different galectins.

Considering the binding results altogether, gal-3 and gal-4 induced up to 10-fold higher signal intensity than gal-7 or gal-1. Moreover, gal-3 and gal-4 binding was detectable even at the lowest protein concentrations, while in the case of gal-7 and gal-1, the binding was only present at the highest proteins concentrations. When gal-3 and gal-4 were used, a lower unspecific signal was observed. Altogether, these results show that gal-3 and gal-4 seemed to be more suitable and more efficient for microarray assays. However, for the sake of the results in chapter 1, gal-7 and gal-1 were still used in all the following assays.

Two different galectin concentrations (20 and 50 µg/ml) were used. As suggested above, it can happen that an excessive amount of protein saturates the signal, creating an interpretation error. The binding of the two concentrations of galectin was tested on the whole cells (**Fig. 3**). Gal-3 bound to the control proteins proportionally to its concentration (**Fig. 3A, B, C, D**). In most of the cases the binding signal was higher when gal-3 was used at 50 µg/ml compared to 20 µg/ml. Interestingly when the binding was tested with the lowest control protein concentration, 0.03 mg/ml, the results were inverted, with the lowest gal-3 concentration often giving the highest signal (**Fig. 3A**). The same pattern of results was obtained with gal-4 (data not shown), proving the similar behaviour of the two galectins regarding the binding to microarrays. Gal-7, on the other hand, behaved in an opposite way (**Fig. 3E, F, G, H**). The lowest gal-7 concentration, 20 µg/ml, was always the one causing the highest signal. Anyway, even when the highest binding was considered for gal-7, it is still 2- to 10-fold lower compared to gal-3 or gal-4. These results evidence how tricky it may be to choose the optimal concentration to deliver the best results.

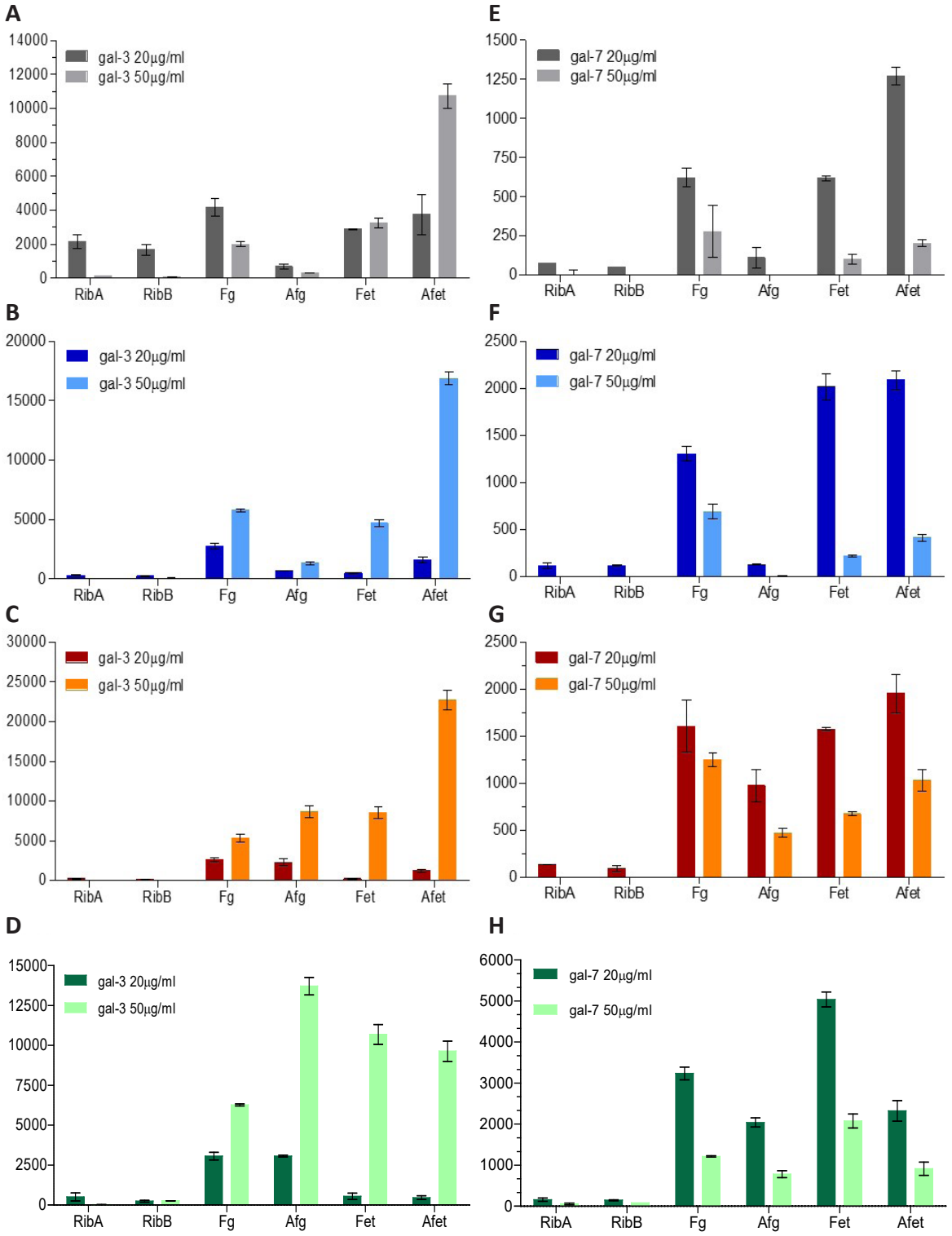


Figure 3: *Testing different galectin concentrations for binding control proteins.* The fluorescence intensity of the binding of two different galectin concentrations was compared for gal-3 (**A, B, C, D**) and gal-7 (**E, F, G, H**). The four control protein concentrations – 0.03 mg/ml (**A, E);** 0.1 mg/ml (**B, F**); 0.3 mg/ml (**C, G**); 1 mg/ml (**D, H**)- were plotted in different graphs for clarity. The intensity values are the average ±SD of three spots. Please note that the scale of the fluorescence intensity may change among different graphs.

In view of the results above, the two protein concentrations (20 and 50 µg/ml) were kept in the last protocol optimization procedure, this time using the whole yeast cells arrays (**Fig. 4**). In the case of gal-3 and gal-4, the highest concentration was the one giving the strongest binding signal (**Fig. 4A, B**), while in the case of gal-7 the opposite happened (**Fig. 4C**). Results in Fig. 3 also showed that PFA-fixed cells bound galectins generally less efficiently than cells in suspension, except in the case of gal-3 binding to BY4741 *Δras2* (**Fig. 4A**) and gal-7 binding to W303-1A *Δras1* (**Fig. 4C**), which bound more efficiently. So the difference in binding ability between PFA-fixed cells and cells in suspension was not as clear as in the case of ConA binding.

Considering all the optimization steps above described, the final procedure consisted of:

1) galectin concentrations:

- 50 µg/ml- gal-3, gal-4 and gal-1,
- 20 µg/ml- gal-7,

2) inhibition controls:

- 50 or 20 µg/ml galectin + 75 mM lactose + 1 mg/ml AFet,

3) negative control:

- No galectin.

This was applied to printed spots of:

1) whole yeast cells, all of which at four $O.D._{600}$ 10, 6, 3 and 1:

- *S. cerevisiae* BY4741 wt, *Δras1, Δras2* (PFA-fixed and in TBS suspension),
- *S. cerevisiae* W303-1A wt, *Δras1, Δras2* (PFA-fixed and in TBS suspension),
- *C. albicans* BPW17 (only PFA-fixed),

2) subcellular fractions, all at four proteins concentrations of 10, 3, 1 and 0.3 mg/ml:

S. cerevisiae W303-1A wt and *Δras2*,

C. albicans BPW17 wt

- cell wall,
- cytoplasm,
- membranes,
- extracellular matrix,

Differently from yeast whole cells, gal-7 concentration used for binding to yeast cell fractions was 50 µg/ml, because it performed better in this specific experiment.

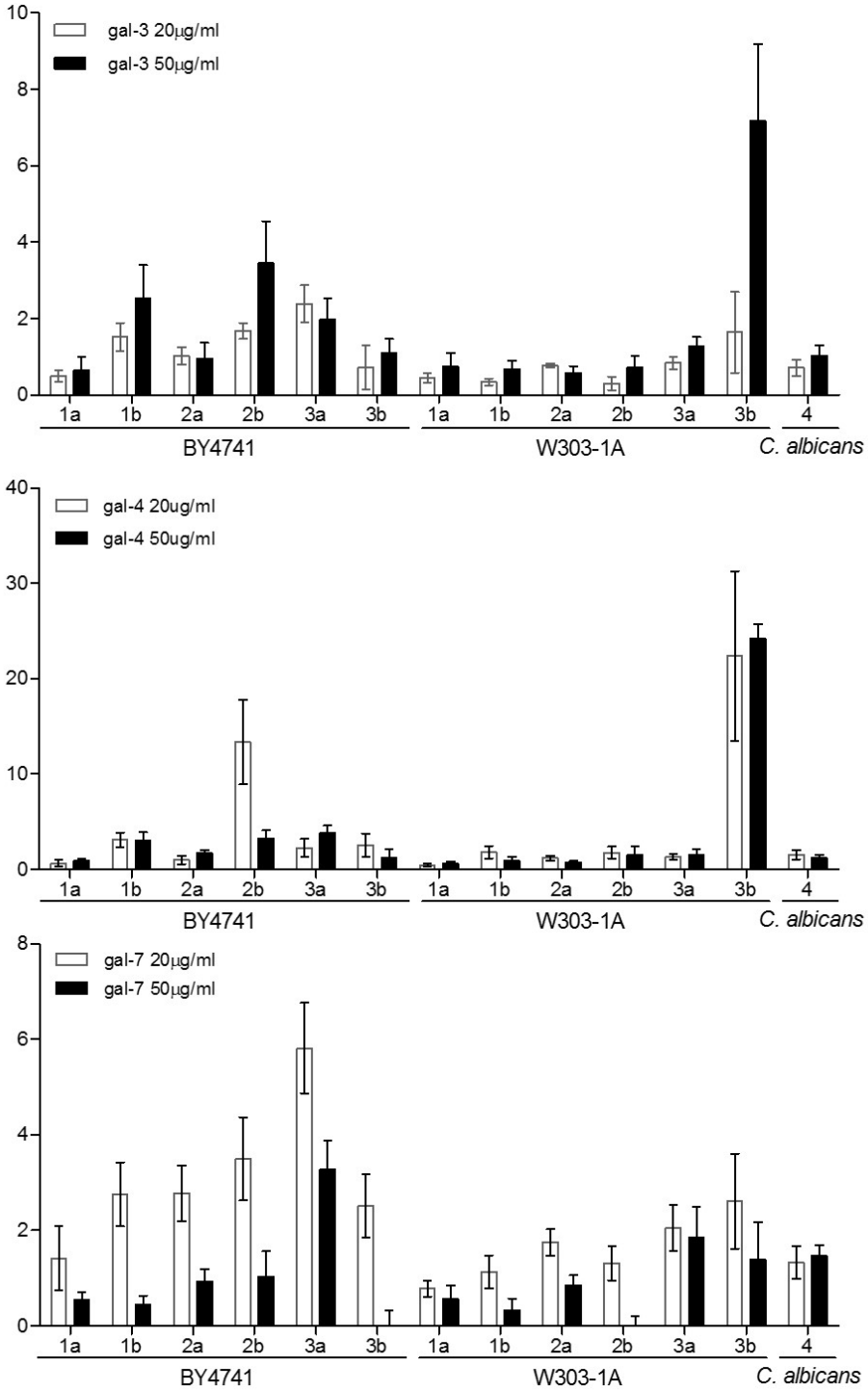


Figure 4: *Testing galectin concentrations for binding whole cells.* The normalized fluorescence derived from the binding of two different galectin concentrations was compared for gal-3 (**A**), gal-4 (**B**) and gal-7 (**C**). Samples are denominated as in Fig 1. The values of the normalized intensity are the average ±SD of 6 to 12 printed spots. Please note that the scale of the fluorescence intensity may change among different galectins.

Every galectin shows a different specificity for yeast cell surface

The binding of each galectin to the whole cell was evaluated using PFA-fixed cells and the cells in TBS suspension. Results present the two types of samples plotted separately (**Fig. 5**), in view of the different binding ability previously observed (**Fig. 5A, B, C, D and Fig. 5E, F, G, H**). It was clear at first sight that the binding specificity of galectins to both PFA-fixed and alive cells was smaller compared to ConA. In the case of gal-1 binding to living cells there was no significant difference between the signal from the binding of the galectin and the controls (**Fig. 5H**). In the case of gal-3 binding to PFA-fixed cells the signals derived from the controls were higher than the one coming from the binding (**Fig. 5A**). This means that other components of the mixture used to reveal the binding signal might be interacting with the surface of the cell, in which case the signal is not decreased by the addiction of specific galectins inhibitors. The results in which the signal deriving from the controls was comparable or higher than the one deriving from putative galectin binding were considered unspecific and not further taken into account.

Generally, living cells delivered a stronger signal than cells fixed in PFA (**Fig. 5A, B, D vs Fig. 5E, F, H**), with exception of gal-7, which bound better to PFA-fixed cells (**Fig. 5C, G**). Actually, gal-7 behaved differently from the other galectins used. It worked better at a lower concentration and with PFA-fixed cells. Regarding *S. cerevisiae* genetic background, it was evident that BY4741 interacted better with the galectins than W303-1A (**Fig. 5A, B, C, D**). This difference was particularly evident in the case of PFA-fixed cells. *C. albicans* showed a behaviour comparable to W303-1A. These results show not only that galectins are able to bind to the yeast cell surface components, but also that this binding is strain-dependent. Importantly, the results also show that the binding to *S. cerevisiae* displays higher intensity than the binding to *C. albicans,* agreeing with the results from chapter 1, and opposing the ones from Jouault *et al*. (2006) and Kohatsu *et al*. (2006). The present results clearly show that the genetic background from different yeast species and strains impacts on the cell surface components, altering the putative recognition by external agents, galectins or others, and consequently the immune response. Jouault *et al*. (2006) and Kohatsu *et al*. (2006), based only on that gal-3 was not able to bind to *S. cerevisiae*, suggested that gal-3 could lead the

discrimination by the immune system of pathogenic yeasts (Jouault *et al*., 2006). The present results clearly show that such a hypothesis requires extensive challenging with other yeast strains, in particular pathogenic, and other culture conditions.

In what regards the possible involvement of the RAS/cAMP/PKA pathway in yeasts response to galectins, the experiments were repeated using *S. cerevisiae RAS1* and *RAS2* deleted strains from both BY4741 and W303-1A. A trend in behaviour was observed in PFA-fixed cells, and to a lesser extent in TBS-suspended cells. The signal deriving from wt cells was lower than the one from *Δras1*, and this was in turn lower than the signal deriving from *Δras2* (**Fig. 5A, B, C, D**).

a) Gal-3 bound strongly only to W303-1A *RAS2* mutant cells (both PFA-fixed and TBS-suspended) (**Fig. 5A, E**). This was particularly so since gal-3 binding to all other yeast strains was considered unspecific due to the high intensity of the controls (**Fig. 5A, E**).

b) Gal-4 generally produced a higher intensity and specificity than gal-3 (**Fig. 5B, F**), particularly strong in the case of TBS-suspended W303-1A *Δras2*. With the exception of gal-4 binding to W303-1A *Δras2*, gal-4 generally bound more efficiently to BY4741 than to W303-1A.

c) Gal-4 and gal-7 generally bound more efficiently to yeast whole cells than gal-3 and gal-1, with the exception of gal-3 binding to W303-1A *Δras2,* which was significant, even if not as high as gal-4 binding to W303-1A *Δras2.* Gal-7 was the galectin better performing in this assay (**Fig. 5C, G**), if we exclude the exception of gal-4 binding to W303-1A *Δras2*. The signals obtained using gal-7 were significant in almost all the cases, with the exception of PFA-fixed W303-1A wt (**Fig. 5C**) Gal-7 performed equally well (**Fig. 5C, G**). The *Δras2* mutant displayed again the strongest binding. This time though it was the PFA-fixed mutant from the BY4741 background (**Fig. 5C**).

d) Finally, gal-1 showed an opposite behaviour compared to gal-7 (**Fig. 5D, H**). Gal-1 caused the weaker signal of all galectins, especially when tested on TBS-suspended cells (**Fig. 5H**). However, even if the intensity was low, the signal coming from PFA-fixed cells was significantly different from control in the case of BY4741 *Δras1* and *Δras2* mutants, W303-1A *Δras2* and *C. albicans* (**Fig. 5D**), contrarily to what happened using gal-3.

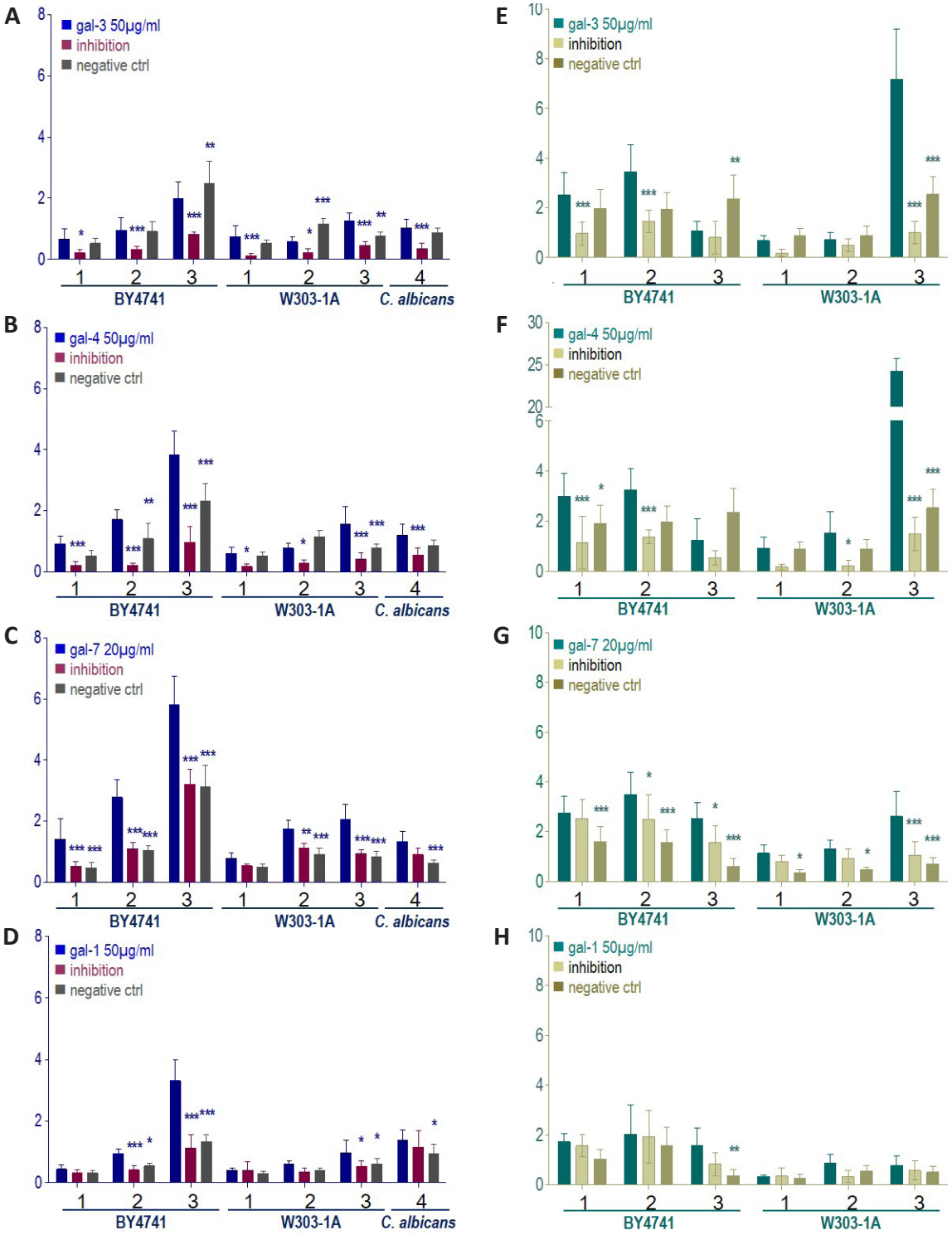


Figure 5: *Galectins binding to yeasts whole cells*. The normalized fluorescence intensity resulting from the binding of different galectins to yeasts whole cells was plotted, together with the signal obtained from the galectins in solution with their inhibitors, lactose and AFet, and with the negative control. PFA-fixed cells (**A, B, C, D**) were analysed separately from cells in TBS suspension (**E, F, G, H**). Gal- 3 (**A, E**), gal-4 (**B, F**), gal-7 (**C, G**) and gal-1 (**D, H**) were plotted in separate graph. The values of the normalized intensity are the average ±SD of 6 to 12 printed spots. Please note that the scale of the fluorescence intensity changes for PFA-fixed cells and cells in TBS suspension.

All taken, it stands out that the most receptive yeast strain to human galectins is *RAS2* mutant, being it from BY4741 or W303-1A genetic backgrounds. The galectin that presented the worst results was gal-3. The inability of this galectin to deliver clear results derived from controls yielding a high unspecific signal (**Table 2**).

Table 2: Schematic representation of the binding ability of gal-3, gal-4, gal-7 and gal-1 to yeasts whole cells. Binding from 0.5 to 2 is represented by +, from 2to 4 by ++, higher than 4 by +++. Binding equal/ lower than the negative control is indicated by ns (non-significant). The cell representing the highest binding intensity for a specific galectin is placed in a grey cell.

yeast	***S. cerevisiae***												***C. albicans***
genetic background	**W303-1A**						**BY4741**						**BPW17**
strain	wt		*Δras1*		*Δras2*		wt		*Δras1*		*Δras2*		wt
storage	**PFA**	**TBS**	**PFA**	**TBS**	**PFA**	**TBS**	**PFA**	**TBS**	**PFA**	**TBS**	**PFA**	**TBS**	**PFA**
gal-3	ns	ns	ns	ns	+	++	ns	ns	ns	ns	ns	ns	ns
gal-4	ns	ns	ns	ns	+	+++	ns	++	+	ns	++	ns	ns
gal-7	ns	+	+	+	++	++	+	++	++	++	+++	++	+
gal-1	ns	ns	ns	ns	+	ns	ns	ns	+	ns	++	ns	+

Galectins preferences for yeast sub-cellular fractions

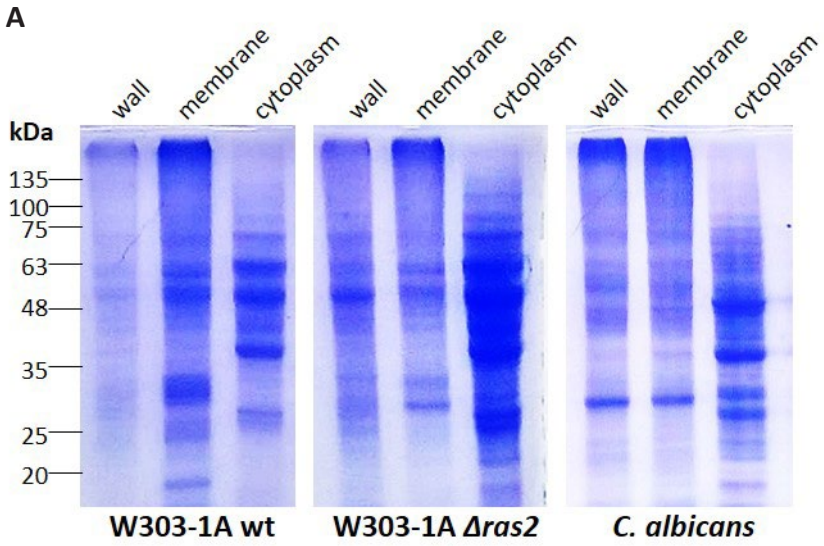
The results obtained with the whole yeast cells did not yield clear results. Some of the strains used were therefore selected to be fractionated into their sub-cellular components – wall, membrane, cytoplasm – in order to concentrate the molecules and possibly to have a binding signal of higher quality. Since galectins are often reported to be located in the extracellular space (Perillo *et al.*, 1998; Compagno *et al.*, 2014), also the ECM produced by the same cells was isolated to test the galectin binding. The strains selected were *S. cerevisiae* W303-1A and *C. albicans*. This choice was based on the need to challenge the results previously published in the literature that, in opposition with the results obtained in the present work (chapter 1), considered *S. cerevisiae* insensitive to gal-3 (Jouault *et al*., 2006; Kohatsu *et al*., 2006). Moreover, the fractionation should allow the recognition of the molecular family of cellular components to which the galectins bind. Previous publications suggested gal-3 bind to *C. albicans* cell wall components (Fradin *et al*., 2000; Jouault *et al*., 2006; Kohatsu *et al*., 2006), even if these are not supposed to contain galactosides (Klis et al., 2006; Free, 2013). In particular, W303-1A was chosen because it was the most used strain in the previous experiments and the one that gave the most significant response to exogenous gal-3 (chapter 1). In view of the results above, also the *RAS2* deletion mutant from the same background was used.

Yeast cells were collected from liquid medium and fractioned. These were quantified and subjected to normalization to ensure that identical amounts of each fraction were printed on the microarray. Every fraction contains different types of molecules in relative different amounts, but all contain proteins. Therefore, it was chosen to quantify the fractions based on the protein content. Each sample was normalized to have 100 µg of protein and run in a gel. The gel was stained with Coomassie Brilliant Blue to highlight the total protein content and the different bands were visualized (**Fig. 6A**). The intensity of the bands was very similar, confirming the normalization of the protein content. As for the different band pattern, the variations were particularly evident between the cytoplasm and the other two fractions.

The ECM was extracted from cultures growing on solid medium (Faria-Oliveira *et al.*, 2014). Also in this case the protein content was quantified and normalized. It was though

not possible to correctly stain and run an SDS-PAGE due to the high ECM contents of glycans, and lipids to a lesser extent.

The different yeast cell fractions were printed on the microarrays in 4 concentrations (0.3 mg/ml, 1 mg/ml, 3 mg/ml and 10 mg/ml), 10-fold higher compared to the control proteins RbA, RbB, Fg, AFg, Fet and AFet. The efficiency of using sub-cellular fractions for microarray printing and lectins binding was first verified using ConA. All the fractions successfully bound to this lectin and the binding correlated to the printed protein concentration in most of the cases, with the exception of ConA binding to the membranes of all the yeasts, the wall of W303-1A wt and the ECM of *C. albicans* (**Fig. 6B**). Moreover, the binding was significant for all the samples, since the signal from inhibition and negative control was lower than the one from ConA binding (**Fig. 6B**, middle and lower panel). As expected, the weakest signal originated from the cytoplasm fractions. ConA bound with higher intensity to *C. albicans* ECM compared to *S. cerevisiae* ECM, while the opposite happened for cell wall and membrane fractions (**Fig. 6B**). Identically, ConA bound with higher intensity to W303-1A *Δras2* ECM than to the wt ECM, while the opposite happened for cell wall and membrane fractions (**Fig. 6B**). These results show that the fractions obtained from the different yeast cell components, as well as ECM, can be used for the microarray investigation about galectins interaction with yeasts. The binding of the four galectins to the yeast cell components of *S. cerevisiae* W303-1A wt and *Δras2,* and *C. albicans* wt strains, was assessed.



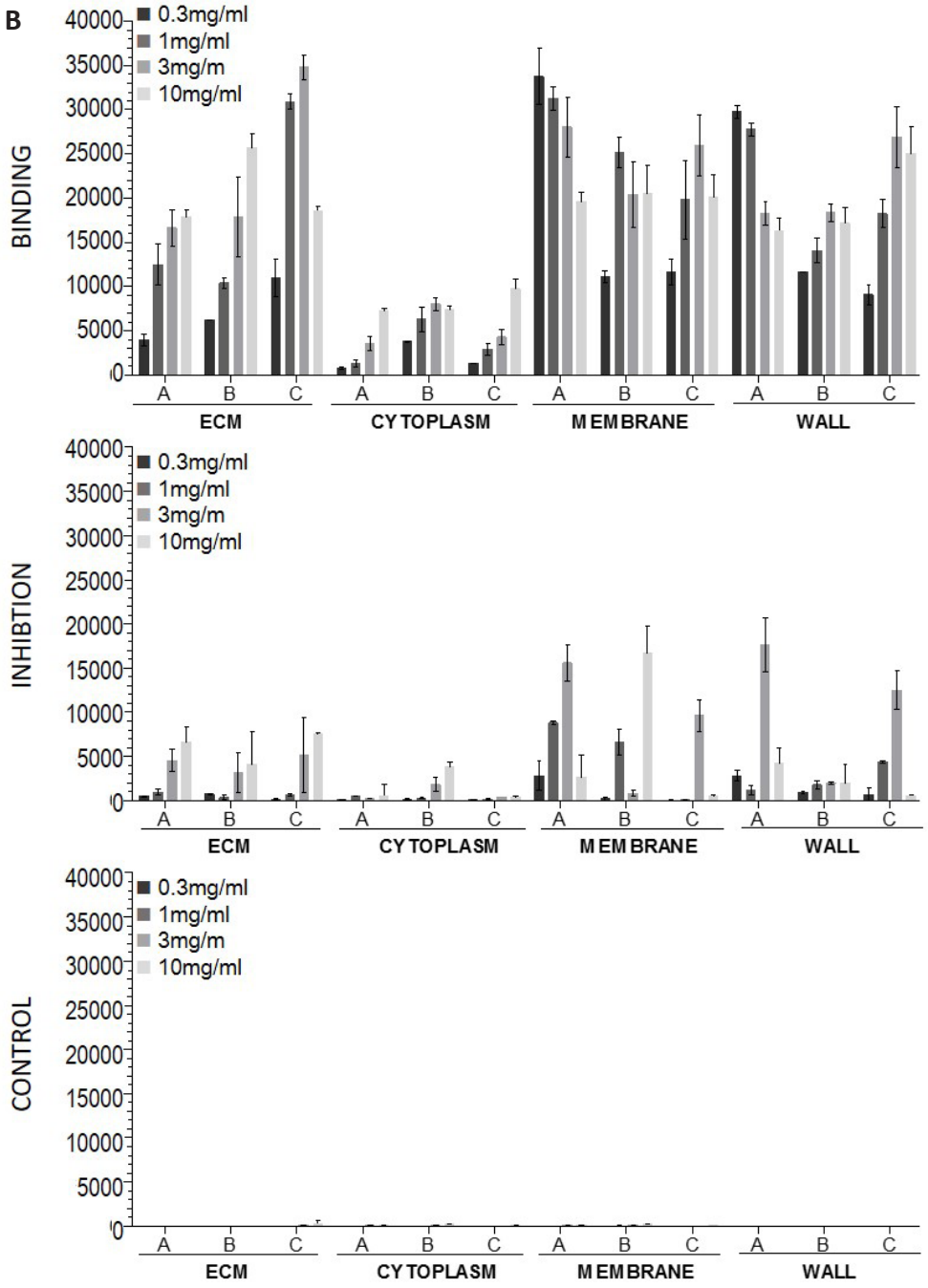


Figure 6: *Testing of the yeast sub-cellular components*. W303-1A wt and *Δras2* and *C. albicans* cellular components – cell wall, membrane and cytoplasm – and ECM were denatured by heat shock (95°C), run in polyacrylamide gel and stained by Coomassie brilliant blue. After proper washing the gel was imaged (**A**), showing the different patterns of the bands. ConA was used to test the proper printing and the binding efficiency of yeast cellular components. The fluorescence intensity resulting from the binding to four cell component concentrations (upper panel) was compared to the binding of ConA inhibited by mannose (central panel) and to the negative control (lower panel) (**B**). "A" and "B" samples correspond to components coming from W303-1A wt and *Δras2*, and "C" from *C. albicans*. The intensity values are the average ±SD of three spots.

S. cerevisiae *W303-1A vs* C. albicans *BPW17*

As mentioned before, different studies reported the inability of gal-3 to bind to *S. cerevisiae*, hypothesizing that this protein could be responsible for discriminating between pathogenic and non-pathogenic yeasts (Jouault *et al.*, 2006; Kohatsu *et al.*, 2006). Each galectin presented a different binding ability in the regards of *S. cerevisiae* and *C. albicans* (**Fig. 7**), but, importantly, all of them could bind both *S. cerevisiae* and *C. albicans* membrane and cell wall fractions. As general consideration, it can be noticed that the intensity of the binding of each galectin varied greatly, being particularly strong for gal-4 (**Fig. 7B**) and much less intense for gal-1 (**Fig. 7D**).

Gal-3, gal-4 and gal-7 showed some common features regarding their binding to yeast components. The binding signal from the cytosolic compartment of both *S. cerevisiae* and *C. albicans* was weak and very unspecific. The inhibition and negative control columns were often comparable or even higher than the actual binding (**Fig. 7A, B, C**), demonstrating the fact that galectins do not bind to intracellular component of the yeast cell, or at least they do to a lesser extent compared to other cellular fractions. Another common characteristic was that the binding to cell wall and membrane was always higher for *S. cerevisiae* than to *C. albicans*, contradicting the previous report (Jouault *et al*., 2006; Kohatsu *et al*., 2006).

a) Gal-3 bound strongly to *S. cerevisiae* cell wall and membranes and to *C. albicans* ECM (**Fig. 7A**). The binding to *C. albicans* ECM was significant, but much lower than the one observed for *S. cerevisiae* wall and membranes. In summary, gal-3 highest signal derived from S. cerevisiae wall and membranes (**Fig. 7A**).

b) Gal-4 showed a particular high affinity for the ECM (**Fig. 7B**), not observed with any other galectin. In particular, gal-4 gave the strongest signal when bound to *C. albicans* ECM. The signals from wall and membrane were lower than the one caused by the binding to ECM in the case of *C. albicans,* with a difference of 6-7-fold for the 10 mg/ml fraction, while in the case of *S. cerevisiae* the binding to ECM, wall and membrane was very similar. So gal-4 mostly bound to the glycans of the ECM of *C. albicans*, while it binds homogenously to ECM, wall and membrane of *S. cerevisiae*.

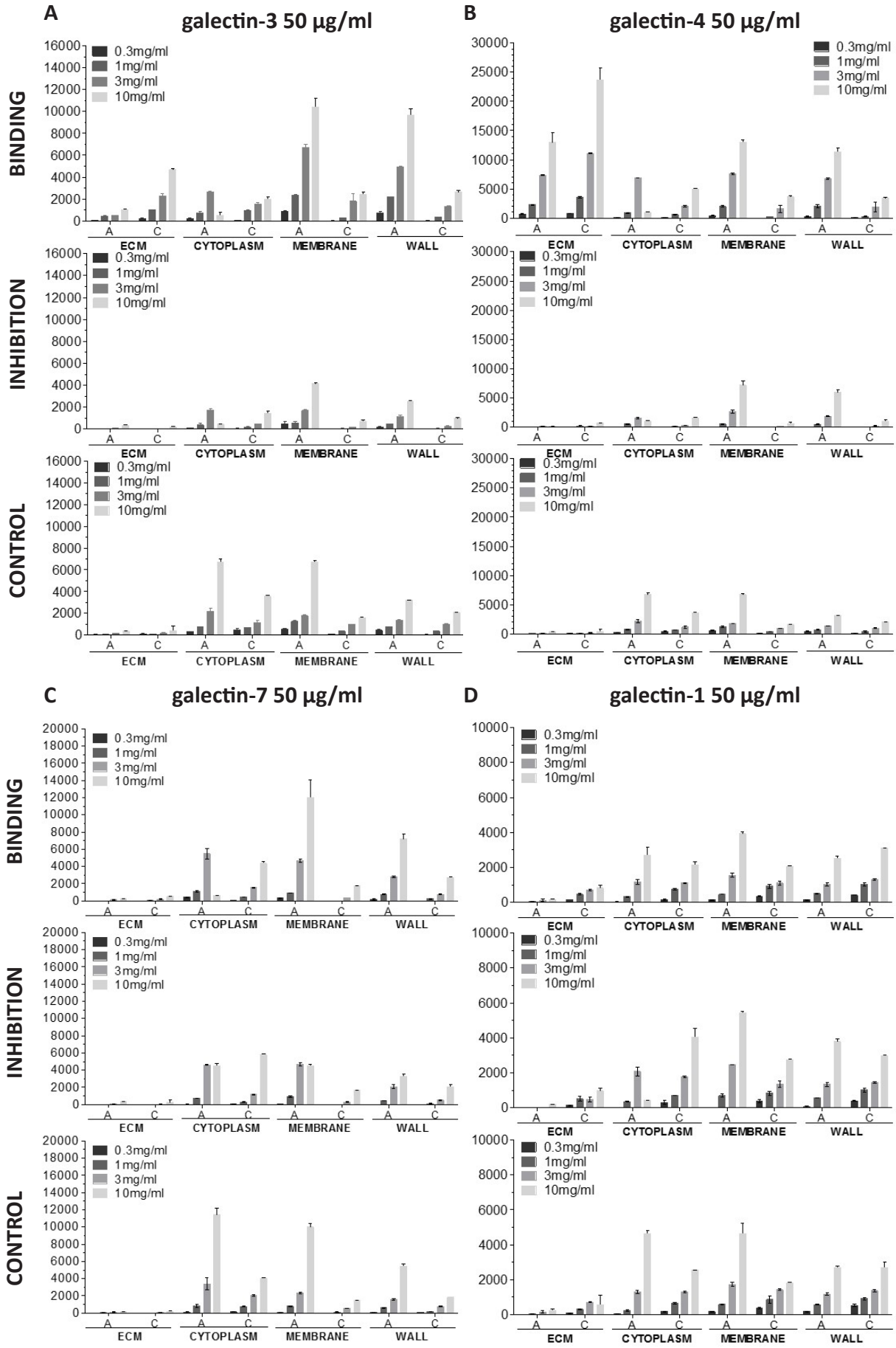

A
galectin-3 50 µg/ml
B
galectin-4 50 µg/ml
C
galectin-7 50 µg/ml
D
galectin-1 50 µg/ml
BINDING
INHIBITION
CONTROL
0.3mg/ml
1mg/ml
3mg/ml
10mg/ml
A
C
ECM
CYTOPLASM
MEMBRANE
WALL

Figure 7: *Galectins binding to yeast cellular components* – S. cerevisiae *compared to* C. albicans. The binding of gal-3 (**A**), gal-4 (**B**), gal-7 (**C**) and gal-1 (**D**) to the cellular components of *S. cerevisiae* and *C. albicans* was analysed. In every case the fluorescence intensity of the binding (upper panels) was compared to the fluorescent resulting from the galectins inhibited by lactose and AFet (central panels) and to the negative control (lower panels). The intensity values are the average ±SD of the three spots printed with the same cellular components concentration. Please note that the scale of the fluorescence intensity is maintained when binding, inhibition and negative control are compared, but it may change among different galectins.

c) Gal-7 showed no affinity at all for ECM and overall a weaker binding ability to all the yeast cell components compared to gal-3 and gal-4 (**Fig. 7C**). In addition, gal-7 only bound to *S. cerevisiae* outer fractions and not to *C. albicans*, whose signal was unspecific. In particular, gal-7 bound with much higher affinity to *S. cerevisiae* cell membrane compared to the wall, contrarily to what happened for gal-3 and gal-4, which bound almost at the same level to the two components (**Fig. 7C**).

d) Gal-1 generally presented a really low intensity compared to the other galectins tested, together with a low specificity. The control binding intensity was higher than the gal-1 binding for all the fractions analysed, proving a really scarce ability of gal-1 to bind to yeast components (**Fig. 7D**).

In summary, these results denied the hypothesis that gal-3 can bind only to *C. albicans* and, on the contrary, they proved that the binding to cell wall and membrane of *S. cerevisiae* cells are stronger. They confirmed the inability of gal-1 to bind to any yeast cell components. Moreover, they showed for the first time that each galectin has a specific affinity for a distinct cellular fraction, confirming one more time the high specialization of galectins binding.

W303 wt vs Δras2

The binding ability of the different galectins was analysed taking into consideration the putative differences between W303-1A wt and *Δras2* (**Fig. 8**). The *RAS2* mutant has been found to not respond to exogenous gal-3 in previous experiments (chapter 1), therefore it is interesting to understand if the responses to the exogenous galectins depend somehow on their binding ability. As in the previous analysis comparing *S. cerevisiae* and *C. albicans*, gal-1 binding was highly unspecific and weak (**Fig. 8D**) and it was therefore not further considered. The other three galectins bound better to the wt cell membrane than to *Δras2* membrane and to the cell wall of *Δras2* than wt (**Fig. 8A, B, C**). None of the galectins presented a strong signal from the cytoplasmic fraction, which actually caused significant signal in the controls, proving to be unspecific. Gal-4 was the one that provoked the better ECM binding to both wt and *Δras2* (**Fig. 8B**). Gal-7 did not bind at all to ECM (**Fig. 8C**), and gal-3 only bound to *Δras2* ECM.

Gal-4 bound at a similar level to all the cell components and the differences between wt and *Δras2*, even if present, were not so evident (**Fig. 8B**). Gal-3 binding to wt membrane was around 1.5-fold higher than to *Δras2* membrane, while it bound to wt wall with less affinity than to *Δras2* wall (**Fig. 8A**). Also gal-7 signal from *Δras2* wall was higher than the one from the wt wall (2.3-fold) (**Fig. 8C**). These results are strong indications that *RAS2* deletion causes perturbations in the components of the external layers of the cells, altering the binding ability to the galectins. Moreover, they agree with literature in that each galectin has a peculiar specificity toward certain ligands, implicating different binding to the different cell fractions.

In summary, gal-4 was the best performing galectin when considering the totality of the fractions and the yeasts, specifically in the regards of ECM binding, bound almost exclusively by gal-4. Overall, galectins bound better to the cell wall and membrane of *S. cerevisiae* than *C. albicans*, and W303-1A *Δras2* bound equally or better to all the galectins than the wt. Gal-1 did not perform at all in this experiment (**Table 3**).

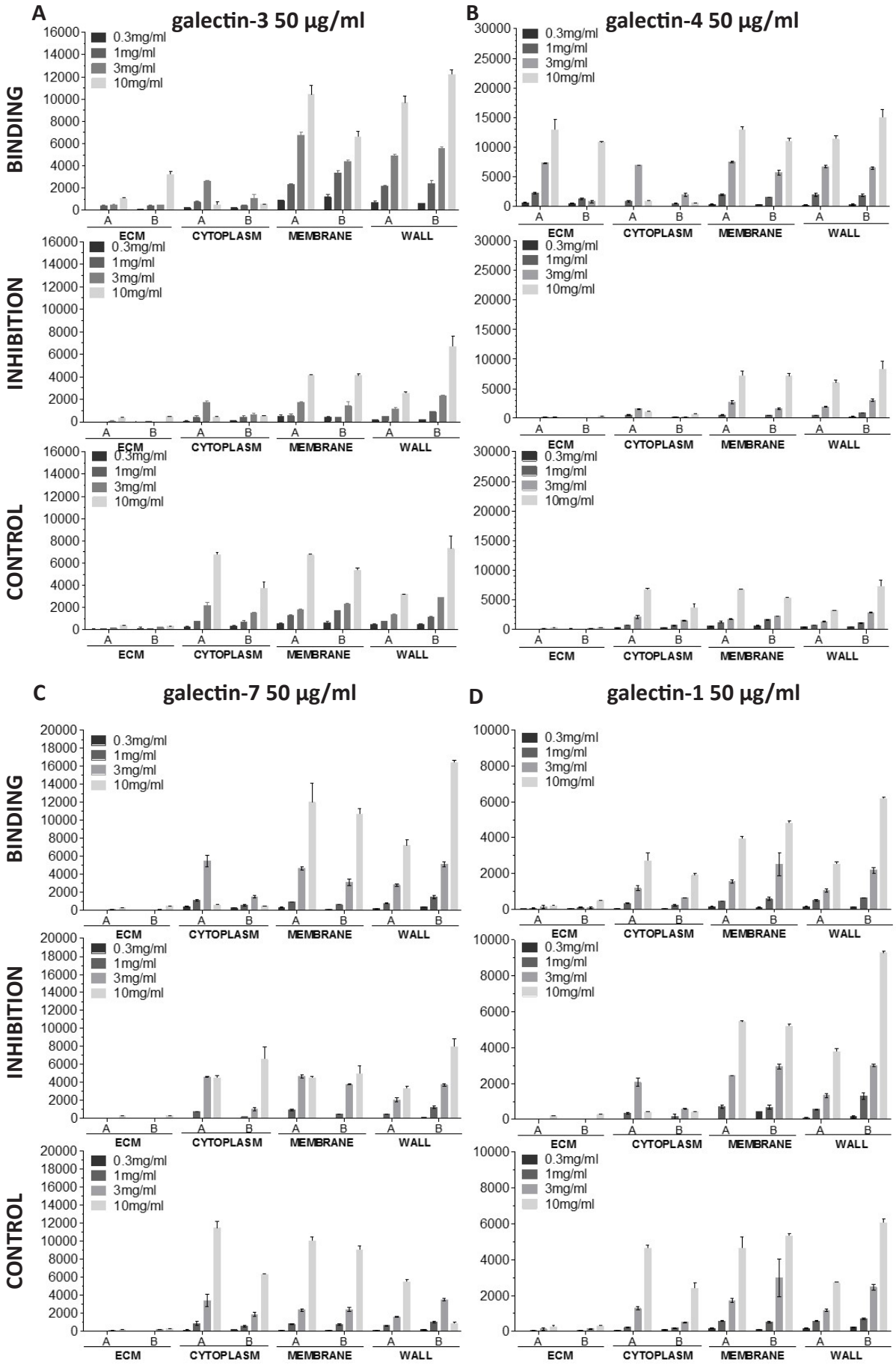

A
galectin-3 50 µg/ml
B
galectin-4 50 µg/ml
C
galectin-7 50 µg/ml
D
galectin-1 50 µg/ml
BINDING
INHIBITION
CONTROL
0.3mg/ml
1mg/ml
3mg/ml
10mg/ml
A
B
ECM
CYTOPLASM
MEMBRANE
WALL

Figure 8: *Galectins binding to* S. cerevisiae *cellular components – wt compared to* Δras2 *mutant.* The binding of gal-3 (**A**), gal-4 (**B**), gal-7 (**C**) and gal-1 (**D**) to the cellular components of *S. cerevisiae* W303-1A wt and *Δras2* was analysed. In every case the fluorescence intensity of the binding (upper panels) was compared to the fluorescence resulting from the galectins inhibited by lactose and asialofetuin (central panels) and to the negative control (lower panels). The intensity values are the average ±SD of the three spots printed with the same cellular components concentration. Please note that the scale of the fluorescence intensity is maintained when binding, inhibition and negative control are compared, but it may change among different galectins.

Table 3: Schematic representation of the binding ability of gal-3, gal-4, gal-7 and gal-1 to yeasts sub-cellular fractions and ECM. Binding from 500 to 5000 is represented by +, from 5001 to 10000 by ++, from 10001 to15000 by +++, higher than 15001 by ++++. Binding lower than the 500 threshold or equal/lower than the two controls is indicated by ns (non-significant). The values taken into consideration are the one obtained with the highest fraction concentration printed (10 mg/ml). The fraction representing the highest binding intensity for a specific galectin is placed in a grey cell.

yeast	***S. cerevisiae* - W303-1A**								***C. albicans* - BPW17**			
strain	wt				*Δras2*				wt			
fraction	**ECM**	**wall**	**membr**	**cyt**	**ECM**	**wall**	**membr**	**cyt**	**ECM**	**wall**	**membr**	**cyt**
gal-3	ns	++	++	ns	+	+++	++	ns	+	+	+	ns
gal-4	+++	+++	+++	ns	++	+++	++	ns	++++	ns	+	ns
gal-7	ns	+	+++	ns	ns	++++	+++	ns	ns	ns	ns	ns
gal-1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Discussion

In this work for the first time the binding of various galectins to yeast cell surfaces was analysed using microarrays technology. With this methodology, multiple ligand targets were screened for the binding of several galectins, covering all the three galectins subgroups, in a fast and efficient way. Ligands included (i) whole yeast cells from two different species (*S. cerevisiae* and *C. albicans*), different genetic backgrounds (BY4741 and W303-1A) and different strains (wt, *Δras1* and *Δras2*), and (ii) yeast sub-cellular fractions (cell wall, membranes, cytoplasmic contents and ECM). A big amount of new data on galectins specificity and on yeast cell surface and binding ability was obtained.

As far as our knowledge goes, the binding ability of galectins for yeast cell outer layers, wall and membrane, was never analysed, probably due to the fact that yeast cell wall is reported not to contain any galactoside glycan (Klis *et al.*, 2006; Free, 2013), for which galectins have highest affinity (Cooper and Barondes, 1999; Compagno *et al.*, 2014). The only case reported is the binding of gal-3 to β-1,2-linked oligomannosides on the cell wall of one strain of *C. albicans* (Fradin *et al.*, 2000), and the lack of binding to cells of *S. cerevisiae* (Kohatsu *et al.*, 2006), putatively because *S. cerevisiae* does not express those type of mannans on the cell wall (Fradin *et al.*, 2000; Jouault *et al.*, 2006). This work showed that, on the contrary, gal-3 is able to bind to *S. cerevisiae* outer cell components, in a more efficient way than to *C. albicans*. In addition, it was shown that also gal-4 and gal-7 bind *S. cerevisiae* wall and membrane, gal-4 to a bigger extent than gal-3. Therefore, the hypothesized specificity of gal-3 for one particular component of *C. albicans* cell wall, unshared with any other galectin (Kohatsu *et al.*, 2006), seemed to be contradicted by the present results. Indeed, not only gal-3, gal-4 and gal-7 bound to *S. cerevisiae*, but this binding was more significant than the one to *C. albicans.* The difference observed between the present work and the previous reports in the literature may be due to the utilization of different yeast genetic backgrounds, both of *S. cerevisiae* and *C. albicans*. As mentioned in the introduction, the composition of yeast cell wall may vary greatly depending on environmental conditions and growth phases (De Nobel *et al.*, 1990; Smits *et al.*, 1999; Smith *et al.*, 2000; Aguilar-Uscanga and Francois, 2003;

Lagorce *et al.*, 2003; Klis *et al.*, 2006), so it is conceivable that different genetic backgrounds in different culture conditions present a differently composed cell wall. In agreement, galectins generally bound more to whole cells of BY4741 than of W303-1A, confirming the influence of genetic background in the glycan composition of the outer layers of the yeast cell to which the binding occurs. In addition, the experimental conditions of the binding assays used in the present work were very different from the ones used by Kohatsu *et al*. (2006). These authors performed a pre-incubation of gal-3 with the yeast cells at 4°C using a much higher concentration of gal-3.

Both these conditions may have influenced the binding capacity of gal-3 to *S. cerevisiae* and *C. albicans*. The present results also suggest that galectins actually bind to more than one specific component, contrarily to what is reported in the literature (Fradin *et al.*, 2000; Jouault *et al.*, 2006; Kohatsu *et al.*, 2006). The fact that they can bind to *S. cerevisiae*, which lacks the β-1,2-linked oligomannosides from *C. albicans*, suggests that galectins can bind as well to different glycans.

When analysing the binding of galectins to yeast cell fractions, it was immediately clear that they bound to the outer cell fractions of the cell, *i.e.* the wall and membrane. Only gal-4 could also bind to the ECM and none of them bound to the cytoplasmic fraction. In mammalian cells the role of intracellular galectins is mainly exerted by protein-protein interactions and not by binding sugars (Rabinovich and Croci, 2012). Galectins may not present the same affinity for yeast intracellular proteins. In accordance, the signal derived from the cytoplasmic fraction was residual and unspecific. Results actually are consistent with galectins binding to components of the cell wall most efficiently. Since the outer layers consist of mannoproteins and do not contain β-galactosides (Klis *et al.*, 2006; Free, 2013), galectins are expected to bind to the yeast wall proteins mannosides (Smits *et al.*, 1999; Klis *et al.*, 2006; Free, 2013). Actually, gal-10 has already been reported to have a higher affinity for mannosides than for galactosides (Yang *et al.*, 2008; Cummings and Liu, 2009). More difficult to assess is the binding of galectins to the membrane fraction. The possibility that it contains glycolipids most probably has to be considered.

With this work it was possible to compare the binding of galectins to yeast whole cells

to the one to yeast sub-cellular fractions. The signal originating from yeast whole cells was hypothesized to derive from binding to yeast outer layer components, more precisely the wall, which was confirmed by finding the highest binding to the correspondent sub-cellular fraction as discussed above. However, the efficiency of binding to whole cells was lower compared to the fractions, especially in the case of W303-1A wt and *C. albicans*, and sometimes the results were even discordant, as in the case of gal-3 and gal-4 binding to W303-1A wt (**Table 4**). This may be due to *in vivo* different presentation of the galectin ligands, as well as their differently available amounts. The fractions were obtained using a higher amount of cells and the components were highly concentrated. As a result, binding to the fractions resulted higher and more specific than binding to whole cells. In addition, cell presentation of glycans is subjected to specific organization in clusters and domains (Gabius *et al.*, 2011; André *et al.*, 2015) and for galectins in solution may be more difficult to recognize those compared to a physiological situation. On the other hand, when concentrated in fractions, glycans are readily available for the binding to galectins. When comparing whole cells with outer cell layers, the binding of gal-3, gal-4 and gal-7 to W303-1A *Δras2* was confirmed in both types of samples. This *S. cerevisiae* mutant strain was the best performing in binding ability to yeast cell wall or membrane (gal-3 and gal-7) (**Table 4**). Therefore, galectin binding to the surface of W303-1A *Δras2* is a very robust result obtained in this work.

In addition to demonstrating the binding ability of galectins to yeast cell and sub-cellular components, this work showed the great diversification in galectins binding specificity. Galectins are categorized as lectins with high affinity for β-galactosides, but the specificity of each member of the family differs (Compagno *et al.*, 2014). This specificity seems not to be correlated to the subgroup to which the galectin belongs. Gal-7 and gal-1 are both *proto-type* galectins, still they showed a very different binding pattern, gal-1 almost not performing at all, compared with gal-7 that bound strongly to *S. cerevisiae* wall and membrane fractions, as well as to the whole cells. On the other hand, gal-7 showed a binding profile to sub-cellular fractions very similar to the one of gal-3, a *chimera* type galectin.

RAS pathway integrity influenced galectin binding ability. In general, *Δras2* was the best performing among the two *RAS* mutants and the wt, followed by *Δras1* and, finally, by the

wt. Concomitantly, W303-1A *Δras2* cell wall fraction was the best performing in the binding of both gal-3 and gal-7. This may be explained by the fact that Ras pathway, in particular through Ras2, which is the most frequently expressed among the two Ras proteins (Breviario *et al.*, 1986; 1988), controls cell wall composition and rearrangement upon stress stimuli (Imazu and Sakurai, 2005).

Table 4: Comparison between the binding ability of gal-3, gal-4, gal-7 and gal-1 to yeast whole cells and yeasts sub-cellular outer fractions (cell wall and membrane). Binding intensity is indicated as in Table 2 and 3, respectively.

yeast	***S. cerevisiae* - W303-1A**								***C. albicans* - BPW17**		
strain	wt				*Δras2*				wt		
sample	whole cell		fraction		whole cell		fraction		whole cell	fraction	
fraction	**PFA**	**TBS**	**wall**	**membr**	**PFA**	**TBS**	**wall**	**membr**	**PFA**	**wall**	**membr**
gal-3	ns	ns	++	++	+	++	+++	++	ns	+	+
gal-4	ns	ns	+++	+++	+	+++	+++	+++	ns	ns	+
gal-7	ns	+	+	+++	++	++	++++	+++	+	ns	ns
gal-1	ns	ns	ns	ns	+	ns	ns	ns	+	ns	ns

In conclusion, with this work it was established that galectins can efficiently bind to yeast cells from *S. cerevisiae* and *C. albicans*, with a higher affinity for the hereby used *S. cerevisiae* strains. Also, the high variety and diversification of galectins binding affinities was highlighted, together with the variation in cell surface composition among different yeast species, genetic backgrounds and even between wt yeast and mutants defective for the Ras signalling pathway. For the first time it was shown that the microarray technology is an efficient approach to test lectins binding to yeast whole cells, and that yeasts can be efficiently printed on microarrays, both alive and fixed, thereby preserving their surface properties. Microarrays have been proven to be an excellent tool to better understand the differential composition of yeast

surface components, as well as the components of sub-cellular fractions, using different lectins with different specificities. In the same way, microarrays can be successfully used to confirm the ligands of galectins and to identify new ones, like in the present work, in which galectins were shown to bind most probably to mannosides.

This method could be further used to unravel the different surface components and binding affinity between pathogenic and non-pathogenic yeasts. The surface molecules are indeed fundamental in the recognition process by the cells of the immune system and for the subsequent onset of cell defence. The usage of microarray could allow the fast determination of differences in lectins and antibodies specificity toward different yeast cell, therefore helping the recognition of factors that determine pathogenicity. A microarray-derived high-throughput analysis of large batteries of yeast clinical isolates could enlighten this very important issue. In the particular case of the present work, a future step would be to dissect the sub-cellular fractions into multiple purified components, to identify the specific yeast molecular targets of galectins, as a step towards clarifying the specificity of these proteins.

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Chapter 3

Construction of a *Saccharomyces cerevisiae*-based Platform Expressing Human galectin-3 and KRAS

Abstract

The utilization of humanized yeast to study the role of human proteins in the model organism *S. cerevisiae* has been well established. Namely, it has been particularly relevant to uncover the roles and modes of action of proteins involved in pathogenesis and disease, mainly cancer onset and progression. This work aims to build a *S. cerevisiae*-based platform expressing two human proteins, galectin-3 and KRAS, whose interaction is relevant for the progression of several types of cancer. KRAS is a well-known oncogene belonging to the RAS family, which has two homologues in yeast, Ras1 and Ras2. Gal-3 is a carbohydrate-binding protein belonging to galectins family, highly conserved in different organisms, but not present in yeast. Two different common laboratory genetic backgrounds of *S. cerevisiae*, W303-1A and BY4741 were transformed with expression vectors harbouring KRAS or gal-3. Both the wild type and the strains lacking either *RAS1* or *RAS2* were identically used. It was shown that gal-3 can be successfully expressed in yeast and the protein is retained in the first phases of logarithmic growth. Moreover, it was shown that gal-3 localizes in the cytoplasm, often clustered in discrete concentrated points. It was also shown that gal-3 expression in wild type yeast influences growth rate, which increased in W303-1A and decreased in BY4741. On the other hand, gal-3 modulates *RAS* mutants chronological life span, which increased in *Δras2* and decreased in *Δras1*. Additionally, human KRAS expression in wt yeast, *i.e.* expressing both endogenous Ras proteins, caused a generalized decreased stress resistance, probably caused by the hyperactivation of Ras/cAMP/PKA pathway that is generated by the presence of an extra Ras protein. In addition, the results obtained when expressing human gal-3 suggest a more prominent role of Ras1 in yeast growth than previously described. Finally, it was shown that the two *S. cerevisiae* genetic backgrounds behaved very differently in what regards the effects generated by gal-3 expression and in response to various stress stimuli, highlighting the importance of the choice of the genetic background in the determination of clear results, and the need for backing them up by testing several strains. This is particularly important when the results from a given study have biomedical/pharmacological implications.

Introduction

Galectin-3 is a member of the galectin family, a sub-group of lectin proteins specialized in the recognition of β-galactoside glycans. Galectins are highly conserved proteins in evolution, with orthologues in many organisms, including birds, amphibians, fish, worms, sponges and fungi. However, galectins do not have orthologues in the model yeast *S. cerevisiae* (Hughes, 1997; Cooper and Barondes, 1999; Dodd and Drickamer, 2001). Galectin-3 is the most extensively studied among galectins, due to the high variety of processes in which it is involved, both inside and outside the cells. Importantly, gal-3 is the only *chimera* type member of the galectin family (Hirabayashi and Kasai, 1993; Rabinovich, 1999), having a unique structure, composed by a carbohydrate recognition domain (CRD) at the C- terminal and a non-carbohydrate binding domain at the N- terminal, atypically long and proline and glycine rich (Hsu *et al.*, 1992; Agrwal *et al.*, 1993). The presence of two distinct domains with different binding properties allows the interaction of gal-3 with a number of intra- and extra-cellular partners. Besides being located both in extra- and intra-cellular environment, also inside the cell gal-3 is found in different locations and generally its role depends on the localization. In the nucleus, gal-3 is a component of the splicing machinery (Dagher *et al.*, 1995) and it contributes to the activation of transcription factors such as CREB, which induces the expression of proteins relevant for cell cycle progression (Kim *et al.*, 1999), whereas when localized at the cell surface gal-3 is involved in cell adhesion, binding to glycans in the extracellular matrix or on the plasma membrane of other cells (Dumic *et al.*, 2006).

Extracellular gal-3 has also an important role in innate immunity, recognizing specific glycan patterns on the cell surface. Gal-3 is mostly involved in the recognition of self-glycans, helping to distinguish between pathogens and self-cells, but sometimes it also binds to microorganisms' glycans present in their cell wall. That is the case of *Klebsiella pneumoniae, Neisseria meningitidis* and *Neisseria gonorrhoeae,* among others (Sato and Nieminen, 2004; Rabinovich and Toscano, 2009). In the cytoplasm, intracellular gal-3 can interact with a high variety of proteins, contributing to the regulation of important cellular processes, such as apoptosis, cell cycle progression and immune escape (Dumic *et al.*, 2006). In particular,

cytoplasmic gal-3 seems to have a prominent pro-survival and anti-apoptotic role in cells, contributing to tumour formation and progression (Califice *et al.*, 2004; Krzeslak and Lipinska, 2004; Newlaczyl and Yu, 2011; Song *et al.*, 2014; Ahmed and AlSadek, 2015; Wang and Guo, 2016).

Particularly relevant for cancer progression and protection from apoptosis is the interaction of gal-3 with the oncoprotein KRAS. Gal-3 and KRAS have been shown to directly interact in a variety of cell types and this interaction mediates the translocation of both proteins to the inner leaflet of the plasma membrane, where gal-3 maintains KRAS nanoclusters organization, functional for its activity (Elad-Sfadia *et al.*, 2002; Shalom-Feuerstein *et al.*, 2005; Ashery *et al.*, 2006; Shalom-Feuerstein *et al.*, 2008). Gal-3 and KRAS interaction and reciprocal regulation have been proven to be critical for different types of cancer progression, including pancreatic (Song *et al.*, 2012; Seguin *et al.*, 2014), thyroid (Levy *et al.*, 2010), breast (Shalom-Feuerstein *et al.*, 2005; Shalom-Feuerstein *et al.*, 2008) and colon (Wu *et al.*, 2013). This is mainly due to an increased activity of KRAS mediated by gal-3, which contributes to maintain KRAS in nanoclusters on the cell membrane, in an activated state.

KRAS is a well-known oncoprotein belonging to the RAS superfamily of small GTPases. KRAS, similarly to all RAS proteins, is a molecular binary switch that can exist in an inactive state, bound to GDP, and an active state, bound to GTP (Bar-Sagi and Hall, 2000; Malumbres and Barbacid, 2003). KRAS is activated by pro-survival signals that are transduced by tyrosine kinase receptors (TKRs). Once the ligand binds to TKRs, they dimerize and autophosphorylate, offering the proper platform for adaptor proteins that in turn recruit KRAS to the plasma membrane (Martinelli *et al.*, 2009; Normanno *et al.*, 2009; Saif, 2010). Activated KRAS can signal to a variety of downstream proteins, including the RAF/ERK/MEK signalling cascade and PI3K (phosphatidylinositol 3-kinase) (Shields *et al.*, 2000; Rajalingam *et al.*, 2007). Similarly to gal-3, also KRAS has mainly a pro-survival role in cells, promoting cell cycle progression (Pruitt and Der, 2001; Coleman *et al.*, 2004) and modulating apoptosis (Chang *et al.*, 2003; Cox and Der, 2003). Consequently, it is not surprising that activating mutations of KRAS are among the most common in tumours. Actually, *KRAS* is the most frequently mutated (~85%) among all RAS isoforms, which together are mutated in approximately 20% of human cancers

(Downward, 2003). In particular, *KRAS* mutations are particularly frequent in colorectal and pancreatic cancer (Forbes *et al.*, 2011).

RAS proteins structure and activity is highly conserved among different organisms, including the yeast *S. cerevisiae* (Wennerberg *et al.*, 2005). In yeast, there are two Ras homologues, Ras1 and Ras2, which cover the same cellular functions, but are differently expressed. For example, *RAS1* is expressed only at very low levels after mid-logarithmic growth phase and when yeast is grown on non-fermentable carbon sources, whereas *RAS2* is always expressed at high levels (Breviario *et al.*, 1986; 1988). Yeast Ras proteins do not activate the same proteins as mammalian KRAS, but the outcomes of Ras activation and the triggers for Ras activation are very similar. Indeed, yeast Ras are activated upon nutrient signalling, especially glucose, and, once activated by GTP binding, they in turn activate adenylate cyclase. The main function of adenylate cyclase is to produce the second messenger cAMP (3',5'-cyclic adenosine monophosphate), which can remove the inhibition from protein kinase A (PKA) (Gibbs and Marshall, 1989; Tamanoi, 2011). PKA phosphorylates a high number of targets, mainly leading to ribosome biogenesis, cell cycle progression, increased cell size and consequently increased growth rate (Zaman *et al.*, 2008; 2009; Busti *et al.*, 2010). Therefore, similarly to mammalian RAS proteins, yeast Ras proteins promote cell growth and survival. However, yeast Ras proteins also control stress response, mainly by downregulating the genes usually involved in the cell defence.

Glucose signals to the cell the availability of nutrients and the absence of threatening conditions, activating Ras, so that the whole cell metabolism is shifted in order to increase growth and to decrease the energy usually reserved for cell defence (Tamanoi, 2011). The same genes involved in stress response are involved in controlling ageing in yeast, characterized by high level of reactive oxygen species (ROS) and nutrient depletion. So, Ras proteins are also involved in the response to specific ageing-inducing stresses, usually accelerating it (Longo, 2003, 2004; Longo and Fabrizio, 2012). Therefore, activating mutations in yeast *RAS2*, the most expressed between Ras proteins, decrease chronological life span and increase sensitivity to stress (Fabrizio *et al.*, 2003; Hlavata *et al.*, 2003), at the same time increasing growth rate and cell size (Baroni *et al.*, 1989; Mitsuzawa, 1994; Jorgensen *et al.*, 2002).

As mentioned above, yeast and mammalian Ras proteins are highly homologous (DeFeo-Jones *et al.*, 1983; Dhar *et al.*, 1984; Powers *et al.*, 1984) and they have been shown to be able to activate reciprocal downstream targets. Indeed human HRAS protein, a different isoform compared to KRAS, was able to complement the phenotypes caused by the absence of *RAS2*, activating adenylate cyclase (Clark *et al.*, 1985; DeFeo-Jones *et al.*, 1985; Kataoka *et al.*, 1985; Toda *et al.*, 1985; Toda *et al.*, 1986; Broek *et al.*, 1987; Ho and Bretscher, 2001). Conversely, a yeast-mammalian hybrid *RAS* gene was able to cause a phenotype resembling the one of *RAS*-transformed cells in mouse (DeFeo-Jones *et al.*, 1985). Even if the ability of *HRAS* to complement for *RAS2* is well documented, little is known about the capability of other human RAS isoforms, such as KRAS, to do the same, mostly because in the middle '80s, when most of the research in this regard was made, the studies were mainly focusing on HRAS (Cox and Der, 2010). Moreover, very few studies addressed the role of Ras1 or its possible complementation by a human RAS protein.

Yeast, humanized through the expression of *KRAS*, both wild type and mutant ($KRAS^{G13D}$, $KRAS^{G12D}$, $KRAS^{G12V}$), was used to investigate the role of *KRAS* mutation in autophagy (Alves *et al.*, 2015), a physiological process relevant for cancer progression when deregulated (Chen and Karantza, 2011). KRAS was shown to function in yeast and activate yeast downstream proteins involved in the same processes of mammalian RAS targets. This evidence is particularly important in view of the present work, which aims to build an *S. cerevisiae*-based platform for the study of processes associated to the interaction between galectin-3 and KRAS, and the effect of pharmacological drugs that demand that interaction. The utilization of humanized *S. cerevisiae* as model to study proteins involved in human pathogenesis, specifically cancer, is well documented (Nitiss and Heitman, 2007; Pereira *et al.*, 2012; Laurent *et al.*, 2016). This approach envisages the heterologous expression in yeast of human proteins, which in this way can be studied in a neutral environment, without the redundancy of pathways and proteins usually encountered in human cells. In addition, the utilization of yeast has the advantage of easy genetic manipulation, fast generation time and cheap maintenance, especially exploitable for high-throughput studies (Mager and Winderickx, 2005; Botstein and Fink, 2011). Yeast can express human proteins that have a yeast counterpart, like in the

case of RAS proteins, as well as proteins without a yeast orthologue, like the case of gal-3. In both cases there are advantages and disadvantages. If yeast has a counterpart of the human protein, it is more likely that the human proteins activate a specific and recognizable signalling pathway, making easier to recognise its function and the role of possible mutations. On the other hand, the absence of orthologue makes sure that the protein has no interference from yeast endogenous proteins, but can also induce protein degradation or absence of recognisable phenotypes. An excellent example in this regard is the study of the tumour suppressor p53 in yeast, which allowed the discovery of important protein regulation mechanisms (Pearson and Merrill, 1998; Hu *et al.*, 2001) and protein mutations functions (Di Como and Prives, 1998; Robert *et al.*, 2000; Kato *et al.*, 2003).

In addition to the role in understanding and defining protein functions, using humanized yeast has been important in drug screening. Indeed, the expression of some human proteins can produce a recognizable phenotype in yeast, which can then be screened for suppression by other human proteins or chemical compounds (Simon and Bedalov, 2004; Menacho-Marquez and Murguia, 2007; Matuo *et al.*, 2012). Easy recognizable phenotypes, such as decreased growth, have been used in cases like the one of human poly (ADP-ribose) polymerases (PARP) proteins inhibitors. PARP encompass a family of proteins involved in DNA repair and apoptosis (Jubin *et al.*, 2016). Their expression in yeast decreased viability, therefore several compounds were tested for the ability of rescuing this phenotype (Perkins *et al.*, 2001). This approach has also been successfully used in the discovery of inhibitors of the pro-apoptotic protein Bax that resulted in uncovering the Bax inhibitor 1, , HMGB1, the bifunctional apoptosis regulator and the Calnexin orthologue Cnx1 (Torgler *et al.*, 1997; Xu and Reed, 1998; Zhang *et al.*, 2000a; 2000b; Brezniceanu *et al.*, 2003). Therefore, the usage of *S. cerevisiae* for understanding the role of human proteins involved in human pathogenesis, in particular cancer progression, and for efficiently testing drugs capable to inhibit or activate these proteins is possible and advisable. In addition, the amenability of yeast and the efficient visualization of yeast phenotypes make this model organism particularly suitable for drug screening, in particular in high-throughput scale, as already mentioned.

In this work, the construction of a yeast-based platform containing yeasts expressing either

human galectin-3 or human KRAS was achieved. These are steps required to further build a strain efficiently expressing both human gal-3 and KRAS. Two different genetic backgrounds were chosen, the common laboratory strains BY4741 and W303-1A. In addition, the human cDNAs were cloned in yeast strains lacking either yeast *RAS* genes. This was done in order to help the recognition of specific phenotypes caused solely by human KRAS, as well as to improve the probability of finding usable gal-3-derived phenotypes in yeast, in view of the known involvement of yeast Ras proteins in the processes of cell growth and ageing. The intracellular localization of gal-3 in yeast was checked using a GFP tag and the phenotypes on yeast growth rate and ageing derived from gal-3 expression were described for the first time. Similarly, the effects of human KRAS on yeast growth rate and various stress response was analysed in the presence and absence of either yeast *RAS* genes, in order to establish which phenotypes are caused by KRAS expression, and which by the loss of *RAS1* or *RAS2*. With further work, the yeast platform and the disclosed phenotypes could be used to screen KRAS and gal-3 exclusive inhibitors and, more importantly, inhibitors of their interaction, particularly relevant for cancer progression.

Materials and methods

Strains and growth conditions

The yeast strains used in this study are listed in **Table 1**. Yeasts were maintained on solid YPD medium (1% yeast extract, 1% peptone, 2% glucose and 2% agar) at 4°C and refreshed for 48h before every assay. Overnight YPD batch cultures (liquid:air ratio of 1:2.5 and orbital agitation at 200 rpm) were used for transformation. Transformants were maintained at 4°C on solid YNB medium (1.7 g/l w/v yeast nitrogen base, 0.5% ammonium sulphate, 2% glucose and 2% agar) supplemented with the appropriate amino acids and refreshed on the same medium for 72 h before every assay. The *Escherichia coli* LX1Blue (endA1 gyrA96 (nalR) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB+ lacIq Δ(lacZ)M15] hsdR17(rKmK+)) used for cloning procedures was maintained and cultivated according to standard procedures.

Table 1: *Saccharomyces cerevisiae* strains used in this study.

yeast strain	genotype
BY4741 wt	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*
BY4741 *Δras1*	Isogenic to BY4741 but *ras1::KanMX4*
BY4741 *Δras2*	Isogenic to BY4741 but *ras2::KanMX4*
W303-1A wt	*MATa leu2Δ3 leu2Δ112 ura3Δ1 trp1Δ1 his3Δ11 his3Δ15 ade2Δ1 can1Δ100*
W303-1A *Δras1*	Isogenic to W303-1A but *ras1::HIS3*
W303-1A *Δras2*	Isogenic to W303-1A but *ras2::KanMX4*

Basic cloning procedures

*p416GDP*LGALS3. The human galectin-3 cDNA (*LGALS3*) was amplified by PCR from the bacterial expression plasmid pET28a+ (MyBioSource) using the *LGALS3* specific primers FW_*LGALS3* (forward) and RV_*LGALS3* (reverse) (**Table 2**). Digestion of *LGALS3* cDNA and p146GDP plasmid was performed with EcoRI and XhoI restriction enzymes (NZYTech). Ligation of *LGALS3* cDNA into p416GDP (Addgene) was performed using T4 DNA ligase (NZYTech) using standard procedures.

*p416GDP*LGALS3GFP. *yEGFP* cDNA was amplified from pYES2GFP, a construction already

available (Tulha J. *et al.*, unpublished results), using the primers FW_GFP and RV_GFP (**Table 2**). yEGFP was fused into p416*LGALS3* using the flanking homology PCR cassette method (Orr-Weaver *et al.*, 1981).

Table 2: Primers used for the construction and the verification of the plasmids that express gal-3 and gal-3GFP. In the case of primers used to add homologous regions to perform homologous recombination, the annealing part of the primers is underlined, while the homology part is plain text.

primer	sequence
FW_*LGALS3*	5'-TTGGCCGAATTCATGGCAGACAATTTTTCG-3'
RV_*LGALS3*	5'-TTGGCCCTCGAGTTATATCATGGTATATGAAG-3'
FW_GFP	5'-TGGTGACATAGACCTCACCAGTGCTTCATATACCATGATATCTAAAGGTGAAGAATTATT-3'
RV_GFP	5'-GGAGGGCGTGAATGTAAGCGTGACATAACTAATTACATGATTATTTGTACAATTCATCCA-3'
FW_4XX	5'-CCAGAACTTAGTTTCGACGG-3'
RV_4XX	5'-GTGAATGTAAGCGTGACATAACTAATTACATGA-3'

*p426GDP*KRAS. The human *KRAS* cDNA was obtained from pLenti-KRAS^{wt}, kindly supplied by Instituto Português de Oncologia, Porto, Portugal. This was digested with the restriction enzymes HindIII and XhoI (NZYTech) to extract the KRAS cDNA. Ligation of *KRAS* cDNA into p426GDP (Addgene) was done using T4 DNA ligase (NZYTech) using standard procedures.

Chromosomal insertion of KRAS - *Strategy 1* (**Fig. 5**). *KRAS* was amplified from the previously constructed p426*KRAS* plasmid using the primers FW_*RAS1* and RV_*RAS1* (**Table 3**) for insertion in the *RAS1* locus, and FW_*RAS2* and RV_*RAS2* (**Table 3**) for insertion in the *RAS2* locus. The PCR amplified cDNA of *KRAS* presented 40 bp of homology with the flanking region to *RAS1* or *RAS2* locus, used to favour homologous recombination in *Δras1* and *Δras2* yeast mutants, respectively. Transformants were selected on the basis of loss of geneticin resistance and confirmed by colony PCR using the primers FW_*KRAS* and RV_*KRAS* (**Table 3**).

Chromosomal insertion of KRAS- *Strategy 2* (**Fig. 7**). Histidine gene and promoter (*HIS3*) were amplified by PCR from p413GDP (Addgene) using the primers FW_*KRASHIS3* and RV_*HIS3CYC1* (**Table 3**). The primers added 40 bp of homology to the 3'-end of *KRAS* and 5'-end of CYC1 terminator for the homologous recombination of *HIS3* downstream *KRAS* in

the p426*KRAS* plasmid. The chimera gene *KRASHIS3* was amplified by PCR from the plasmid p426*KRASHIS3* using the primers FV_*RAS1* and RV_*HIS3RAS1* or FV_*RAS2* and RV_*HIS3RAS2* (**Table 3**), which added 40 bp of homology for the insertion of the amplicon in *Δras1* or *Δras2* mutant, respectively. The transformants were selected by the ability to grow on minimal medium not supplemented with histidine, and then tested by colony PCR using the primers FW_*RAS1*gen or FW_*RAS2*gen and RV_*HIS3*middle.

Chromosomal insertion of KRAS- *Strategy 3* (**Fig. 9**)*. KRAS* cDNA was amplified by PCR from p426*KRAS* using the primers FV_*RAS1* or FV_*RAS2* and RV_KanX*KRAS* (**Table 3**). Kanamycin resistance cassette (KanX) was amplified from pUG6, kindly donated by Johannson B. (Centre of Molecular and Environmental Biology, University of Minho, Portugal) using the primers FW_KanX*KRAS* and RV_KanX*RAS1* or RV_KanX*RAS2* (**Table 3**). The *KRAS* fragment obtained with FV_*RAS1* and RV_KanX*KRAS* added homology for the recombination of *KRAS* in *RAS1* locus, putatively fused together with KanX fragment obtained with FW_KanX*KRAS* and RV_Kanx*RAS1*. The fragments of *KRAS* and KanX obtained using the other two pairs of primers (FV_*RAS2* and RV_KanX*KRAS;* FW_KanX*KRAS* and RV_KanX*RAS2*) presented homologous regions for the recombination in *RAS2* locus. The transformants were selected by the ability to grow on medium supplemented with geneticin and then tested by colony PCR with the primers FW_*KRAS* and RV_KanXmiddle.

Obtaining transformants

The transformation of *E. coli* LX1Blue and *S. cerevisiae* strains listed in **Table 1** were performed according to standard procedures (Green and Sambrook, 2012). Competent cells of *E. coli* were prepared following the Hanahan method (Hanahan, 1983) and transformed with a $CaCl_2$/heat shock-based method. Transformants were selected through ampicillin resistance. Plasmid extraction was done using the GenElute Plasmid Miniprep kit (Sigma-Aldrich), following the manufacturer instructions.

S. cerevisiae cells were transformed by the improved lithium acetate/SS carrier DNA/PEG method (Gietz *et al.*, 1995), with modifications. The heat shock was prolonged up to 45 min, and the recovery time at 30°C was extended up to 8 h. Transformants were selected on

YNB supplemented with specific amino acids according to auxotrophy requirements. The p416*LGALS3*GFP and the p426KRASHIS3 plasmids obtained from homologous recombination transformations were purified from the yeast transformants using the GenElute Plasmid Miniprep kit (Sigma-Aldrich), which required adjustments. Prior to lysis, 20 µl of lyticase (3.3 U/ml) were added to the yeast solution, incubated for 30 min at 37°C, and subjected to 5 min of constant vortexing together with 200 µl of glass beads. Success of the transformation was verified by plasmid digestion or PCR followed by agarose electrophoresis in the case of bacterial transformation, and by colony PCR in the case of yeast transformation.

Western blot analysis

Protein extraction. Total protein extracts were obtained according to Burke *et al.* (2000). Yeast cells were grown overnight, inoculated in fresh media and collected at two different growth time points: mid exponential phase ($O.D._{600}$ 0.5) and late exponential ($O.D._{600}$ 0.8). The cells were collected by centrifugation, and 0.2 M NaOH/2% β-mercaptoethanol was added to the pellet, further incubated on ice for 10 min. Proteins were precipitated with 20% trichloroacetic acid (TCA) for 15 min and washed twice with cold acetone. The dried pellet was solubilized in 2x Leammli buffer (Laemmli, 1970).

SDS-PAGE and blotting. The samples were run in a 5% stacking gel and 12% resolving polyacrylamide gel until complete separation. The proteins were then blotted on a PDVF (polyvinylidene difluoride) membrane, treated with the appropriate primary and secondary antibody, and revealed by chemiluminescence (ECL Bio-Rad). Anti-GFP and anti-Pgk1 were used as primary, and anti-mouse as secondary antibodies. All antibodies were purchased from Sigma-Aldrich. Densitometry analysis was performed using the free software ImageJ (http://imagej.nih.gov/ij/), and protein expression levels were normalized to the level of Pgk1.

Table 3: Primers used for the construction of the plasmid expressing *KRAS*; primers used for the construction and the verification of the chromosomal insertion of *KRAS* in the place of *RAS1* or *RAS2*. In the case of primers used to add homologous regions to perform homologous recombination, the annealing part of the primers is underlined, while the homology part is plain text.

primer	sequence
FW_*KRAS*	5'-GCGAAGCTTATGACTGAATATAAACTTGTGGTAGTTGGA-3'
RV_*KRAS*	5'-GCGCTCGAGCATAATTACACACTTTGTCTTTGACTTCTT-3'
FW_*RAS1*	5'-GATTGAACAGGTAAACAAAATTTTCCCTTTTTAGAACGACATGACTGAATATAAACTTGTGG-3'
RV_*RAS1*	5'-GCAATCAAAACCATGTCATATCAAGAGAGCAGGATCATTTTTACATAATTACACACTTTGTC-3'
FW_*RAS2*	5'-ATTGGCAAAAGCTTAACTTTCCTCTATATGTCTTTTTTTTATGACTGAATATAAACTTGTGG-3'
RV_*RAS2*	5'-GTTCTTTTCGTCTTAGCGTTTCTACAACTATTTCCTTTTTATTACATAATTACACACTTTGTC-3'
FW_*RAS1*gen	5'-CTTTTTCAACTCATCGCAAGATTAT-3'
RV_*RAS2*gen	5'-TGGGTAAACATTTATAGTACCCCAA-3'
RV_*KRAS*middle	5'-GGACCATAGGTACATCTTCAGAGTCC-3'
RV_*KanX*middle	5'-CTGCAGCGAGGAGCCGTAAT-3'
RV_*HIS3*middle	5'-ATCATATGGTCCAGAAACC-3'
FW_*KRASHIS3*	5'- AAAGAAGAAAAAGAAGTCAAAGACAAAGTGTGTAATTATGTATCCTTACGACGTGCCTGACTACGCCTAACTAGTACACTCTATATTTTT- 3'
RV_*HIS3CYC1*	5'- GGGGGGCCCGGTACCCAATTCGCCCTATAGTGAGTCGTACTACATAAGAACACCTTTGG--3'
RV_*HIS3RAS1*	5'-GCAATCAAAACCATGTCATATCAAGAGAGCAGGATCATTTCTACATAAGAACACCTTTGG-3'
RV_*HIS3RAS2*	5'-TTCTTTTCGTCTTAGCGTTTCTACAACTATTTCCTTTTTACTACATAAGAACACCTTTGG-3'
RV_KanX*KRAS*	5'-CGTCAAGACTGTCAAGGAGGGTATTCTGGGCCTCCATGTCTTACATAATTACACACTTTGTCTTT-3'
FW_KanX*KRAS*	5'-GAAGAAAAAGAAGTCAAAGACAAAGTGTGTAATTATGTAAGACATGGAGGCCCAGAATACCCTCC-3'
RV_KanX*RAS1*	5'-GCAATCAAAACCATGTCATATCAAGAGAGCAGGATCATTTCAGTATAGCGACCAGCATTCACATA-3'
RV_KanX*RAS2*	5'-TTCTTTTCGTCTTAGCGTTTCTACAACTATTTCCTTTTTACAGTATAGCGACCAGCATTCACATA-3'

Fluorescence microscopy

A Leica Microsystems DM-5000B epifluorescence microscope with DIC (differential interference contrast) and light filter setting specific for GFP visualization and a 100x/1.0 oil-immersion objective was used, in order to visualize the chimera protein gal-3GFP.

Growth rate measurement

The yeast cultures were inoculated overnight at 30°C, 200 rpm, in YNB supplemented with the appropriate amino acids. The overnight grown culture was used to inoculate fresh YNB to an $O.D._{600}$ of 0.05 – T0. The culture was then monitored for 28 h, reading $O.D._{600}$ every hour for the first 14 h, and every 4 h after that. Specific growth rate (μ_g) was estimated from the logarithmic growth phase. At least three independent experiments were performed.

Chronological life span

The yeast cultures were inoculated overnight at 30°C, 200 rpm, in YNB supplemented with the appropriate amino acids. The overnight grown culture was inoculated in fresh YNB medium to an $O.D._{600}$ of 0.1. After 24 h of growth at 30°C, 200 rpm, an aliquot of 1 ml of culture was taken and serially diluted 1:10 in dH_2O four times. The last dilution was used to plate 8 drops of 40 µl on YPD. The plate was incubated 48 to 72 h at 30°C, until the colonies were visible, and the colonies originating from each drop were counted. This was considered T0. Subsequently, the same procedure was repeated every 60-96 h, until no colonies were able to grow (~2 weeks). The survival was estimated as a percentage of the media of the number of colonies obtained in each drop at each time point (8 drops) in comparison with T0- 100%. Four to five independent experiments were performed to ensure statistical significance.

Serial drop test

Serial drop test was performed from mid-exponential YPD (stress stimuli in **Table 4**) or YNB (stress stimuli in **Table 5**) culture. Five 1:10 serial dilutions were made and 5 µl of each dilution were plated on YPD or YNB plates supplemented with the appropriate stress inducers (**Table 4** and **5**). The dilutions were plated as well on YPD or YNB agar medium

without stressor as control. Results were imaged after 48 h (YPD) or 72 h (YNB) at 30°C. Every condition was repeated three to four times, to ensure reproducibility.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software. One-way ANOVA analysis was used. The difference between experimental data was considered significant whenever p-value ≤ 0.05.

Table 4: Medium and conditions used in the serial drop test for stress resistance, listed according to the type of stress stimuli, with YPD as basal medium.

STRESS	BY4741	W303-1A
temperature	YPD - 37°C	YPD - 37°C
carbon source	YPE (2% ethanol, 1% yeast base, 1% peptone, 2% agar)	YPE (2% ethanol, 1% yeast base, 1% peptone, 2% agar)
	YPG (2% glycerol, 1% yeast base, 1% peptone, 2% agar)	YPG (2% glycerol, 1% yeast base, 1% peptone, 2% agar)
osmotic stress	1.3 M NaCl	1.3 M NaCl
	1.5 M KCl	1.5 M KCl
wall stress	10 mM caffeine	10 mM caffeine
	10 mM caffeine + 1 M sorbitol	10 mM caffeine + 1 M sorbitol
other stresses	6% ethanol	6% ethanol
	65 mM acetic acid (pH 4.5)	50 mM acetic acid (pH 4.5)

Table 5: Medium and conditions used in the serial drop test for stress resistance, listed according to the type of stress stimuli, with YPD as basal medium.

STRESS	BY4741
temperature	YNB - 37°C
carbon source	YNB-ethanol (2% ethanol, 0.5% ammonium sulfate, 1.7 g w/v yeast nitrogen base and 2% agar)
	YNB-glycerol (2% glycerol, 0.5% ammonium sulfate, 1.7 g w/v yeast nitrogen base and 2% agar)
osmotic stress	1 M NaCl
	1.5 M Sorbitol
oxidative stress	0.5 mM H_2O_2

Results

1. Platform construction and verification

Designing the LGALS3 expressing plasmids

Galectins are lectin proteins that do not exist in *S. cerevisiae* (Kasai and Hirabayashi, 1996; Dodd and Drickamer, 2001). However, yeast can express other type of lectins, such as calnexin, L-type and P-type lectins (Dodd and Drickamer, 2001). To the extent of our knowledge, only one study was performed using *S. cerevisiae* to express human galectins, in particular galectin-1,-3 and-4 (Ryckaert *et al.*, 2008). The purpose of that study was the surface presentation of the galectins on the outer side of the yeast cell, forcing therefore the localization of the galectins with a specific genetic strategy. The yeast cells were used as Surface Display Technology tools to analyse the interaction between lectins and carbohydrate in a living environment capable of mimicking the natural multivalent presentation of lectins on cell surface (Ryckaert *et al.*, 2008). In the present study, on the other hand, the localization of gal-3 should be intracellular, as the one of KRAS, so that the two proteins can interact. The choice was to transform the yeast with a constitutive plasmid expressing gal-3 without addressing the protein to any specific cell compartment, allowing spontaneous physiological localization. This presented beforehand several challenges. Gal-3 could (i) not be produced, (ii) be synthetized but immediately degraded for absence of recognition, or (iii) it could be toxic to the yeast, either alone or after combining with the expression of *KRAS*. Moreover, the spontaneous localization of the protein could not be adequate for interacting with KRAS.

The first step was to assess the capability of the yeast to express and synthetize gal-3 and to verify whether the intracellular localization of the protein was tolerated, allowing the cell to survive. Gal-3 was inserted in a constitutive low copy expression plasmid, p416GPD. A centromeric plasmid such as p416GPD, should promote a low transcription rate of the gene, at around one copy per cell (Christianson *et al.*, 1992; Mumberg *et al.*, 1995), preventing putative cytotoxicity deriving from high amount of the foreign protein. Human *LGALS3* cDNA was amplified by PCR from a commercially acquired plasmid (My BioSource) and the primers

used for amplification (**Table 2**, FW_*LGALS3* and RV_*LGALS3*) were designed in order to add two restriction sites at the terminals of the *LGALS3* fragment. The so-obtained *LGALS3* fragment and the p416GPD plasmid were digested using the same restriction enzymes and subjected to ligation using T4 ligase (**Fig. 1A**). The product obtained was amplified in *E. coli* and further transformed into *S. cerevisiae*, obtaining p416*LGALS3*.

Another plasmid expressing *LGALS3* was designed to express a chimera containing GFP, in order to follow more easily the behaviour of the protein in the yeast environment. *LGALS3* was cloned together with yeast-enhanced GFP (yEGFP). The flanking homology PCR cassette method was chosen to fuse yEGFP into the plasmid already expressing *LGALS3*. yEGFP was amplified by PCR using primers that added 40 bp at each terminal (**Table 2,** FW_GFP and RV_GFP). The 40 bp are homologous to the regions flanking the future position of yEGFP – the 3'-end of *LGALS3* and the 5'-end of *CYC1* terminator (**Fig. 1B**).

The yeast was transformed at the same time with yEGFP and p416*LGALS3*. The recombinant plasmid obtained was extracted from yeast, amplified in *E.coli* and further extracted from *E. coli* for verification by PCR using two different sets of primers. The empty p416GPD plasmid was used in parallel as control. *LGALS3* presence was assessed with the same set of primers used for its construction (**Table 2,** FW_*LGALS3* and RV_*LGALS3*), while the presence of *LGALS3*GFP was assessed with the forward primers for *LGALS3*, FW_ *LGALS3*, and the reverse for GFP, RV_GFP (**Table 2**). The three plasmids (p416 Ø, p416*LGALS3* and p416*LGALS3*GFP) were also used as templates for amplification with a different set of primers, which anneal to the regions flanking its multicloning site, *i.e*. the end of the GPD promoter and the beginning of the *CYC1* terminator (**Table 2,** FW_p4XX and RV_p4XX, respectively). This consisted in a further control, done to determine if the genes were not only inserted in the plasmid and correspondent to the right size, but also if they were in the proper position inside the plasmid. The amplified fragments resulted as expected (**Fig. 2A**). The fragment correspondent to *LGALS3* (**Fig. 2A,** lanes 1 and 2) has around 800 bp, matching the size of the *LGALS3* cDNA. The same results were obtained using the p4XX primers, confirming the position of *LGALS3* inside the cloning site of the plasmid. *LGALS3*GFP fragment also measured the right size, around 1400 bp – 753 bp *LGALS3* + 714 bp GFP – and was found in the right location in the

plasmid (**Fig. 2A,** lanes 3 and 4). The specificity of the primers was confirmed by the fact that no amplification was obtained when the empty plasmid was used as DNA template (**Fig. 2A,** lanes 5 and 6).

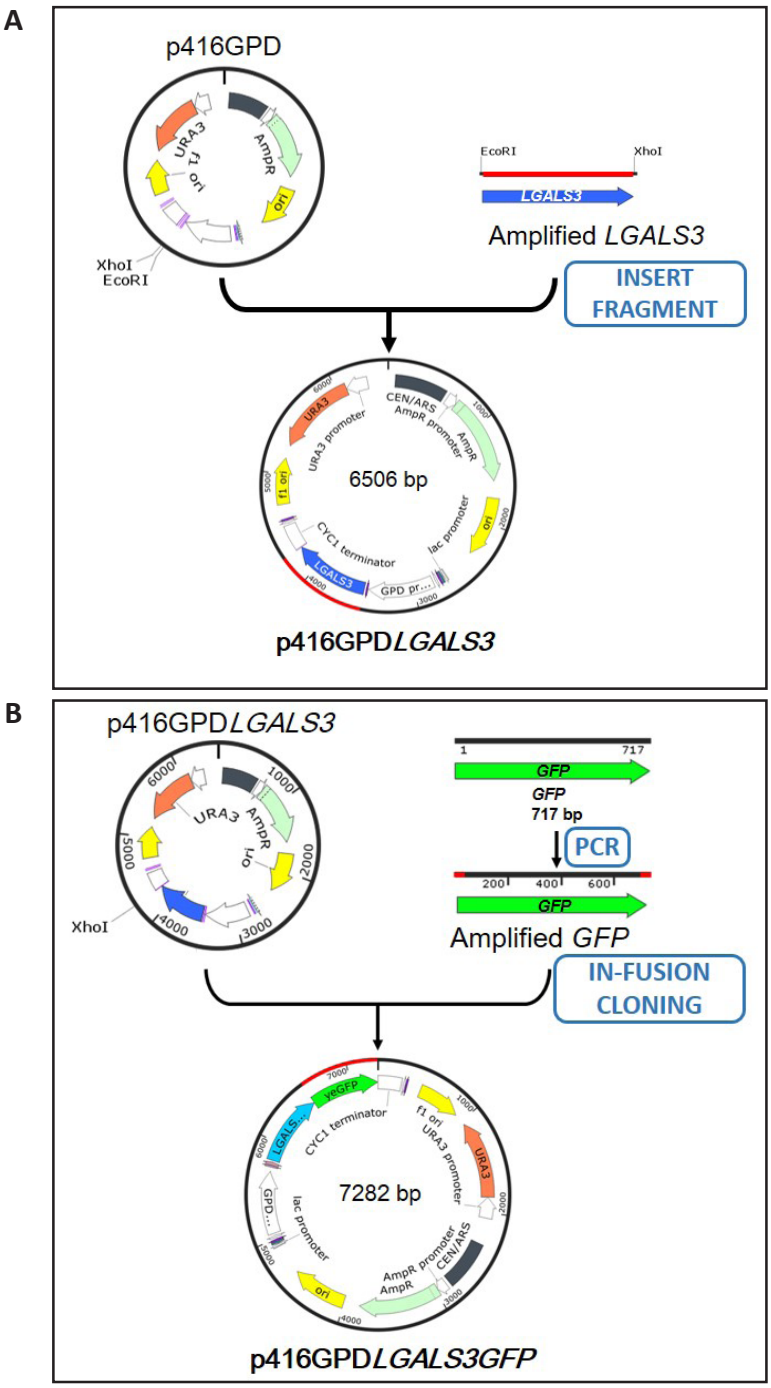
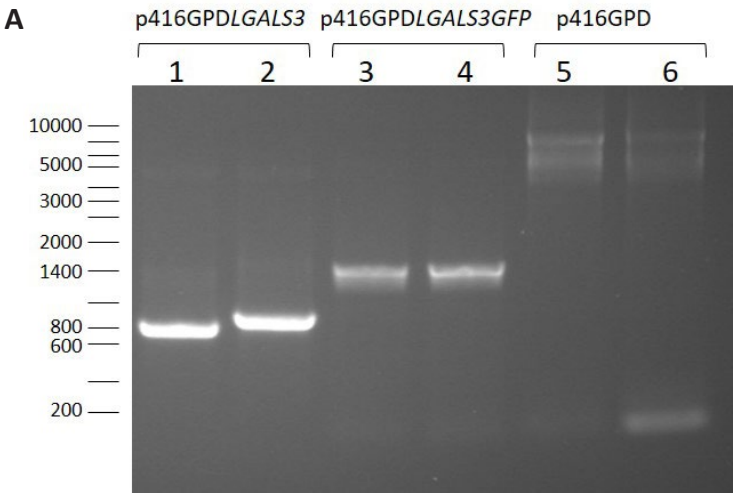


Figure 1: *Designing the plasmid expressing galectin-3*. *LGALS3* cDNA was amplified by PCR, digested and ligated into the equally digested p4216GPD plasmid by T4 ligase, obtaining the p416GPD*LGALS3* plasmid (**A**). *yEGFP* was amplified by PCR and fused into p416*LGALS3* plasmid by homologous recombination, originating p46GPD*LGALS3GFP* (**B**).

As recipient yeasts, both *S. cerevisiae* BY4741 and W303-1A strains were used. This aimed at avoiding putative drawbacks deriving from one specific genetic background (Cohen and Engelberg, 2007). These strains were chosen for two reasons. Firstly, they are well known for their very different phenotypic responses, in particular in the viability/stress resistance tests planned for the complete platform (Petrezselyova *et al.*, 2010; Kokina *et al.*, 2014). Secondly and importantly, these strains different auxotrophic markers implicate different amino acid synthesis pathways, which may impact, even if indirectly, on the Ras pathway performance and therefore on the effect of human *KRAS* expression. Bearing this in mind, *RAS1* and *RAS2* mutants from both genetic backgrounds were added to the construction of the platform. These mutants were used in the attempt to impede a strong interference of yeast Ras proteins functions in the expression and putative roles of the heterologously expressed human *KRAS*. In *S. cerevisiae*, it is not possible to avoid the presence of both yeast Ras proteins, since the double deletion mutant is unviable (Kataoka *et al.*, 1984; Tatchell *et al.*, 1984). This way, the *LGALS3* recipient yeast strains were six: W303-1A wt, *Δras1* and *Δras2*, and BY4741 wt, *Δras1* and *Δras2*. All these strains were transformed with p416 empty plasmid, p416*LGALS3* and p416*LGALS3*GFP. The efficacy of the transformation with p416*LGALS3* was verified by colony PCR, using primers specific for *LGALS3* (**Table 2**, FW_*LGALS3* and RV_*LGALS3*). All the transformants tested successfully demonstrated the presence of *LGALS3* DNA (**Fig. 2B**).



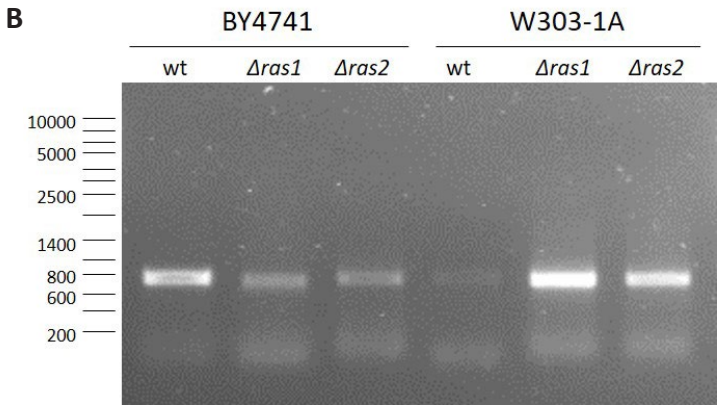
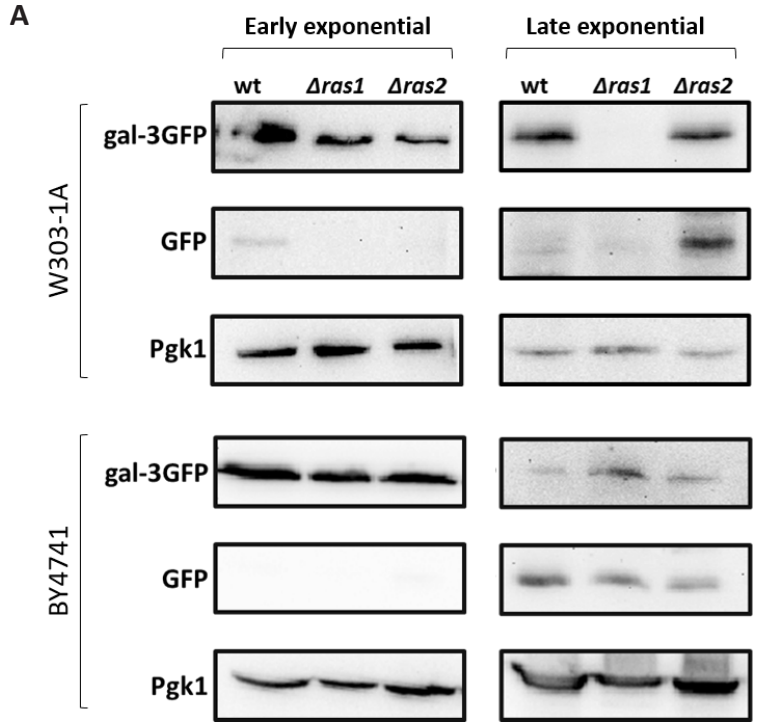


Figure 2: *Verification of the construction of the plasmids expressing gal-3 and of their transformation into* S. cerevisiae. The two plasmids harbouring L*GALS3*, p46GPD*LGALS3* and p416GPD*LGALS3GFP* were verified by PCR. p416GPD*LGALS3* was amplified with primers for *LGALS3* cDNA (lane 1) and for GPD promoter and CYC1 terminator (lane 2), obtaining in both cases a product corresponding to the size of gal-3, around 750 bp. p416GPD*LGALS3GFP* was amplified with the primers for the chimera gene *LGALS3GFP* (lane 3) and for GPD promoter and *CYC1* terminator (lane 4). The products observed corresponded to the chimera gene *LGALS3GFP*, around 1400 bp. Controls correspond to the empty plasmid p416GPD, used as template for the primers used for *LGALS3* (lane 5) and promoter and terminator (lane 6). No amplification was obtained, meaning that the primers are specific for the gene. The fragment obtained using the primers for the plasmid is too small to be visualized (**A**). *S. cerevisiae* BY4741 and W303-1A wt, *Δras1* and *Δras2* were transformed with p416GPD*LGALS3* and the correct insertion of the plasmid was verified by colony PCR. (**B**). The band sizes indicated correspond to the Ladder III (NZYTech).

On the other hand, the efficacy of the transformation with p416*LGALS3GFP* was verified by fluorescence microscopy and western blot (WB) analysis using anti-GFP antibody (**Fig. 3**). Actually, WB was performed not only to confirm the expression of *LGALS3GFP* in *S. cerevisiae,* but also to compare the efficiency of *LGALS3GFP* expression between *S. cerevisiae* genetic backgrounds. Cells from two growth phases of batch cultures on glucose were used, early/middle log ($O.D._{600}$ ~0.5) and middle/late log ($O.D._{600}$ ~0.8). Protein extraction procedure was verified using an antibody against Pgk1, a protein ubiquitously expressed in yeast. Results showed that gal-3GFP chimera is identically expressed in both W303-1A and BY4741 backgrounds (**Fig. 3A**). Moreover, in the late log phase (**Fig. 3A**, right) the intensity of the chimera band was fainter than in the early log phase, while the GFP-alone band was stronger (**Fig. 3A**, left), which suggests that the galectin-3 undergoes increased degradation as growth

proceeds. Interestingly, in the late log phase, the expression of the chimera protein in W303-1A wt was higher than in the mutants that also showed different expression level. Indeed, the expression decreased from wt and *Δras2* to *Δras1* (**Fig. 3A**, upper right panel), which almost did not express the chimera protein. Actually, *Δras2* showed a higher expression, but an increased amount of degraded galectin-3 compared to the wt (**Fig. 3A,** upper right panel), indicating that this mutant was less tolerant to the chimera. In opposition, the BY4741 genetic background did not present such differences (**Fig. 3A**, lower panels).

Early log cells were further analysed by fluorescence microscopy. The localization of gal-3GFP did not differ significantly between W303-1A and BY4741 (**Fig**. **3B**). The chimera localized mainly in the cytoplasm. Especially in the two mutants lacking either *RAS1* or *RAS2*, its distribution was clearly punctuated. Gal-3GFP appeared clustered in small brightly green fluorescent dots (**Fig. 3B**, *Δras1* and *Δras2* BY4741 and W303-1A). A similar distribution is usually observed for proteins that localize in the Golgi (http://yeastgfp.yeastgenome.org/), but it could also derive from the formation of protein multimers, or proteins accumulation in vesicles (Babst *et al.*, 2002; Smaczynska-de Rooij *et al.*, 2012).



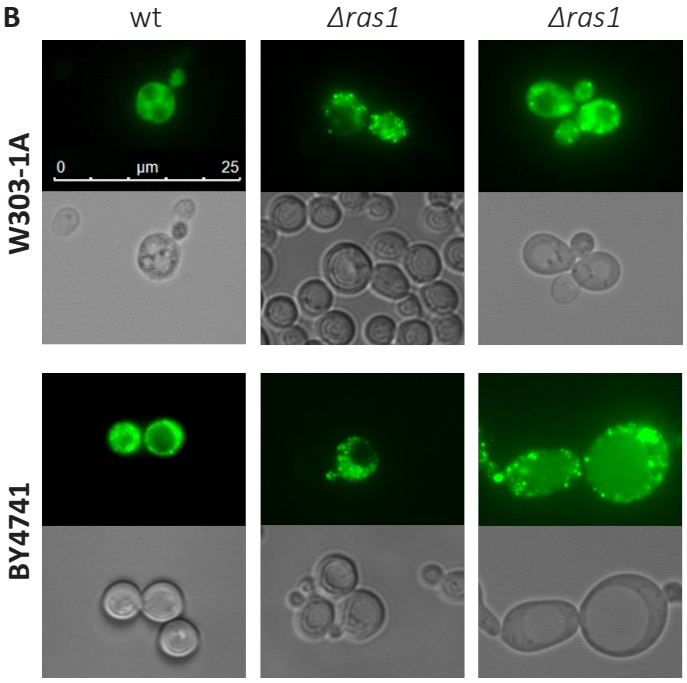


Figure 3: *Galectin-3GFP expression and localization in* S. cerevisiae. The expression of the chimera protein gal-3GFP in *S. cerevisiae* strains W303-1A and BY4741, wt and lacking either *RAS1* or *RAS2*, was obtained through transformation with p416*LGALS3GFP* plasmid and detected by WB using protein extracts obtained in two different yeast growth phases, early log ($O.D._{600}$ ~0.5) and late log (O.D.600 ~0.8). The blot images are representative of at least two independent experiments (**A**). The protein expression was confirmed by fluorescence microscopy using early log phase cells of strains W303-1A and BY4741 wt as well as *Δras1* and *Δras2* mutants. Figure depicts representative images of each (**B**).

Designing the KRAS expressing yeasts

The insertion of *KRAS* in *S. cerevisiae* was planned in two different ways, by plasmidic transformation and by chromosomal insertion. The plasmidic transformation offers a direct comparison with the yeasts expressing *LGALS3* also deriving from a plasmid construction, while the chromosomal insertion is intended for the double transformation with *LGALS3* and *KRAS*. The transformation with plasmid requires the maintenance of the yeast on minimal medium supplemented by the proper amino acids, without the selective marker necessary to maintain the plasmid expression. On the other hand, the chromosomal transformation is more stable and, once the human gene is inserted in the yeast chromosome, the yeast can be cultivated in any condition, depending alone on the regulation from the correspondent promoter. In addition, these two cloning modes also generate different expression levels. In

the case of plasmid transformation, the expression directly depends on the type of plasmid chosen – centromeric or 2µ- and by its promoter. On the other hand, when a gene is inserted in a chromosome, one of two choices may be done, either cloning together with the gene an exogenous promoter replacing the full *locus*, or cloning only the gene, keeping the control of an endogenous yeast promoter, subjecting the foreign protein to the same expression regulation as the substituted gene.

Designing the KRAS expressing plasmids

The p426GPD plasmid was chosen for the *KRAS* plasmid transformation strategy. Unlike the case of galectin-3, the expression of human RAS proteins in yeast is well documented (Clark *et al.*, 1985; Kataoka *et al.*, 1985), leading to consider that KRAS does not induce significant cytotoxicity. Therefore, a 2µ plasmid with a strong promoter (GPD) was chosen, which produces around 5-fold higher amounts of protein compared to a centromeric plasmid (Mumberg *et al.*, 1995), resulting in more than one plasmid copy per cell.

The *KRAS* cDNA was amplified by specific primers (**Table 3,** FW_*KRAS* and RV_*KRAS*) that added one restriction site at each end of the DNA fragment, to allow the insertion in the plasmid. The plasmid and the *KRAS* fragment were digested with specific restriction enzymes (HindIII and XhoI) and ligated by T4 ligase (**Fig. 4A**). The so-obtained recombinant plasmid was amplified in *E.coli*, subsequently extracted and verified by PCR. Identically to above, the empty plasmid p426GPD was used as negative control and amplification was done with two different sets of primers. One set of primers corresponded to the one used to amplify *KRAS* in the first place (**Table 3,** FW_*KRAS* and RV_*KRAS*), and the other was the one specific for p4XX plasmid, also used to verify *LGALS3* harbouring plasmids (**Table 2,** FW_p4XX and RV_p4XX). The band obtained matched *KRAS* size, 567 bp (**Fig. 4B**, lane 1), and *KRAS* was correctly inserted between the GPD promoter and the *CYC1* terminator (**Fig. 4B**, lane 2). Once the proper construction of the p426*KRAS* plasmid was verified, it was used to transform W303-1A and BY4741 wt, *Δras1* and *Δras2*. The correct insertion of the plasmid harbouring human KRAS was demonstrated by colony PCR using the primers that amplify KRAS (**Table 3,** FW_*KRAS* and RV_*KRAS*) (**Fig. 4C**).

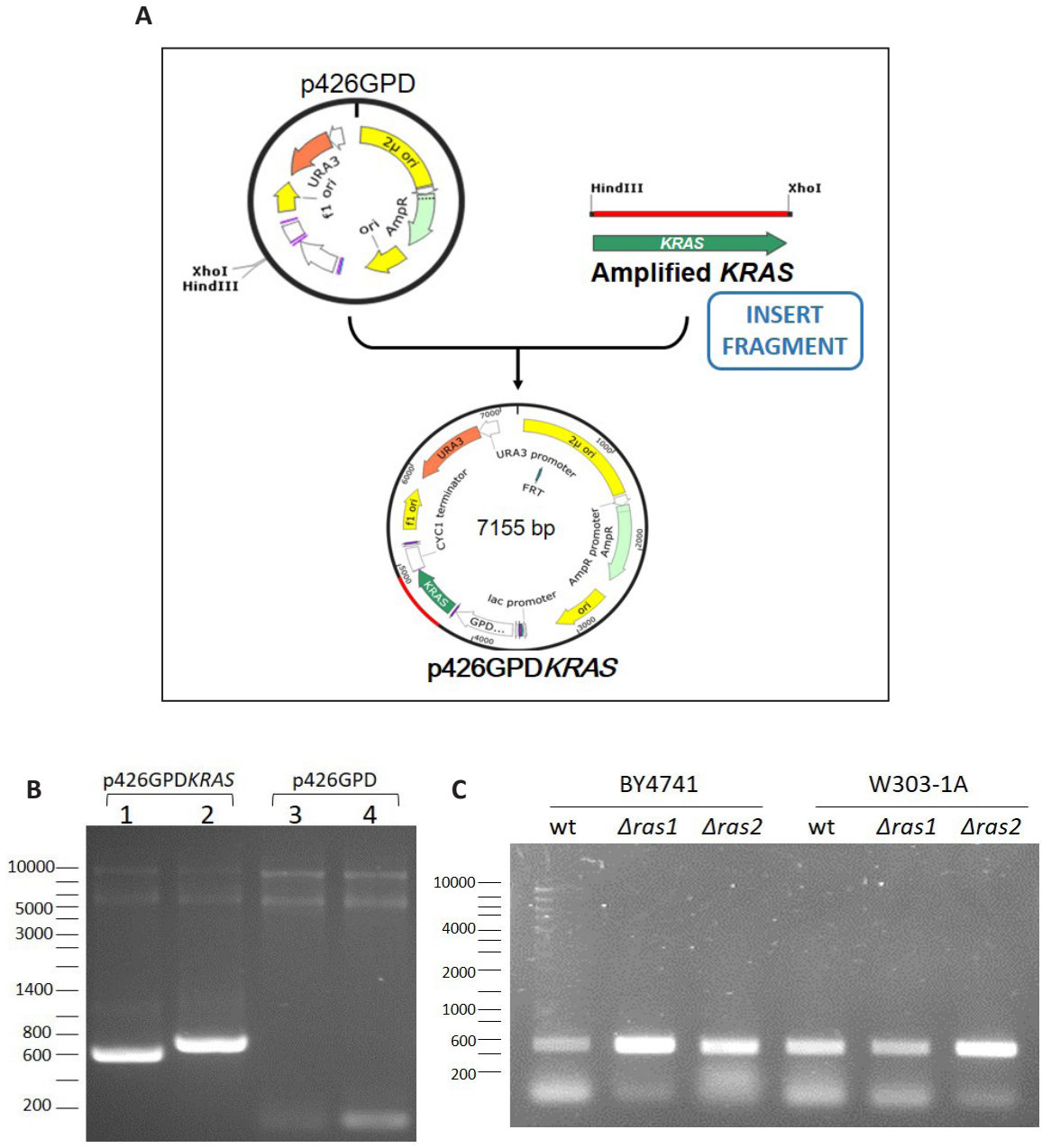


Figure 4: *Designing the plasmid expressing KRAS, its construction and transformation validation*. Human *KRAS* cDNA was amplified by PCR, digested and ligated into the equally digested p426 by T4 ligase, obtaining p426GPD*KRAS* (**A**). p426GPD*KRAS* was used as PCR template for primers that amplify *KRAS* cDNA (lane 1) and primers that anneal to GPD promoter and *CYC1* terminator (lane 2). Both amplifications gave as result a band corresponding to the size of *KRAS* gene, approximately 600 bp. The empty plasmid p426GPD was amplified with the same sets of primers to confirm their specificity. In lane 3 is show the result for the *KRAS* primers, in lane 4 the one for promoter and terminator (**B**). *S. cerevisiae* BY4741 and W303-1A wt, *Δras1* and *Δras2* were transformed with p416GPD*KRAS* and the correct insertion of the plasmid was verified by colony PCR. All the strains showed a band corresponding to *KRAS* gene, proving the successful transformation (**C**). The band sizes indicated correspond to the Ladder III (NZYTech).

Designing the KRAS chromosomal insertion

The chromosomal insertion of *KRAS* was planned, as mentioned above, to substitute yeast *RAS1* and *RAS2* genes in their *loci* under the control of their own promoters. Several different strategies were implemented in order to achieve the construction of the yeast chromosome containing human *KRAS*. In all cases, the transformation was the Flanking Homology PCR Cassette method. This method takes advantage of the high spontaneous recombination rates that occur in *S. cerevisiae* between two sequences of homologous DNA (Orr-Weaver *et al.*, 1981; Wendland, 2003). Usually, a homology region around 40 bp is enough to ensure correct recombination, but sometimes it may be necessary to extend this region due to some particular structure of the chromatin (Hegemann *et al.*, 2006).

Strategy 1*: insertion of* KRAS *in the place originally occupied by* RAS1 *and* RAS2 *genes in* RAS *mutants.*

In the first strategy, the two yeast *RAS* mutants from both *S. cerevisiae* BY4741 and W303-1A were used. The proper insertion of *KRAS* is expected to occur in the place of the cassette used to delete each *RAS* gene (**Fig. 5**). In the case of BY4741 *Δras1* and *Δras2* and W303-1A *Δras2*, the cassette is kanamycin-based, conferring resistance to geneticin. In W303-1A *Δras1*, the *RAS1* was deleted using the *HIS3* gene instead. *KRAS* cDNA was amplified using 60 bp primers. 40 bp out of 60 bp were homologous to the flanking regions of *RAS1* and *RAS2*, while the other 20 bp were necessary for the annealing of the primers to *KRAS* gene during the PCR cycles. Since *RAS1* and *RAS2 loci* are in different chromosomes, respectively XV and XIV, (Kataoka *et al.*, 1984; Tatchell *et al.*, 1984), two different sets of primers were used to amplify the *KRAS* gene introducing specific flanking sequences (**Table 3**, FW_*RAS1* and RV_*RAS1*; FW_*RAS2* and RV_*RAS2*). The amplified *KRAS* was used to directly transform the yeast mutants.

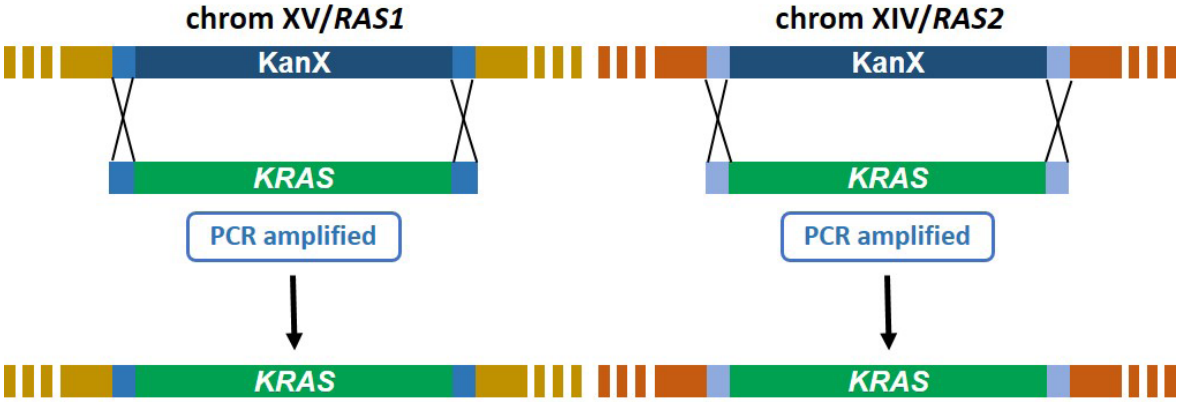


Figure 5: *Strategy 1 for the chromosomal insertion of* KRAS. *KRAS* cDNA was amplified with primers that add at the terminals of the amplified sequence homology regions to the flanking DNA of *S. cerevisiae RAS1* or *RAS2*. The insertion was performed in BY4741 and W303-1A *Δras1* and *Δras2*.

The yeasts were firstly grown on rich medium, while the selection for the gene insertion was done in parallel on rich medium, alone or complemented with geneticin, or medium without histidine in the case of W303-1A *Δras1*. Colonies were replicated in both types of media. The colonies not able to grow in the presence of geneticin or without histidine were selected. These colonies are supposed to harbour cells where either the KanX cassette or the *HIS3* gene was replaced with the human *KRAS*. Once selected, the colonies were further tested for the actual presence of *KRAS* by colony PCR, using the forward and reverse primers for *KRAS* (**Table 3**, FW_*KRAS* and RV_*KRAS*) (**Fig. 6A**). The colonies positive for *KRAS* were tested to determine the position of *KRAS* inside the yeast genome. A primer annealing around 350 bp upstream the gene insertion site (**Table 3**, FW_*RAS1*gen or FW_*RAS2*gen) was paired with either a primer annealing in the middle of the *KRAS* gene sequence, or in the middle of KanX cassette/*HIS3* gene (**Table 3**, RV_*KRAS*middle, RV_KanXmiddle, RV_*HIS3*middle). None of the colonies selected showed amplification when using the reverse primer annealing to *KRAS*, but they all did when using the reverse for either KanX or *HIS3* (**Fig. 6B**), showing that *KRAS* gene was inserted in the yeast genome, but not in the place of *RAS1* or *RAS2* as it should, and therefore not under the control of the proper promoter. Due to the time consuming procedure used to select positive colonies with this transformation protocol, a different strategy was adopted.

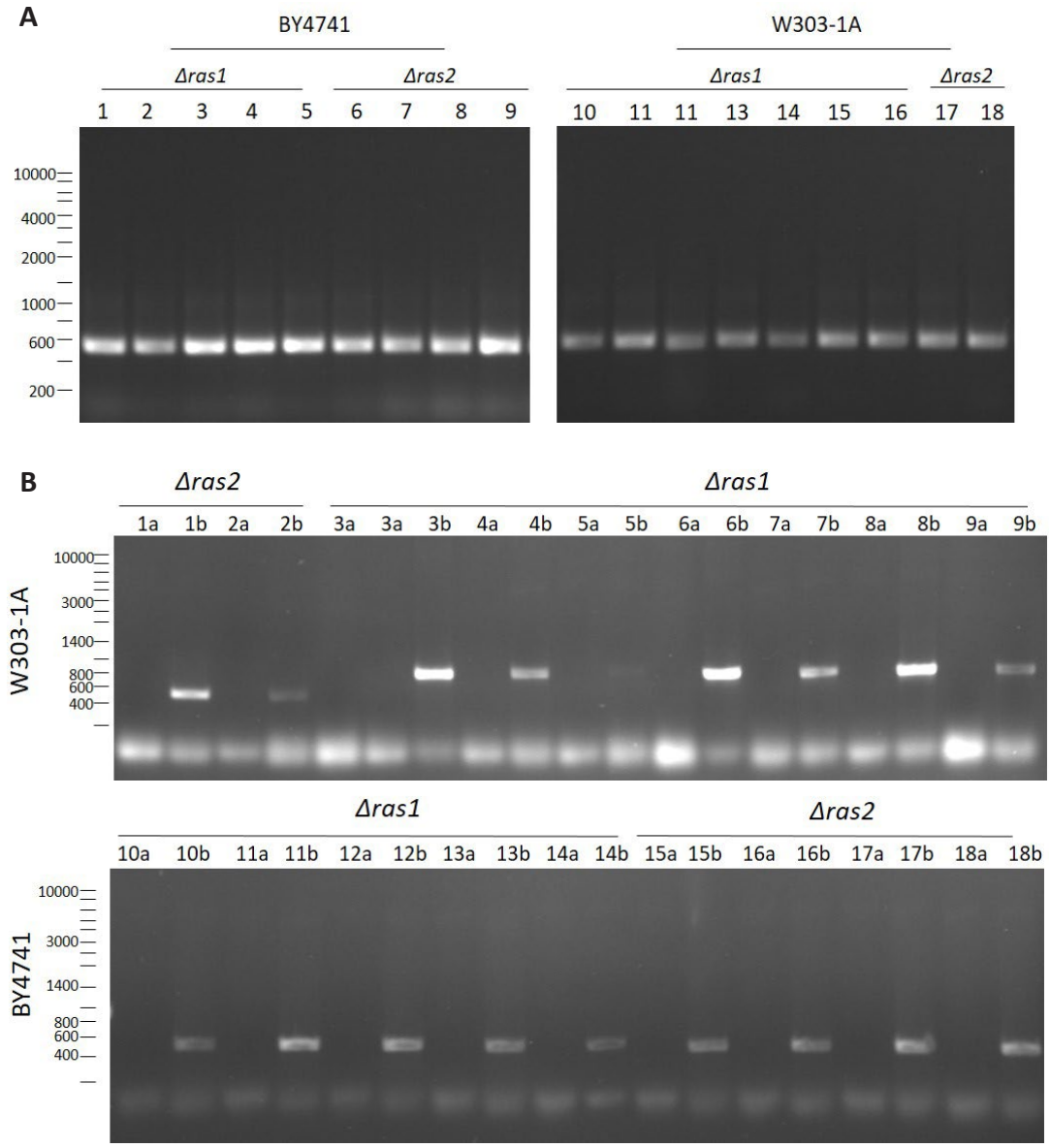


Figure 6: *Validation of KRAS chromosomal insertion following the transformation strategy 1*. Colonies showing ability to grow on selective marker were verified for the presence of *KRAS* by colony PCR. All the bands obtained are the same size of the *KRAS* gene, around 600 bp (**A**). For verification of *KRAS* position in the chromosome, different sets of primers were used. For BY4741 *Δras1* and *Δras2* and W303-1A *Δras2*, the FW primer anneals 250 bp upstream the 5'-end of the gene and the RV primer anneals either 170 bp downstream the 5'-end of KanX cassette or 340 bp downstream the 5'-end of *KRAS* gene. For W303-1A *Δras1*, the FW primer anneals as before, 250 bp upstream the 5'-end of the gene, and the RV primers either 384 bp downstream the 5'-end of the *HIS3* gene or 340 bp downstream the 5'-end of *KRAS* gene (**B**). The band sizes indicated correspond to the Ladder III (NZYTech). Results from successful and unsuccessful transformations are indicated with respectively a and b. Since the primers amplified the selective marker (KanX or *HIS3*) that should be deleted by the insertion of *KRAS*, when there is PCR product the transformation of *KRAS* into the yeast genome is unsuccessful.

***Strategy 2**: insertion of the amplicon* KRAS *+* HIS3 *in the place occupied by* RAS1 *or* RAS2 *gene.*

A direct selection method was preferred, with the fusion of the gene of interest together with a selective marker. This method is usually used to perform gene deletion (Langle-Rouault and Jacobs, 1995), but in the present case, *KRAS* was used instead to replace *RAS1* or *RAS2* in the wt strain. *KRAS* was first cloned in-frame upstream the *HIS3* gene and promoter, and the so-obtained amplicon inserted in the yeast genome, selecting for growth in the absence of histidine (**Fig. 7**). The strategy used to fuse the *HIS3* gene with *KRAS* was to insert *HIS3* DNA in the plasmid already harbouring *KRAS*, p426*KRAS*, once more through the Flanking Homology PCR Cassette method. *HIS3* was amplified by PCR from the empty plasmid p413GDP, whose selective marker is *HIS3* (Mumberg *et al.*, 1995). The primers used (**Table 3,** FW_*KRASHIS3* and RV_*HIS3CYC1*) added 40 bp at each end of *HIS3*. The 5'-end was homologous to the 3'-end of *KRAS* gene, and the 3'-end to the 5'-end of *CYC1* terminator. The DNA fragment was transformed into BY4741 wt strain together with p426*KRAS*.

The correct insertion of histidine in the plasmid was selected on minimal medium without histidine. The medium did not contain uracil as well, to select only colonies that incorporated the p426*KRAS* plasmid. The grown colonies were tested for the presence of the *KRASHIS3* fragment by colony PCR (**Fig. 8A**).

The plasmid p426*KRASHIS3* was subsequently extracted from the yeast cells and used as template for the amplification of the amplicon needed for the following transformation. In this case, the wt strains of both BY4741 and W303-1A were used. The insertion of *KRASHIS3* was planned to occur in the *loci* of *RAS1* or *RAS2*. For that purpose, the *KRASHIS3* fragment was amplified with FW_*RAS1* or FW_*RAS2* (**Table 3**), and with reverse primers that anneal to the 3'-end of *HIS3* and are homologous to the flanking region of the 3'-end of either *RAS1* or *RAS2* (**Table 3**, RV_*HIS3RAS1* and RV_*HIS3RAS2*). The obtained DNA fragment was used to directly transform yeast. The transformants were selected on minimal medium without histidine and tested for the presence of *KRAS* by colony PCR using FW_*KRAS* and RV_*KRAS* primers (**Table 3**) (**Fig. 8B**).

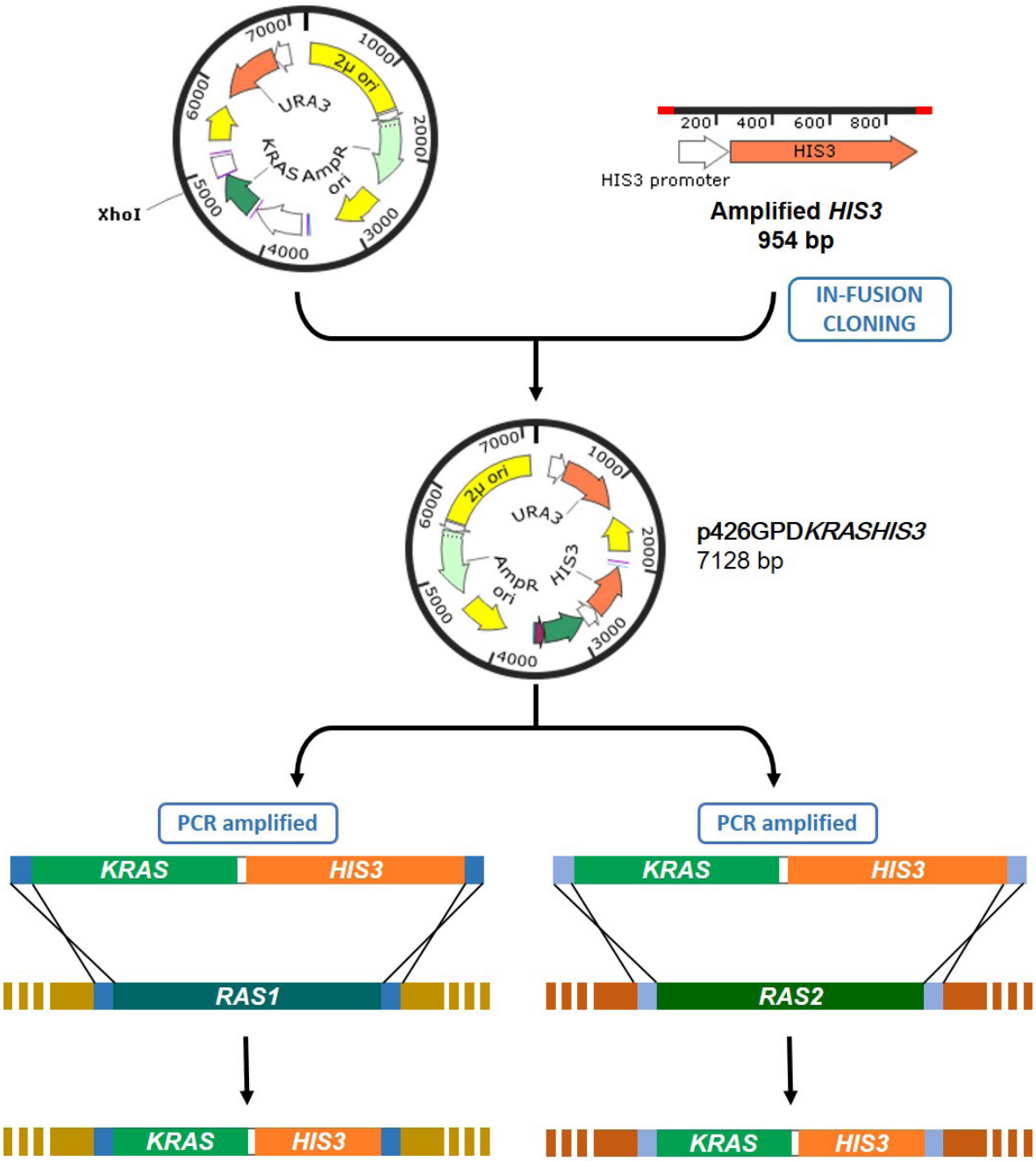


Figure 7: *Strategy 2 for the chromosomal insertion of* KRAS. *HIS3* gene, together with its promoter, was amplified by PCR adding around 40-45 extra bases homologous to the flanking region of *KRAS* in p426GPD*KRAS*. The so-obtained fragment and p426GPD*KRAS* were transformed together in *S. cerevisiae*, obtaining p426*KRASHIS3*. The plasmid was transformed into *E. coli* cells and used as *KRASHIS3* PCR template. The primers used add extra nucleotides homologous to the flanking regions of *RAS1* and *RAS2* in the yeast chromosome. The fragment obtained was used to transform *S. cerevisiae* BY4741 and W303-1A wt. Transformants were retrieved according to *HIS3* auxotrophy. Each mutant strain was transformed separately with the fragment containing either *RAS1* or *RAS2* flanking regions targeting either *locus*.

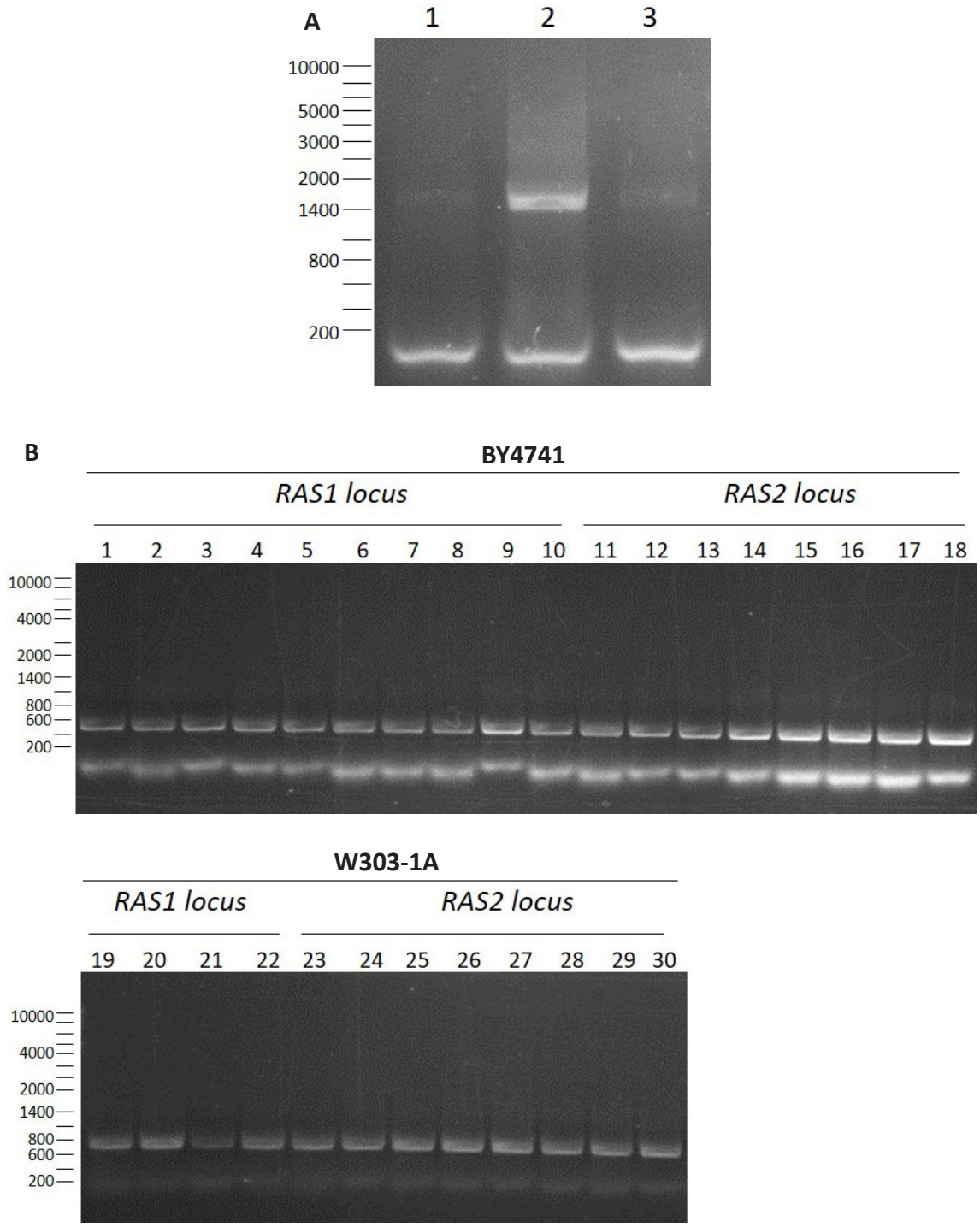


Figure 8: *Validation of the chromosomal insertion of* KRAS *following the transformation strategy 2*. Colonies of transformants tested by colony PCR for the presence of *KRASHIS3* fragment (1400 bp = 564 bp of *KRAS* + 847 bp of *HIS3* and its promoter). Lanes 1 and 3 tested negative, lane 2 tested positive (**A**). Transformants of *S. cerevisiae* BY4741 and W303-1A were tested for the presence of *KRAS* by colony PCR. Gel electrophoresis results show the expected *KRAS* band of 564 bp (**B**). The band sizes indicated correspond to the Ladder III (NZYTech).

When the colonies that contained *KRAS* were further tested for the proper localization of *KRAS*, the results were similar to the ones obtained with the transformation Strategy 1. There was no amplification when using the primers for chromosome XV or XIV- FW_*RAS1*gen and FW_*RAS2*gen, respectively (**Table 3**)- paired with a reverse primer annealing in the middle of *KRAS* gene (**Table 3**, RV_*KRAS*middle). One of the main problems encountered when using this transformation strategy was the high number of colonies obtained, which indicates that the selective marker chosen was not effective, allowing a high percentage of cells to escape selection. Indeed, all of the putative transformant colonies tested (≥ 20/strain) resulted negative.

Strategy 3: *simultaneous insertion of* KRAS *and KanX in the place occupied by* RAS1 *or* RAS2 *gene.*

A third transformation strategy was therefore chosen, with the utilization of a stronger selective marker, geneticin. The KanX cassette was thus fused with the *KRAS* gene. Instead of beginning by constructing an amplicon with the fusion of the two genes as done before, the two DNA fragments (*KRAS* and KanX) were transformed simultaneously into yeast (**Fig. 9**).

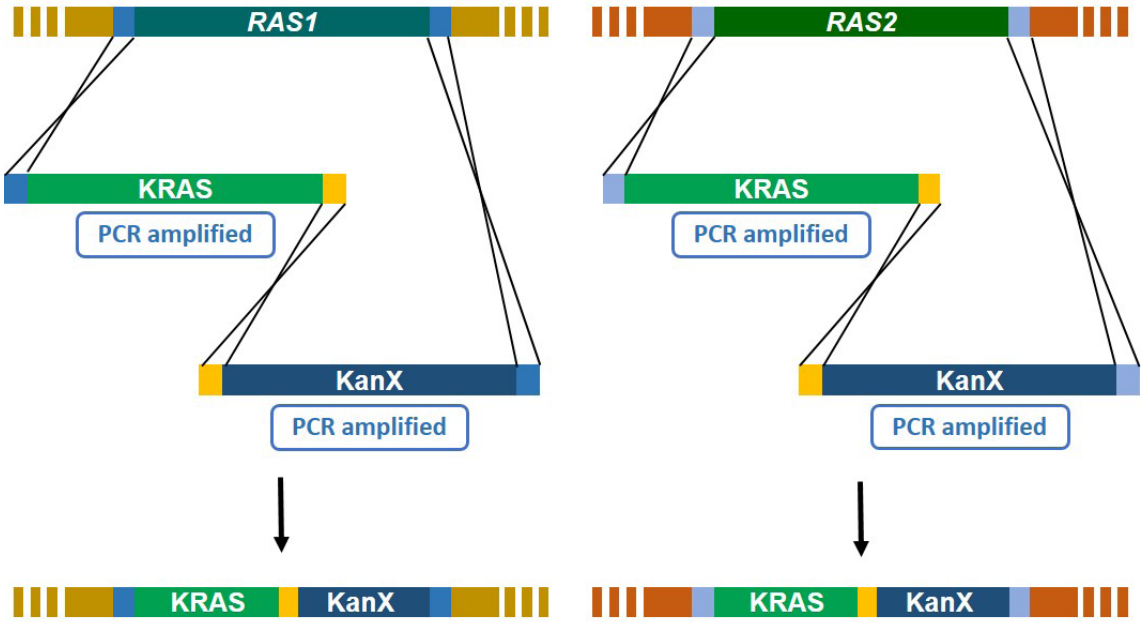


Figure 9: *Strategy 3 for the chromosomal insertion of* KRAS. *KRAS* and KanX DNA fragment were amplified by PCR, adding specific region of homology to promote homologous recombination with either *RAS1* or *RAS2 locus*. The fragments were transformed together into *S. cerevisiae*, to substitute *RAS1* and *RAS2* with the *KRAS* and KanX.

KRAS was amplified with primers that added homology for the flanking region of *RAS1* or *RAS2* (**Table 3**, FW_*RAS1* or FW_*RAS2*) and for the 5'-end of KanX cassette (**Table 3**, RV_KanX*KRAS*). KanX was amplified from pUG6 plasmid using primers that added homology for the 3'-end of *KRAS* (**Table 3**, FW_KanX*KRAS*) and for the flanking region at the 3'-end of *RAS1* or *RAS2* (**Table 3**, RV_KanX*RAS1* or RV_KanX*RAS2*).

Further verifications confirmed the presence of *KRAS* in the yeast genome (**Fig. 10A**). Apparently, it is though not located correctly, as shown by the absence of amplification when using the primer that anneals upstream the 5'-terminal of the gene (**Table 3**, FW_*RAS1*gen or FW_*RAS2*gen) (**Fig. 10B**). A further attempt to successfully obtain a yeast properly transformed, planned for future work, would be to extend the homology region, in order to ensure the fusion with the right part of the yeast genome. The present state of the collection of constructions that constitute the *S. cerevisiae*-based platform for the study of processes and the effect of pharmacological drugs that demand the interaction between galectin-3 and KRAS is listed in **Table 6**.

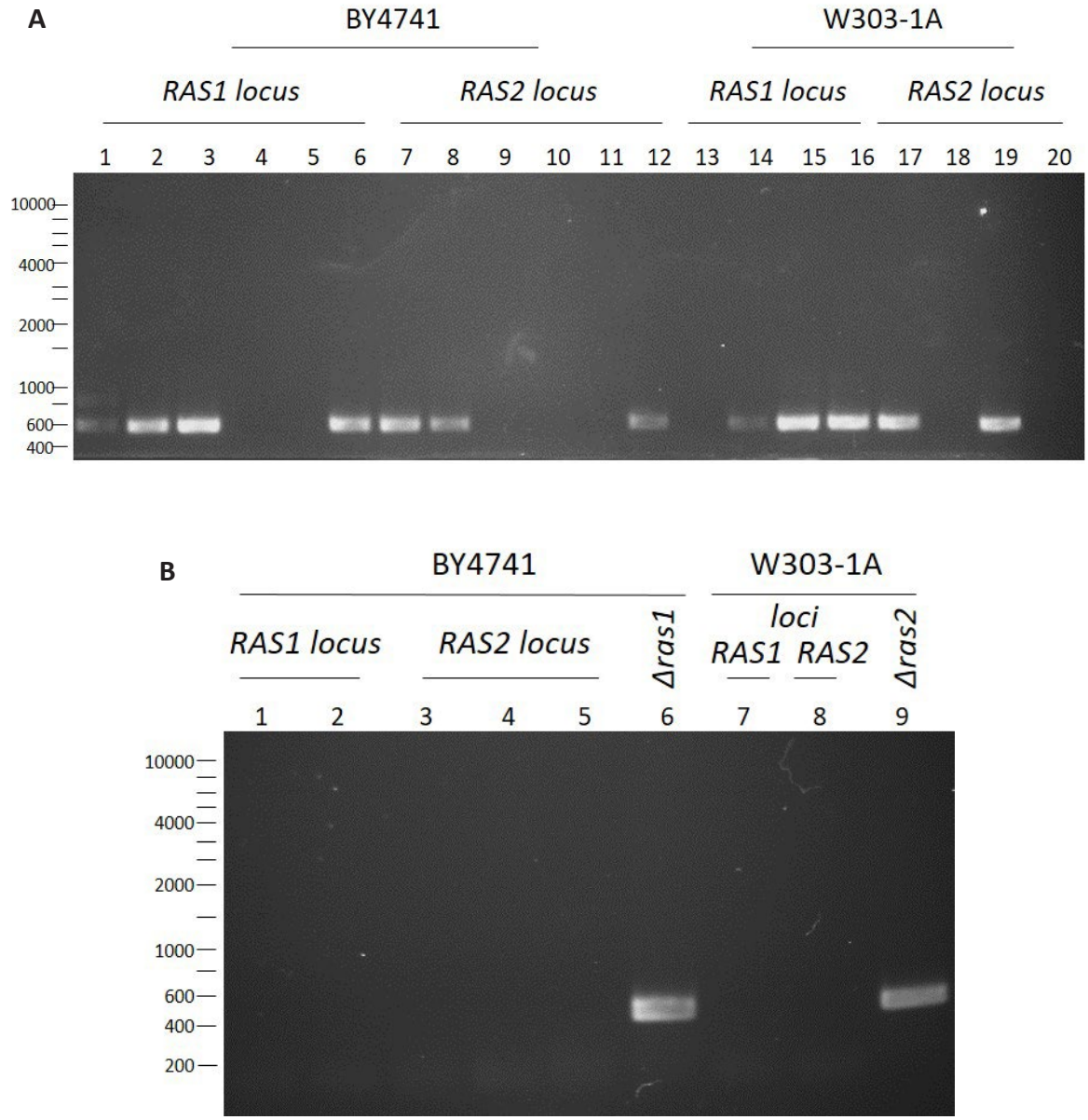


Figure 10: KRAS *chromosomal insertion validation following the transformation strategy 3.* Several colonies selected by their ability to grow on medium supplemented with geneticin were verified by colony PCR for the presence of *KRAS* DNA (**A**). The colonies positive for the presence of *KRAS* were verified for positioning of *KRAS* in the chromosome, using the primers for a region upstream the 5'-end of the gene and a region inside the KanX cassette. None of the colonies tested presented amplification using these primers, demonstrating that the insertion of *KRAS* occurred in a different place (**B**). The picture shows a representative agarose gel, in which the PCR products were run. BY4741 *Δras1* and W303-1A *Δras2* were used as DNA template to prove the actual ability of the primers to anneal to the yeast chromosomal DNA.

Table 6: Present state of the platform expressing human galectin-3 and KRAS proteins. c*KRAS* designation indicates the chromosomal insertion of human *KRAS* in the *RAS1* or *RAS2* yeast loci. The constructions indicated with ✓ in the white background were successfully obtained and verified in both genetic backgrounds, while the ones indicated with x in grey background are planned for the future.

genetic background	**W303-1A**					**BY4741**				
strain	wt	*Δras1*	*Δras2*	*Δras1 cKRAS*	*Δras2 cKRAS*	wt	*Δras1*	*Δras2*	*Δras1 cKRAS*	*Δras2 cKRAS*
p416 Ø	✓	✓	✓	x	x	✓	✓	✓	x	x
p426 Ø	✓	✓	✓	x	x	✓	✓	✓	x	x
p416*LGALS3*	✓	✓	✓	x	x	✓	✓	✓	x	x
p426*KRAS*	✓	✓	✓	x	x	✓	✓	✓	x	x
p416*LGALS3GFP*	✓	✓	✓	x	x	✓	✓	✓	x	x

2. Phenotyping of the yeast expressing *LGALS3*

Galectin-3 expression affects differently the growth rate of wt and mutants depending on the yeast genetic background

The growth on glucose of *S. cerevisiae* transformed with p416*LGALS3* was monitored and compared with the recipient strains, in order to detect if the expression of human gal-3, a protein completely alien to yeast, induces any toxicity in the yeast cells. All the yeast strains were identically inoculated on YNB at $O.D._{600}$ 0.05 and allowed to grow at 30°C until stationary phase (approximately 28 h). Statistically validated values of the specific μ_g are presented in **Fig. 11**.

Yeast growth rate varied between the wt and the *RAS* mutants, even in the absence of gal-3. Indeed, W303-1A *Δras1* grew ~15% faster, and *Δras2* ~13% slower than wt (**Fig. 11A**). BY4741, on the other hand, behaved differently. The μg of *Δras1* was still higher than the one of the wt, even though less significantly (~8%) than in W303-1A, but *Δras2* grew ~10% faster than wt as well (**Fig. 11B**). This concurs with previous observations showing that the phenotypes deriving from the deletion of either *RAS1* or *RAS2* differ between genetic backgrounds (see following sections). The transformation of yeasts with the empty plasmid slightly decreased the μg of all strains, except of W303-1A *Δras2*. A decreased fitness of plasmid-transformed strains is expected, considering the energetic costs of plasmid replication. When gal-3 was expressed, the only significant difference in μg was observed between the W303-1A and BY4741 wt strains that responded in an opposite way. W303-1A expressing gal-3 grew 18% faster than the same strain harbouring the empty plasmid (**Fig. 11A**). The correspondent μg (0.270±0.020 h^{-1}) thus became identical to the μ_g of *Δras1* expressing gal-3 (0.275±0.035 h^{-1}). In BY4741 wt, the expression of gal-3 decreased μ_g of 14% (**Fig. 11B**). The *RAS* mutants from either genetic background resulted only mildly affected by the expression of gal-3 and none of those differences were statistically significant. This is not surprising, since a strong relationship between Ras proteins, specifically KRAS, and gal-3 has been well documented in human cells. The interaction between KRAS and gal-3 is particularly important to maintain KRAS signalling nanoclusters, thus promoting cell proliferation and survival (Elad-Sfadia *et al.*, 2004; Shalom-Feuerstein *et al.*, 2008; Levy *et al.*, 2010; Levy *et al.*, 2011; Song *et al.*, 2012).

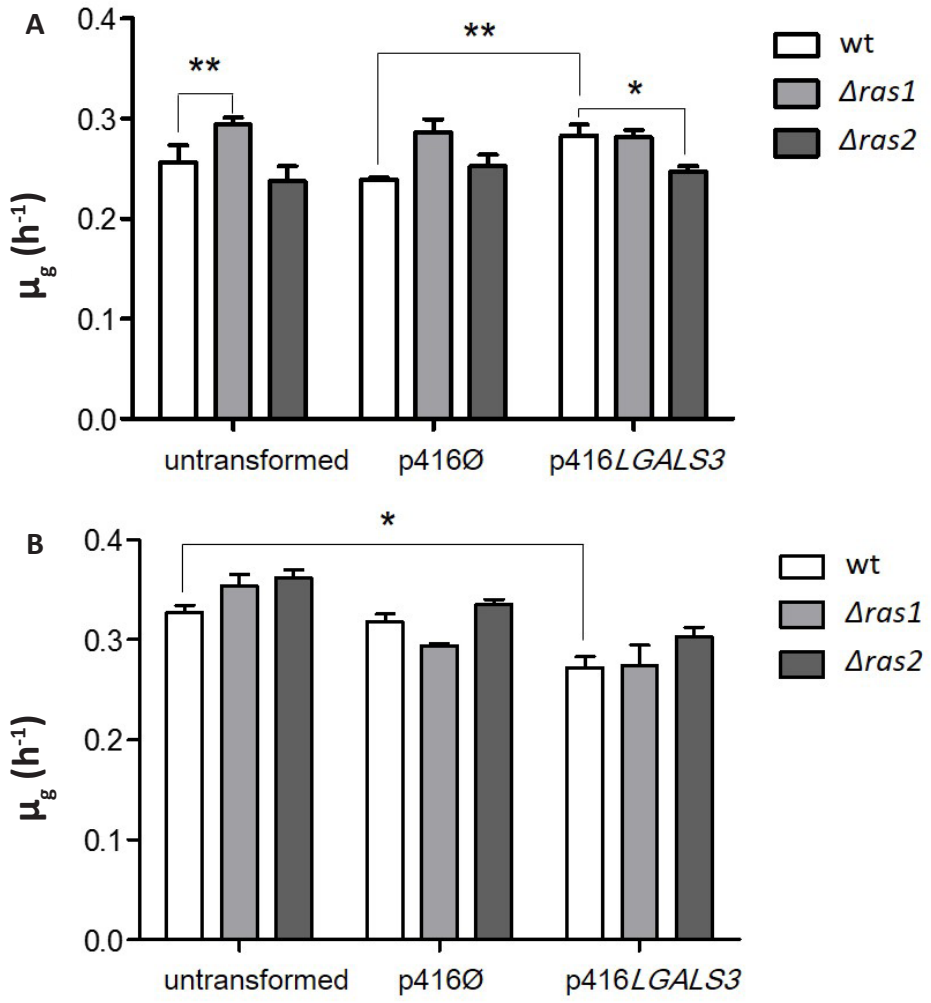


Figure 11: *Specific* μ_g *of* S. cerevisiae *W303-1A and BY4741 transformed with p416*LGALS3. *S. cerevisiae* W303-1A (**A**) and BY4741 (**B**) wt, *Δras1* and *Δras2* transformed with p416*LGALS3* were grown on glucose for 28 h from $O.D._{600}$ 0.05 up to stationary phase. The untransformed yeasts and the yeasts transformed with the empty plasmid (p416 Ø) were used as controls. Growth rates were estimated from log phase. Graphs show the average ±SD of three independent experiments. Statistical significant differences are shown: *(p-value ≤ 0.05) and **(p-value ≤ 0.01).

Chronological life span of S. cerevisiae expressing human LGALS3

Growth rate measures the ability of the yeast to actively replicate when young and in fresh medium. On the other side, the chronological life span (CLS) indicates the capability of the yeast cell to remain viable in stationary phase, without nutrient replacement (Fabrizio *et al.*, 2001; 2003; 2004). There are differences in CLS of yeasts depending on genetic background, different culture conditions and compounds added to the medium. For example, ammonium sulphate, a very common nitrogen source for yeasts, can decrease substantially some strains

CLS (Santos *et al.*, 2012). Also, the deletion of genes involved in pathways that regulate aging alters significantly the CLS of yeast (Fabrizio *et al.*, 2001; 2003; 2004; Powers *et al.*, 2006; Orlandi *et al.*, 2012; Cao *et al.*, 2016). These include genes that regulate nutrient assimilation and consumption, such as *RAS1* and *RAS2* (Sun *et al.*, 1994; Shama *et al.*, 1998; Lee *et al.*, 1999; Fabrizio *et al.*, 2003; Longo, 2003). Given (i) the role of *RAS* genes in the effects caused by human gal-3 expression in *S. cerevisiae* described above, and (ii) the well-documented interaction between gal-3 and RAS proteins in human cells (Elad-Sfadia *et al.*, 2004; Shalom-Feuerstein *et al.*, 2008; Levy *et al.*, 2010; Levy *et al.*, 2011; Song *et al.*, 2012), it was particularly important to determine whether the expression of gal-3 in yeasts introduced any changes in CLS.

W303-1A wt, *Δras1* and *Δras2*, as well as the same strains transformed with p416*LGALS3,* were grown in YNB up to stationary phase (24 h of growth). BY4741 was not used in this assay in view of the absence of response to gal-3 above described. A 24 h time point was chosen as starting point for the test. At that point no further growth was observed since nutrients are exhausted. Therefore, most cells should still be viable though not actively dividing. This was considered the day 0 (T_0 - 100% viable/alive cells). The replication ability of the yeast cells was measured by CFU (colony forming units) every 3 days, until the cells were not able to replicate anymore and no CFU were formed. The values of CFU were normalized with the ones obtained at T0 and considered as percentages of survival and plotted against time to create the ageing curve (**Fig. 12A**). It is immediately visible that the shape of the curve was different depending on the strains, though the time taken to obtain ≤10% of survival was very similar (10 to 14 days).

In the absence of gal-3, W303-1A clearly maintained its ability to duplicate longer than the correspondent *RAS* mutants, especially when compared to *Δras1* (**Fig. 12A**). Moreover, the replication ability of the *Δras1* strain dropped significantly in the first 3-5 days, maintaining a slower decrease thereafter (**Fig. 12A**), in opposition to wt strain whose viability decreased along time more homogenously. The behaviour of the *RAS2* mutant was somehow in between the one of the wt and the one of *Δras1* (**Fig. 12A**). In the first 4-8 days it decreased faster than the wt, but not as fast as *Δras1.* When gal-3 was expressed, the CLS of the wt

strain was almost indistinguishable from the untransformed strain, or the one transformed with the empty plasmid. On the other hand, gal-3 accelerated the ageing of *Δras1* compared to the empty plasmid, while the opposite happened in the case of *Δras2*, gal-3 inducing a significant delay in the aging process of this mutant.

In order to better compare the different strains, the percentage of survival at day 5 was plotted (**Fig. 12B**). This time point was chosen since it represents approximately the middle of the CLS curve of the wt strain and the differences among strains were mostly observed in that ageing phase, not in the total amount of time necessary for complete culture death. Both *Δras1* and *Δras2* mutants, regardless of the transformation with empty plasmid or gal-3, presented a much lower percentage of survival than wt, ranging from -60% to -90% (**Fig. 12B**). Moreover, the wt strain was not affected significantly by the transformation with the empty plasmid, or the gal-3 expressing plasmid. On the other hand, the *RAS* mutants were more severely affected by gal-3 expression and behaved in an opposite manner. The survival of *Δras1* decreased ~66%, while the survival of *Δras2* surprisingly increased 3.5 times (arrows in **Fig. 12B**).

As above, these results suggest a strong relationship between galectin-3 and Ras proteins. Moreover, the different results from the two mutants indicate that the roles of the two Ras proteins may not be interchangeable in the ageing process when gal-3 is present. A divergent role of Ras1 and Ras2 in the yeast ageing process was already described, even though in different *S. cerevisiae* genetic backgrounds (Sun *et al.*, 1994; Shama *et al.*, 1998; Fabrizio *et al.*, 2003; Longo, 2003). This appears to be particularly important since some of these works describe that *RAS2* deletion decreases CLS and *RAS1* deletion increases it (Sun *et al.*, 1994), while others suggest that *RAS2* deletion increases CLS (Marek and Korona, 2013; Garay *et al.*, 2014). This data inconsistency is quite common in the literature referring Ras proteins functions and the phenotypes associated with their absence, suggesting that auxotrophy, *i.e.* mutations in the metabolism of amino acid synthesis, are highly interconnected with the Ras pathway.

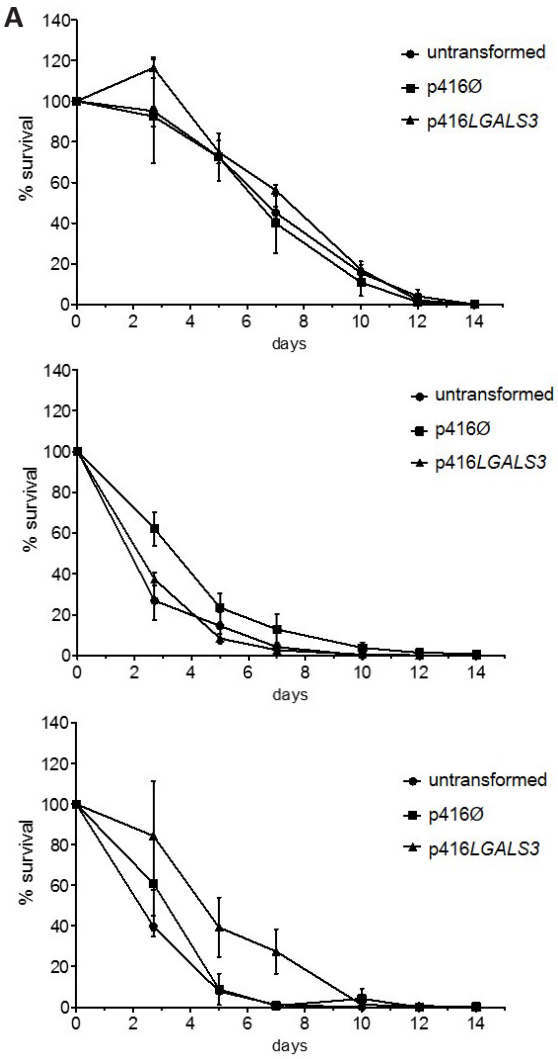
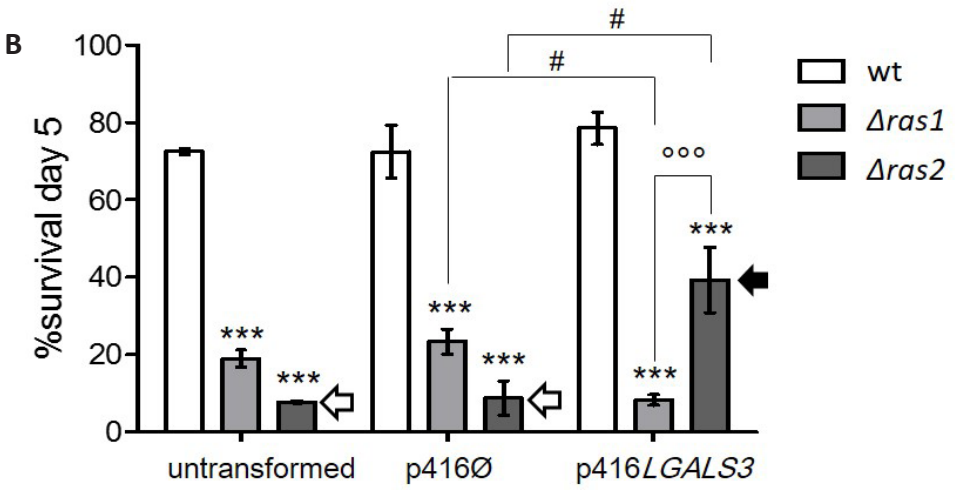


Figure 12: *CLS of W303-1A transformed with p416*LGALS3. *S. cerevisiae* W303-1A wt, *Δras1* and *Δras2* transformed with p416*LGALS3* (▲) were grown on glucose for ≥14 days, *i.e.* until the population was not able to duplicate. The untransformed yeast (●) and the yeast transformed with the empty plasmid (p416 Ø) (■) were used as controls. The number of colonies of each time point was normalized as percentage of survival compared to T_0- 100% of survival, and the average ±SD from at least three independent experiments was estimated (**A**). The average ±SD of the percentage of survival of every strain at day 5 (correspondent to approximately the middle of the aging curve) was plotted (**B**). Statistical significant differences are shown between the wt and the mutant yeasts: ***(p-value ≤ 0.001); the two different mutants: °°°(p-value ≤ 0.001); the differently transformed yeasts: # (p-value ≤ 0.05).



3. Phenotyping of the yeast expressing *KRAS*

Preliminary stress resistance assays in RAS mutants highlight the differences between BY4741 and W303-1A

One of the most time- and cost-effective ways to investigate the ability of *S. cerevisiae* strains and genotypes to resist stress or other environmental constraints is the dropout test. One of the purposes of the construction of the yeast platform expressing *LGALS3* and *KRAS* is to determine the response of these strains to galectins inhibitor compounds. The platform also allows to determine the ability of the human KRAS to complement phenotypes associated with either *RAS1* or *RAS2* deletion. It is known that human HRAS can complement some of the *Δras2* defects (Ho and Springer, 1982; Clark *et al.*, 1985; Morishita *et al.*, 1995), but less is known about KRAS.

The first approach was to standardize the response of the recipient yeast strains to the desired growth conditions, including different carbon sources, stress-inducing physical conditions, or chemicals. As before, *S. cerevisiae* strains BY4741 and W303-1A wt and *RAS* mutants were assayed in parallel. Conditions were chosen according to previously reported *Δras1* and *Δras2* growth defects. *RAS2* deletion mutant is the most frequently investigated. It is reported not to grow on non-fermentable carbon sources (Tatchell *et al.*, 1985; Breviario *et al.*, 1986; Lisziewicz *et al.*, 1990; Petitjean *et al.*, 1990) and at non-permissive high temperature (Ho and Bretscher, 2001; Ma *et al.*, 2012). Moreover, *RAS* mutants have been reported to be affected by caffeine (Kuranda *et al.*, 2006) and hyperosmotic stress (Wei *et al.*, 2009). All the conditions above mentioned were tested. In addition, *RAS* mutants were assayed for resistance to other common stress stimuli, such as high ethanol and acetic acid concentrations (**Table 4**).

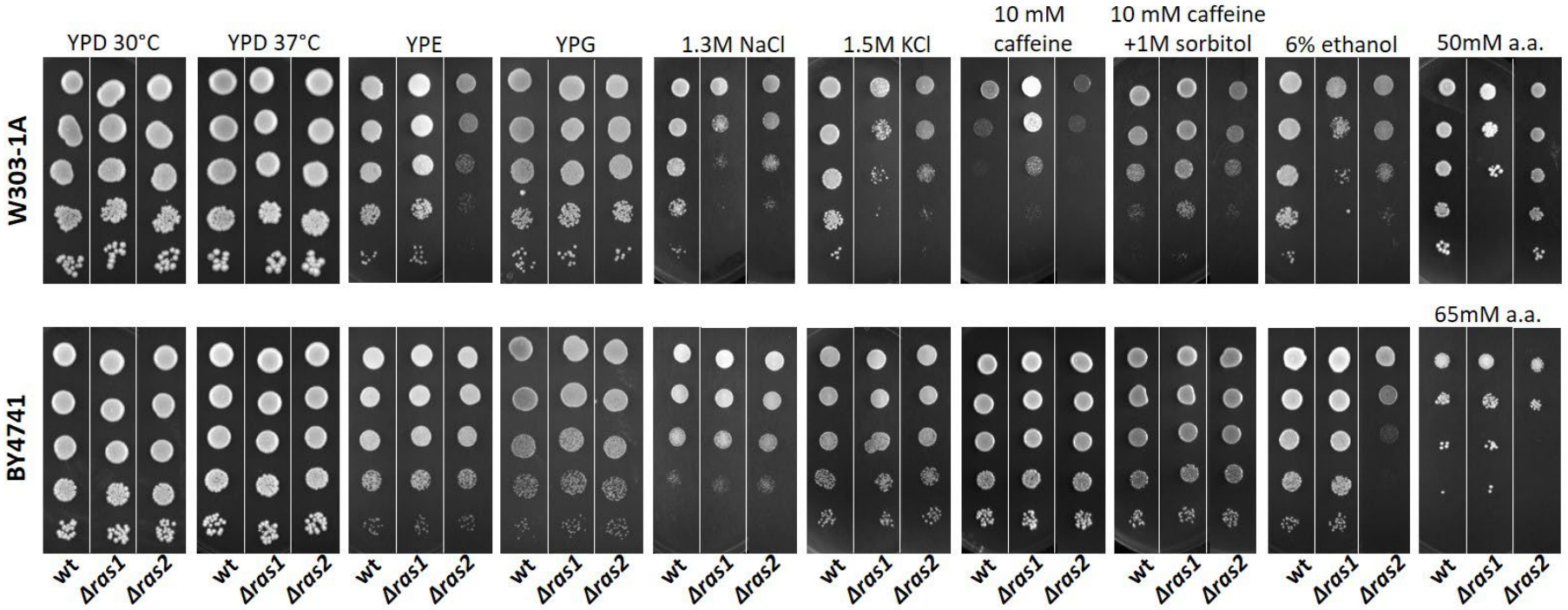
Results turned out different from the literature. Globally, BY4741 was less affected in all conditions than W303-1A. Importantly, both BY4741 and W303-1A-derived *RAS* mutants grew identically to the controls when cultured at 37°C and when cultured using the non-fermentable carbon source glycerol (**Fig. 13**). Also the effect of osmotic stress was different from the expected. While Wei *et al.* (2009) described *Δras2* as more resistant to a 2M NaCl-

induced hyperosmotic stress , the present W303-1A mutants strains showed an increased sensitivity to both 1.3M NaCl and 1.5M KCl, though more pronounced in the *Δras1* than in the *Δras2*. The BY4741 strains were all identically not sensitive to these stress conditions. None of the genetic backgrounds used in the literature was BY4741 or W303-1A. These striking differences in behaviour highlight the important impact of genetic background on the outcome of experiments (Cohen and Engelberg, 2007; Young and Court, 2008; Petrezselyova *et al.*, 2010; Kokina *et al.*, 2014).

None of the BY4741 strains was affected by caffeine, while W303-1A wt and mutants showed a significant growth inhibition, partially recovered with the addition of sorbitol (**Fig. 13**). Interestingly, W303-1A *Δras1* strain was less affected by caffeine and, accordingly, recovered slightly better than the other strains. The differences between BY4741 and W303-1A backgrounds were even more evident in what regards the effects to ethanol and acetic acid. BY4741 *Δras2* grew normally on the non-fermentable carbon source ethanol, while the correspondent W303-1A mutant presented a growth defect (**Fig. 13**). When exposed to 6% ethanol, BY4741 *Δras2* was the mutant showing the most significant growth inhibition, while in the case of W303-1A the most affected one was *Δras1* (**Fig. 13**). These results appear contradictory, but growing on 2% ethanol as carbon source is physiologically very different from growing on glucose in the presence of much higher ethanol concentrations. In these conditions, ethanol functions as a chaotropic agent decreasing substantially the a_w of the surrounding environment. Identical result was observed in the presence of high amounts of acetic acid. Importantly, a highest concentration of acetic acid was used for BY4741 (65 mM), since the concentration used for W303-1A (50 mM, data not shown) caused no significant effects.

Importantly, these results stress the fact that the differences between strains must not be underestimated, since they can significantly alter the results of an assay, and therefore the perception of the role of a protein. Moreover, they give a first indication of the conditions in which the platform expressing *KRAS* and *LGALS3* should be used.

Figure 13: *Serial drop test of BY4741 and W303-1A* RAS *mutants*. *S. cerevisiae* BY4741 and W303-1A wt, *Δras1* and *Δras2* were subjected to stress resistance assay as described in Materials and Methods. The direction of the serial dilutions, from 10^{-1} to 10^{-5}, proceeds from top to bottom. The image is representative of one of three independent experiments.



The following results using *S. cerevisiae* BY4741 expressing human *KRAS* through the p426*KRAS* plasmid are published in the Master thesis on Molecular Genetics of Carneiro E.R., entitled **Heterologous expression of human KRASwt cDNA in *Saccharomyces cerevisiae* and its mutants from the RAS signalling pathway and phenotype screening** (January 2015, CBMA, Uminho, Portugal), and were performed by Carneiro E.R. and Cazzanelli G. during the PhD thesis development. The thesis is publicly available at: http://repositorium.sdum.uminho.pt/handle/1822/35689.

Serial drop test of S. cerevisiae *BY4741 expressing human* KRAS

The first physiological screening of *S. cerevisiae* BY4741 wt and RAS mutants expressing p426*KRAS* was done with the purpose of verifying the capacity of KRAS to complement common yeast phenotypes associated with the deletion of either *RAS1* or *RAS2*. Therefore, the BY4741 wt, *Δras1* and *Δras2* mutants were used to perform a large dropout assay screening. The stress conditions tested were very similar to the ones described above and included non-permissive temperature (37°C), non-fermentable carbon sources, osmotic stress and oxidative stress (**Table 5**). This screening differed from the above described in that tests were made in minimal YNB medium instead of the rich YPD medium. Representative results are shown in **Fig. 14**. Surprisingly, in opposition to the results obtained before in YPD (**Fig. 11**), as well as in opposition with the literature, neither strain (wt or mutant) showed any evident phenotype in none of the stressful conditions tested. This prompted the expansion of the platform to a second genetic background, W303-1A, known to be more responsive (Cohen and Engelberg, 2007; Young and Court, 2008; Petrezselyova *et al.*, 2010; Kokina *et al.*, 2014), and to the use of YPD instead on YNB.

The results, however, showed that the expression of human *KRAS* in yeast, especially in the BY4741 wt, caused a decrease in the strain resistance to high non-permissive temperature, osmotic stress and oxidative stress (**Fig. 14**). Moreover, both wt and *RAS* mutants expressing human *KRAS* were less able to grow on non-fermentable carbon sources like ethanol and glycerol (**Fig. 14**). This diminished stress resistance aligns with previous findings on yeast strains expressing a hyperactive variant of *RAS2* (Fabrizio *et al.*, 2003; Hlavata *et al.*, 2003). The cause was suggested to be a stronger inhibition by PKA of the transcription factors acting on STRE responsive genes (Pedruzzi *et al.*, 2000; Proft *et al.*, 2001; Gorner *et al.*, 2002; Ferguson *et al.*, 2005; Pascual-Ahuir and Proft, 2007). The expression of *KRAS* in a yeast cell

already expressing its own *RAS1* and *RAS2* genes, as it happens in the wt strain, could induce an effect similar to a hyperactivation of the pathway. This would justify why the phenotype was more visible in this strain than in the *RAS* mutants also expressing the human *KRAS*. This strongly suggests that KRAS, once inside the yeast cell, has roles that are probably very close to the ones of the endogenous Ras proteins.

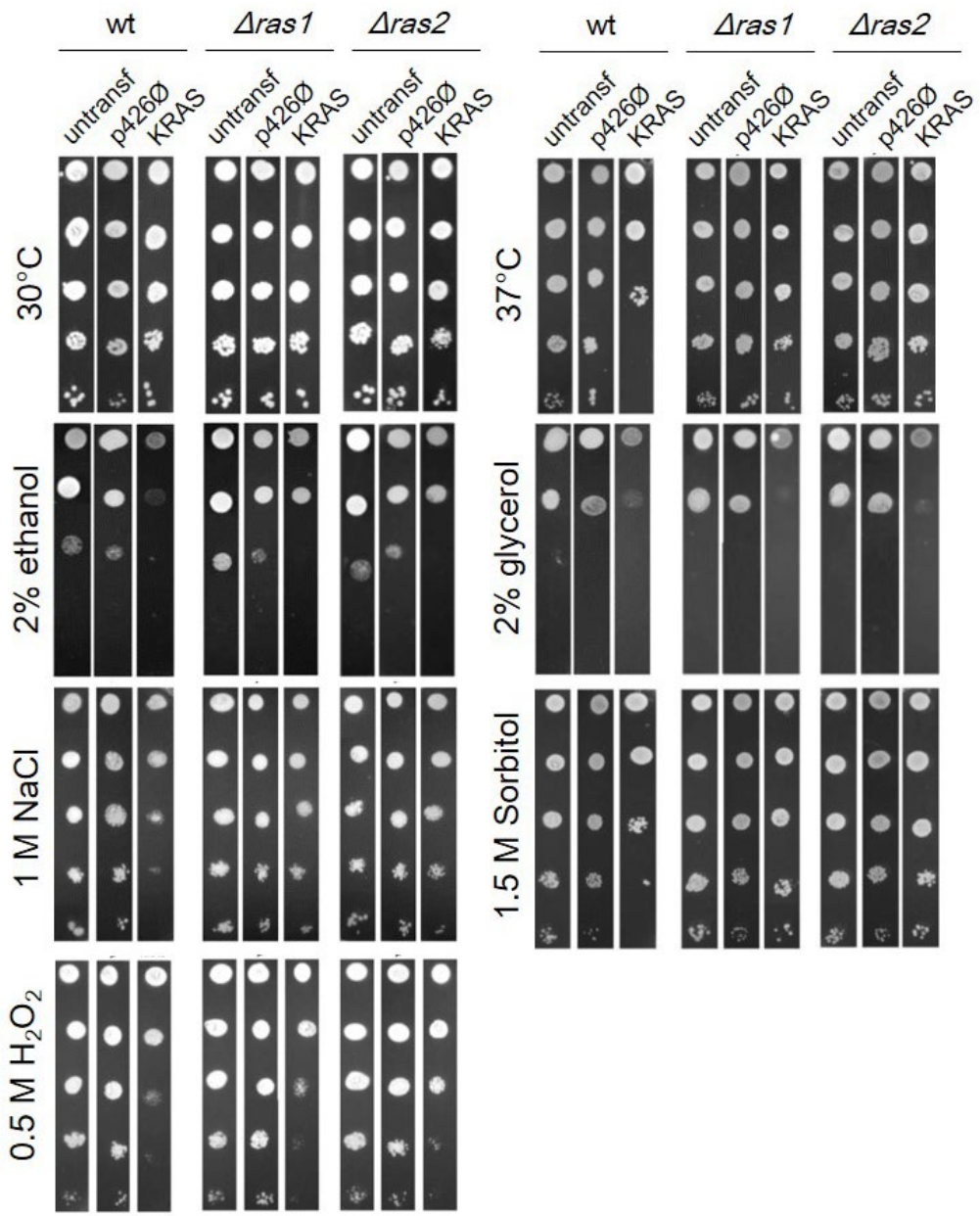


Figure 14: *Serial drop test of BY4741 expressing human* KRAS. *S. cerevisiae* BY4741 wt, *Δras1* and *Δras2* were subjected to stress resistance assay as described in Materials and Methods (stress stimulus described on the side of each box). The direction of the serial dilutions, from 10^{-1} to 10^{-5}, proceeds from top to bottom. The image is representative of one of three independent experiments.

Growth rate measurement of S. cerevisiae *BY4741 expressing human* KRAS

The μ_g of yeast expressing human *KRAS* was measured and compared with the recipient strains (**Fig. 15**). Moreover, the growth rates were also compared to the ones displayed by the BY4741 wt strain transformed with p416*LGALS3* (**Fig. 11B**). Globally, μ_g was slightly lower. However, it is important to take into consideration the bigger standard deviation (SD) obtained in this assay, which somehow masks the differences observed. Interestingly, the μg of the BY4741 *RAS* mutants, which is smaller than the one of the wt, decreases even more when *KRAS* is present. The empty plasmid, on the other hand, surprisingly seemed to contribute to improve the fitness of *Δras1* mutant, whose μg was higher than the ones of wt and *Δras2* also transformed with empty p426. However, none of differences above mentioned were statistically significant, probably because of a larger variability, indicated by the bigger SD values.

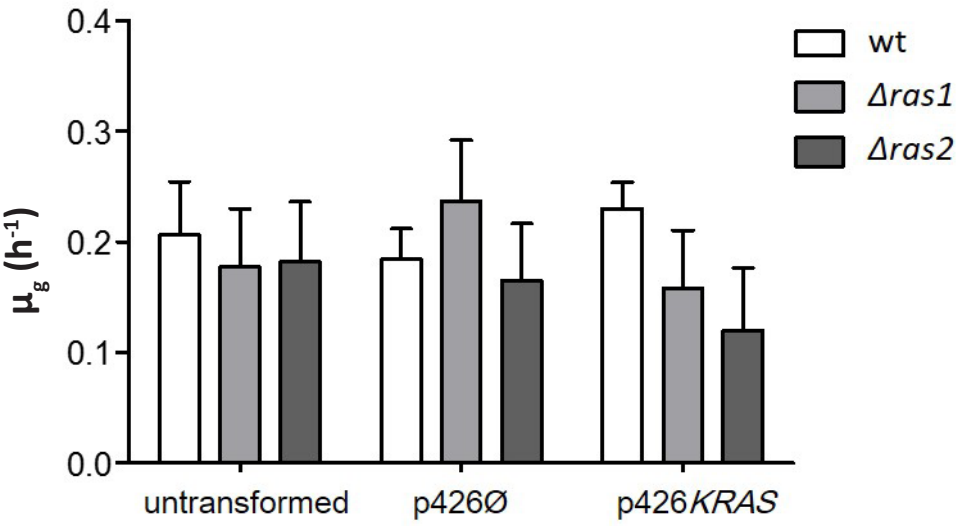


Figure 15: *Specific μ_g of* S. cerevisiae *BY4741 transformed with p416*KRAS. *S. cerevisiae* BY4741 wt, *Δras1* and *Δras2* transformed with p426*KRAS* were grown on glucose up to stationary phase. The untransformed yeasts and the yeasts transformed with the empty plasmid (p426 Ø) were used as controls. Growth rates were estimated from log phase. Graphs show the average ±SD of three independent experiments.

Discussion

In this work we initiated the construction of a *S. cerevisiae*-based platform in which yeast expresses two human proteins, galectin-3 and KRAS, which are known to interact on the inner leaflet of the plasma membrane of human cells (Elad-Sfadia *et al.*, 2004; Shalom-Feuerstein *et al.*, 2005; Ashery *et al.*, 2006; Shalom-Feuerstein *et al.*, 2008). The main purpose of building this platform is to use it as a tool for (i) pharmacological drug screening against KRAS and/or gal-3, and (ii) to enable the study of the interaction between gal-3 and KRAS in an environment free of the interference of the molecular complexity of human cells. Yeast, as an eukaryote that shares with human cell a great deal of proteins and molecular processes, can provide the ideal organism to test and screen a great number of compounds putatively inhibiting gal-3/KRAS interaction, relevant in cancer treatment. The use of yeasts in this regard is fully documented in the literature (Barberis *et al.*, 2005; Mager and Winderickx, 2005), as is the use of yeasts to study human proteins (Sekigawa *et al.*, 2010; Pereira *et al.*, 2012; Laurent *et al.*, 2016).

Yeast has no galectins orthologues (Hughes, 1997; Dodd and Drickamer, 2001). Maybe for this reason, they were expressed in yeast only once (Ryckaert *et al.*, 2008). That study reported the expression of galectins in yeast, targeted to the outer leaflet on the plasma membrane with a highly specific membrane tag. The success of that work suggested that galectins are not toxic to the yeast cell, supporting the endeavour of expressing galectins in yeast with the purpose of retaining them intracellularly. There, gal-3 should be able to interact with KRAS also expressed by the same cells.

In the present work, human *LGALS3* cDNA was successfully cloned into *S. cerevisiae* of two different genetic backgrounds. WB analysis and fluorescent microscopy showed that *S. cerevisiae* is capable of expressing and retaining gal3, and its levels of degradation were minimal in the early logarithmic phase. As confirmation by fluorescence microscopy, no gal-3 was observed in the vacuole of yeast, but most of it localized in the cytoplasm. However, when cells progressed into late exponential phase of growth, degradation increased, matching a decreased expression level. Consistently, less cells showed intense fluorescence with

increasing $O.D._{600}$ measurements (data not shown). The two genetic backgrounds behaved similarly in the first phases of growth, however, some differences were present in the intact protein level of gal-3 in late log phase, both between BY4741 and W303-1A and among wild type and *RAS* mutants. BY4741 seemed to degrade more efficiently gal-3 in all the three yeasts, wt, *Δras1* and *Δras2*, while W303-1A presented some variations in expression levels, having wt a less intense degradation, *Δras2* a more prominent degradation and *Δras1* almost not expressing gal-3 at all.

Studies performed in human cells report a correlation between RAS and gal-3 proteins levels. In particular, cell lines displaying a high level of KRAS always show a correspondently high level of gal-3 (Elad-Sfadia *et al.*, 2004; Shalom-Feuerstein *et al.*, 2005; 2008; Levy *et al.*, 2010). In the present work, the other way around, it was demonstrated that silencing of *KRAS* in colorectal cancer cells caused a mild decrease in gal-3 protein level (chapter 6, this thesis). It may therefore be hypothesized that when the global level of Ras proteins is lower than physiological conditions, *i.e.* in *Δras2* during late exponential phase when *RAS1* expression is partially repressed, gal-3 has a lower amount of intracellular "anchor", gal-3. This may cause the degradation of gal-3, which would be no longer interacting with any yeast partner. This would happen only to a lesser extend in the wt, when only *RAS1* is repressed in late exponential phase (**Fig. 16**).

Significant differences between the two genetic backgrounds, and between wt and *RAS* mutants, were a constant in this work. Indeed, they were observed as well when the yeasts growth rate was analysed. Even without expressing human proteins, W303-1A presented a slower growth in exponential phase compared to BY4741 and also the differences between wt and *RAS* mutants varied from W303-1A to BY4741, having W303-1A *Δras2* the slowest growth, whereas for BY4741 this was represented by the wt strain. These results as many others throughout the present work clearly show that the yeast genetic background chosen to perform the experiments can determine their outcome (Petrezselyova *et al.*, 2010; Kokina *et al.*, 2014). Therefore, similarly to what is common practice in studies using human cells, it would be advisable to routinely test more than one yeast genetic background.

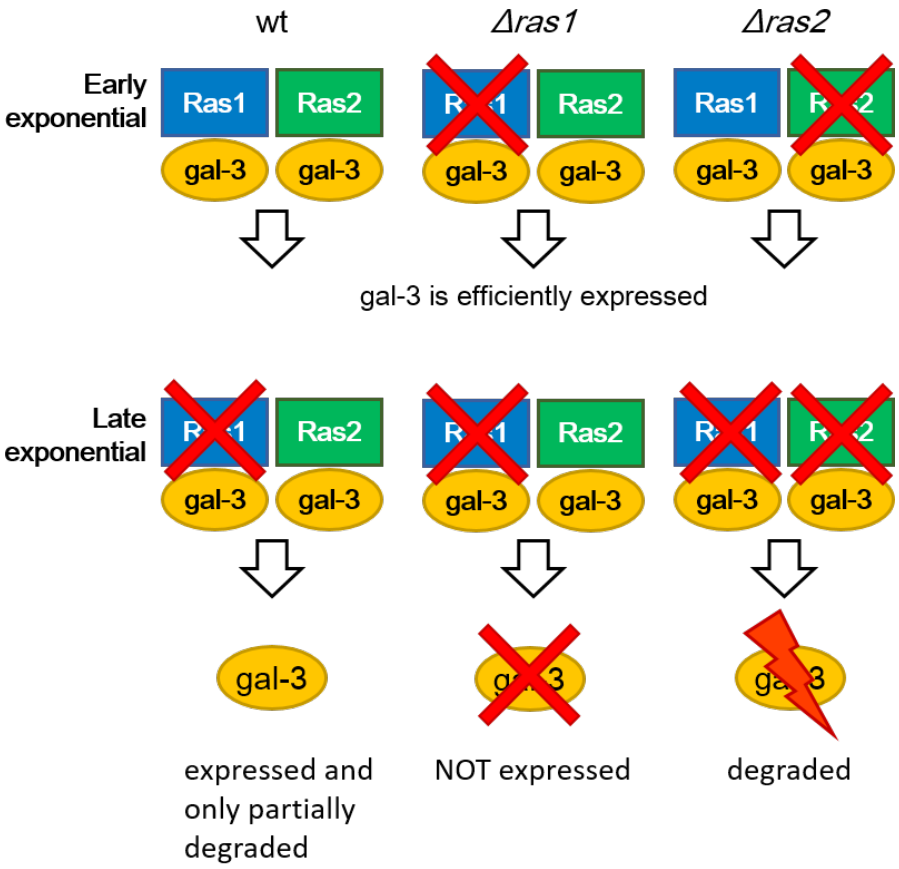


Figure 16: *Schematic representation of the effects caused by* RAS1 *or* RAS2 *deletion on galectin-3 expression in W303-1A in early and late logarithmic phase*. In early exponential phase the presence/ absence of either Ras protein does not influence gal-3 expression level, which is high in all three strains, wt, *Δras1* and *Δras2*. On the other hand, in late exponential phase, *Δras1* does not express gal-3 and *Δras2* mostly degrades gal-3 after expression.

Another clear difference between strains and between wt and *RAS* mutants was observed when yeast expressed gal-3. *RAS* mutants did not show any variation upon gal-3 expression, whereas wt strains from both genetic backgrounds did. They actually showed an opposite effect. W303-1A expressing gal-3 grew faster during exponential growth phase and BY4741 grew more slowly. Since these variations were only seen in wt, it was hypothesized that both *RAS* genes should be present for gal-3 to exert its effects on growth rate. If only one Ras protein was necessary, a phenotype similar to the one triggered in the wt strain should have been also observed in the strain lacking the dispensable Ras protein, which was not the case (**Fig. 17**). The roles of Ras1 and Ras2 are reported to be mostly redundant and overlapping in yeast, the only difference between them being the transcriptional regulation and not different

downstream pathways. Ras1 is reported to be expressed at detectable levels only up to mid-log phase and only on fermentable carbon sources and therefore could not complement for the absence of *RAS2*, which is always expressed at higher levels, in late log phase and on non-fermentable carbon sources (Breviario *et al.*, 1986; 1988). When *RAS1* was put under the regulation of constitutive promoters, its function was perfectly overlapping with Ras2 (Marshall *et al.*, 1987). The activity of Ras1 is often considered dispensable and no clear phenotype has been identified so far, causing also a scarce interest from the researchers for this Ras isoform. However, it is important to remember that some differences in the phenotype regarding ageing were found in *RAS1* and *RAS2* deletion mutants. In particular, the two proteins have been reported to have opposite roles in the yeast ageing process, *RAS1* deletion increasing CLS and *RAS2* deletion decreasing it (Sun *et al*., 1994). These data are not definitive, since other opposing studies reported *RAS2* deletion to be one of the main causes of increased CLS (Fabrizio *et al.*, 2003; Sun *et al.*, 1994; Shama *et al.*, 1998, Longo, 2003), whereas other reported the opposite (Marek and Korona, 2013; Garay *et al.,* 2014). In the present case, both BY4741 or W303 *RAS1* mutants, which express Ras2 protein, did not show any alteration in the μ_g phenotype, contrarily to the wt strains, meaning that the presence of Ras2 protein alone is not sufficient to cause a detectable phenotype in growth rate (**Fig. 17**). It was therefore hypothesized that Ras1 might have a bigger impact on yeast cells than what is expected according to the literature. This concurs with the fact that *Δras1* responded differently than wt and *Δras2* to some stress stimuli, such as acetic acid, high concentration of ethanol, osmotic stress and caffeine (see following sections).

Summarizing, human gal-3 activity on growth rate is mediated by both yeast Ras proteins and causes opposite effect on the wt strains of W303-1A and BY4741. The case of W303-1A seems to be more consistent with what usually happens in human cells, where both gal-3 and KRAS exist. In human cells, gal-3 interacts with KRAS, stabilizing its active GTP-bound form (Elad-Sfadia *et al.*, 2004; Shalom-Feuerstein *et al.*, 2005; Levy *et al.*, 2010; Song *et al.*, 2012) and its positioning on the inner side of the plasma membrane, the proper location for RAS signalling (Elad-Sfadia *et al.*, 2004; Ashery *et al.*, 2006; Bhagatji *et al.*, 2010). Several mammalian RAS partners could successfully interact with yeast Ras protein, for example the

mammalian GTP activating protein NF1 (Gibbs *et al.*, 1988; Han *et al.*, 1991) and proteins involved in the autophagic process (Alves *et al.*, 2015). It is therefore conceivable that yeast Ras proteins might interact as well with gal-3, a well-established KRAS partner, as mentioned above. In support of this hypothesis, the putative structure of RAS proteins inferred from amino acid sequence appears to be very conserved between human and yeast, enough to allow this interaction. This Ras/gal-3 interaction could therefore cause in yeast effects that are similar to the ones occurring in human cells, namely, the stabilization and activation of Ras proteins by gal-3, enhancing Ras signalling, this way increasing the growth rate. W303-1A genetic background was used to further analyse the effects of expressing human gal-3 in yeasts on another important RAS pathway-dependent feature, the CLS. Only W303-1A was used because, according to the results on growth, it apparently mimics more accurately the behaviour of gal-3/KRAS in human cells. The CLS of W303-1A was therefore analysed, and the expression of human gal-3 only affected the ageing of the *RAS* mutants. In particular, gal-3 caused opposite effects on *Δras1* and *Δras2*, decreasing and increasing CLS, respectively. The importance of yeast Ras proteins in mediating human gal-3 effect was thereby emphasized when examining ageing.

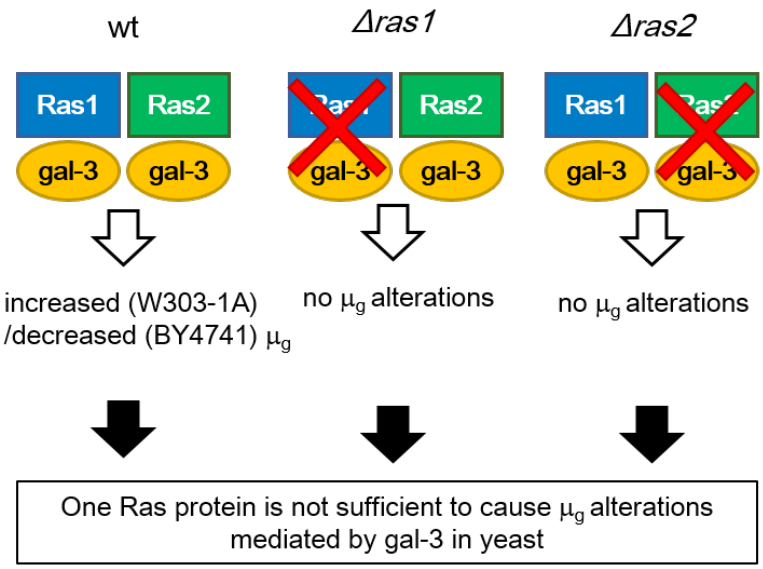


Figure 17: *Schematic representation of the effects caused by galectin-3 expression on W303-1A growth rate.* The absence of Ras1 or Ras2 protein does not cause any significant alteration on W303-1A μ_g when expressing gal-3. On the other hand, the wt strain displays μ_g alteration, showing that both Ras proteins are important in mediating gal-3 effect on μ_g.

The involvement of yeast Ras proteins in ageing processes has been established, even though the specific effect of each Ras isoform is sometimes contradictory in the literature. *RAS1* and *RAS2* deletions were correlated both with decreased CLS (Sun *et al.*, 1994; Marek and Korona, 2013; Garay *et al.*, 2014) and with increased CLS (Sun *et al.*, 1994; Fabrizio *et al.*, 2003). In the present case, both Ras1 and Ras2 seem to have an anti-aging role in yeast, since their deletion significantly decreased CLS, more accentuated in the case of *RAS2* deletion. Gal-3 expression, as mentioned above, caused opposite effects on *RAS* mutants, pronouncedly increasing survival in *Δras2*, and decreasing it in *Δras1*. In this last mutant, it can be hypothesised that gal-3 interacts with Ras2 (the only available Ras protein) promoting an increase in activity that ultimately would cause decreased survival. This phenotype is similar to the one obtained when expressing the constitutively active $RAS2^{val19}$ (Hlavata *et al.*, 2003), which hyperactivates the pathway. Hyperactivated Ras2 downregulates stress response genes, through the transcription factors Msn2 and Msn4, leading to weaker ability to survive stressful environmental conditions (Longo, 2003, 2004; Longo and Fabrizio, 2012). The present results are consistent with the contribution of gal-3 in hyperactivating Ras2, which would falsely signal an abundance of nutrients to the cell, leading to a decrease in survival. On the other hand, the fact that the gal-3-induced phenotype was not identical in the *Δras2* mutant, in spite that the Ras1 and Ras2 should be redundant, could derive from gal-3 being less suitable for interaction with Ras1, or from this interaction causing a downregulation of the Ras signalling pathway. Actually, CLS was shown previously to increase upon deletion of *RAS2* (Longo, 2003). This time, the enhancement of CLS seems to be mediated by gal-3 expression.

The effects of Ras pathway supposed hyperactivation were observed also in the case of human KRAS expression in yeast. An extra Ras protein, in addition to the two endogenous Ras1 and Ras2, triggered in yeast an increased sensitivity to various stress stimuli, such as high temperature, osmotic and oxidative stress, whereas the decrease in viability was less pronounced or non-existent in *RAS* deletion strains. So, apparently, KRAS can activate as well yeast Ras downstream cascade, leading to defective activation of stress response genes. However, KRAS activity may not be sufficient for Ras signalling hyperactivation in the absence

of one of the two endogenous Ras proteins. Moreover, human KRAS additive effect seems to be exclusive for stress response, since no significant effects were observed on growth rate. Taking into account the results from growth rate and CLS of yeasts expressing human gal-3, and growth rate and stress response of yeast expressing human KRAS, relevant aspects concerning gal-3/RAS interaction were found: (i) the roles of Ras1 and Ras2 proteins in yeast might not be completely redundant and overlapping, since the deletion of these genes causes different effects, not only when grown on non-fermentable carbon source, but also in what regards stress response, growth rate and ageing, as well as gal-3 interaction; (ii) gal-3 effects on growth rate and ageing appear to be mediated by Ras proteins in yeast, confirming the great importance of this relationship in mammals, especially in the regards of cell growth; and (iii) the coordination of gal-3 and Ras proteins action in yeast seems to be mediated by divergent mechanisms influencing exponential growth, measured by growth rate, and ageing, measured by CLS. In the case of growth, gal-3 needs both Ras proteins to mediate its effect, either enhancing growth rate, for W303-1A, or decreasing it, for BY4741, whereas in the case of CLS, only yeast lacking one Ras protein showed a phenotype distinguishable from the untransformed yeast. Further confirmation of the two first points came from experiments done in yeast *RAS* mutants to test their stress response.

W303-1A and BY4741 behaved very differently upon stress. BY4741 showed an overall higher resistance to various stress stimuli compared to W303-1A. In two cases a difference between wt and *RAS* mutants was observed (acetic acid and 6% ethanol). The less resistant yeast to these stressors was BY4741 *Δras2*, while *Δras1* behaved identically to the wt. On the contrary, W303-1A *Δras2* responded similarly to the wt and *Δras1* was the most affected strain. At the same time, W303-1A *Δras1* was also more sensitive than *Δras2* to osmotic stress. While *Δras1* showed no variation in growth when cultivated on the non-fermentable carbon source ethanol, it grew less on glycerol. This still confirms the previous reports stating that *RAS1* is less expressed on non-fermentable carbon sources (Breviario *et al.*, 1986; 1988). Glycerol is less assimilated by *S. cerevisiae* than ethanol as carbon source, so in this condition the reduced global levels of Ras proteins – deleted *RAS2* together with reduced expression of *RAS1*- influences more strongly the growth on glycerol, decreasing yeast growth.

These results give more importance to Ras1 in the response to stress stimuli, role that seems to be exclusive for the W303-1A genetic background. Ras1 function in yeast might then be more relevant and not completely overlapping with the one of Ras2 in W303-1A, as proven by the differential response to stress stimuli and to gal-3 expression for CLS, and by the requirement of both Ras1 and Ras2 to mediate gal-3 effect on cell growth rate.

In conclusion, this work provides the foundation of a yeast-based platform expressing human gal-3 and KRAS. Its phenotyping provided the detection of some easily readable phenotypes induced by gal-3 expression, which could be further screened in search for gal-3 inhibitors (**Table 7**). Moreover, results showed that in the lower eukaryote *S. cerevisiae*, in which galectins are not expressed, gal-3 exerts a function mediated by yeast Ras proteins, highlighting the degree of conservation of the Ras proteins and pathway. Additionally, this confirms the pivotal role of gal-3/KRAS interaction for cell growth and the fact that yeast is a suitable model for the study of these two proteins. Finally, the pivotal role of the genetic background was clearly exposed, suggesting the need to routinely use and compare more than one strain to be confident of results. To finalize the yeast-based platform, further work is needed. The final aim was to simultaneously express human gal-3 and KRAS in the same yeast cell. For that purpose it was necessary to insert the KRAS cDNA into yeast chromosome. That was attempted targeting the *RAS1* and *RAS2 loci*, using three different strategies, which did not yield the desired construction. In this way, the study of the interaction between the two human proteins could be achieved.

Table 7: Schematic representation of the phenotypes obtained in *S. cerevisiae* W303-1A and BY4741 wt, *Δras1* and *Δras2* expressing human gal-3 or KRAS. When the expression of the human protein in yeast caused an increase in values compared to the untransformed strain, it is indicated by ↑, when it caused a decrease is indicated by ↓, while when it did not cause any alteration is indicated by =. In case the phenotype was not monitored in this work, it is indicated by nd (not determined).

genetic background	**W303-1A**			**BY4741**		
strain	wt	*Δras1*	*Δras2*	wt	*Δras1*	*Δras2*
phenotype analysed						
	+ human gal-3			+ human gal-3		
μ_g	↑	=	=	↓	=	=
CLS	=	↓	↑	nd	nd	nd
	+ human KRAS			+ human KRAS		
μ_g	nd	nd	nd	=	=	=
temperature (37°C)	nd	nd	nd	↓	=	=
YPG	nd	nd	nd	↓	↓	↓
YPE	nd	nd	nd	↓	↓	↓
osmotic stress	nd	nd	nd	↓	=	=
oxidative stress	nd	nd	nd	↓	↓	↓

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Chapter 4

Uncover the Role of KRAS/galectin-3/p16^{INK4a} Axis Regulation in Colorectal Cancer

Abstract

Galectin-3 belongs to the galectin family, characterized by a conserved carbohydrate-recognition domain. Its role in human cells varies depending on the subcellular localization, the cell type and the proliferation state. It is involved in human carcinogenesis, promoting cell growth, transformation, angiogenesis, invasion and metastasis, and suppressing apoptosis. Its function as pro-survival protein can be exerted through the interaction with KRAS oncoprotein, acting as its scaffold protein, stabilizing and increasing its signalling. KRAS is one of the most frequently mutated protein in colorectal carcinoma. Its mutations lead to increase proliferation and evasion from apoptosis. p16^{INK4a} is a well-known tumour suppressor and it seems to be related with both gal-3 and KRAS, downregulating these proteins. The aim of this study is to better understand the role of the KRAS/gal-3/p16^{INK4a} axis regulation, using the colorectal cancer cell line SW480 and the immortalized cell line NCM460 transfected with FlagKRAS^{wt}. It was shown that the three proteins interact physically with each other and co-localize in colorectal cells, confirming the existence of a KRAS/gal-3/p16^{INK4a} axis. The effect of the silencing by RNA interference of gal-3 and KRAS, both alone and simultaneously, on cell proliferation, apoptosis induction and cell cycle was further analyse. The silencing of KRAS alone decreases cell proliferation and viability, while increases the percentage of death cells, confirming previous results on the role of KRAS in survival. The silencing of gal-3, on the other hand, provokes milder effect, while the silencing of both proteins seems to revert the effect of KRAS silencing, suggesting a possible feedback loop between gal-3 and KRAS. Moreover, the levels of proteins expression were analysed upon gal-3 and/or KRAS silencing and the results suggest that there is a feedback loop regulation in the expression of these proteins. In addition, a first viability test on cell treated with novel galectins inhibitors confirms that when galectins are inhibited, cells slightly increase their mortality, offering a good start for future development and studies on the role of gal-3/KRAS interaction in colorectal cancer.

Introduction

Galectin-3 (gal-3) is a member of the galectin family. Galectins are lectins proteins characterized by a conserved carbohydrate recognition domain (CRD) that has a high affinity and specificity for galactoside glycans (Cooper and Barondes, 1999; Boscher *et al.*, 2011; Compagno *et al.*, 2014). Gal-3 is the only chimera- type galectin and the most studied among them, due to the high variety of its roles and its ubiquitous expression (Hirabayashi and Kasai, 1993; Rabinovich, 1999). It is characterized by a C-terminal carbohydrate binding domain and an N-terminal atypically long domain, proline and glycine rich (Hsu *et al.*, 1992; Agrwal *et al.*, 1993). The N-terminal domain is essential for the biological activity of gal-3, since it allows the oligomerization of the monomeric proteins in multimers and therefore the cross-linking activity of its CRD domain (Massa *et al.*, 1993). Gal-3 has multiple glycan ligands that differ in structure and function. The specificity of gal-3 toward one ligand instead of another depends on sugar-binding affinity, on the phosphorylation status of gal-3 and on the time and space coordination of the ligand expression. Importantly, gal-3 can interact also with unglycosylated protein due to its N-terminal domain (Sato and Hughes, 1992; Hirabayashi and Kasai, 1993). Upon binding to the proper ligand, the conformation of gal-3 changes, with the rearrangement of the backbone loops near the binding site (Agrwal *et al.*, 1993; Umemoto *et al.*, 2003). Gal-3 is ubiquitously expressed in human adults, in particular in epithelial cells, myeloid and amoeboid cells. Gal-3 is synthetized in the cytoplasm, but it is found also on the cell membrane, in the nucleus and in the extracellular space (Dumic *et al.*, 2006). The transcription of its coding gene, *LGALS3* (Raimond *et al.*, 1997), is modulated by various factor, including the proliferation state of the cell (Moutsatsos *et al.*, 1987), infections (Elliott *et al.*, 1991) and tumour progression (Hebert and Monsigny, 1994). Gal-3 protein localization inside the cell and the cell typology in which it is expressed define its biological role. Cytoplasmic gal-3 is involved in various intracellular events, such as regulation of apoptosis, through the interaction of proteins like Bcl-2 (Yang *et al.*, 1996; Fukumori *et al.*, 2006), CD95 (Fukumori *et al.*, 2004), Alix (Liu *et al.*, 2002), Annexin VII (Yu *et al.*, 2002) and Nucling (Liu *et al.*, 2004). Gal-3 is also involved in cell proliferation and differentiation, by interacting with

the oncoprotein KRAS (Elad-Sfadia *et al.*, 2004; Shalom-Feuerstein *et al.*, 2008; Levy *et al.*, 2010; Levy *et al.*, 2011; Song *et al.*, 2012). Moreover, it has been discovered that gal-3 is highly expressed in a variety of tumours and that its expression and localization within the cell are often altered in cancer cells (Cay, 2012). In this perspective, many researches have been focused on the study of the role of gal-3 in different cancer progression, trying to use gal-3 as a diagnostic and prognostic marker (Sanjuan *et al.*, 1997; Kim *et al.*, 1999; Honjo *et al.*, 2000; van den Brule *et al.*, 2000; Danguy *et al.*, 2002; Puglisi *et al.*, 2004; Nakahara *et al.*, 2005; Newlaczyl and Yu, 2011). However, the role of gal-3 in cancer has not been completely unveiled. In the nucleus gal-3 is associated with the ribonucleoprotein complexes (Laing and Wang, 1988), being involved in the spliceosome assembly and in mRNA splicing (Dagher *et al.*, 1995). When gal-3 is localized on the cell surface, it exerts functions like adhesion, interacting with glycoprotein on the extracellular matrix, and recognition of specific glycans pattern on the surface of microorganism or host cells (Krzeslak and Lipinska, 2004; Dumic *et al.*, 2006)

KRAS belongs to the RAS proteins family, which accounts for 36 members and is part of the superfamily of small guanosine triphosphatases (GTPases). They all share a common biochemical mechanism as they work as binary molecular switches that alternate an active state, in which they are bound to GTP, to an inactive state, in which they are bound to GDP (Bar-Sagi and Hall, 2000; Malumbres and Barbacid, 2003; Wennerberg *et al.*, 2005). The switch from GTP to GDP and *vice versa* is mainly regulated by two classes of proteins: the guanine-nucleotide exchange factors (GEFs), which enhance the intrinsically low exchange activity of RAS proteins, and the GTP-ase activating proteins (GAPs), which accelerate the GTPase activity, constitutively low in RAS proteins (Boguski and McCormick, 1993; Zheng and Quilliam, 2003; Rajalingam *et al.*, 2007). RAS proteins work as transducer of pro-survival signals from the extracellular space to the intracellular compartment, through different tyrosine kinases receptors, such as the ones belonging to the ErbB family of related cell membrane receptor, among which the most studied is EGFR (epidermal growth factor receptor). The dimerization of the receptors upon ligand binding triggers a conformational change that activates the catalytic tyrosine kinase domain, enabling the autophosphorylation of the intracellular

carboxyl-terminal domain and therefore its activation. Once phosphorylated, it provides the ideal docking sites for multiple signaling proteins (Martinelli *et al.*, 2009; Normanno *et al.*, 2009; Saif, 2010). Phosphorylated intracellular domain of EGFR recruits guanine tyrosine exchange factors and these proteins remove GDP from RAS proteins, which consequently bind to GTP, resulting in activated RAS which is able to signal downstream (Schulze *et al.*, 2005). Activated RAS proteins can bind and activate at least 20 different effectors, among which the best known and characterized are RAF kinases, phosphatidylinositol 3-kinase (PI3K) and RAL guanine nucleotide dissociation stimulator (RALGDS). Other less studied effectors of RAS proteins are p120^{GAP}, RIN1, Tiam, Af6, Nore1, PLCε and PKCζ (Shields *et al.*, 2000; Downward, 2003; Herrmann, 2003; Rajalingam *et al.*, 2007). Briefly, activated RAF kinases phosphorylate and activate MEK, leading to the activation of MAPK (also called ERK, extracellular signal-regulated kinase). MAPK regulates several transcriptional factors that influence cell cycle progression, proliferation and survival (Malumbres and Barbacid, 2003). In the other pathway, activated PI3K leads to the production of the second messenger lipid phosphatidylinositol (3,4,5)-triphosphate (PIP_3), which activates phosphatidylinositol dependent kinase 1 (PDK1). PDK1 phosphorylates and activates AKT, which in turn promotes proliferation and survival while controlling cell death (Yan *et al.*, 1998; Scheid and Woodgett, 2001a, 2001b). RAS, when interacting with RALGDS, one of four RAS-related RAL proteins, stimulates RAL (RAS-like) GTPases, inducing the activation of phospholipase D1 and CDC42/RAC-GAP-RAL binding protein 1 (RALBP1). These, among other pro-survival functions, promote the progressing of the cell cycle, inhibiting transcription factors implicated in cell cycle arrest such as the FORKHEAD transcription factors (Downward, 2003; Rajalingam *et al.*, 2007). Therefore, RAS proteins have a critical role in human oncogenesis, not only providing crucial signalling in proliferation and survival pathways like autophagy (Furuta *et al.*, 2004; Elgendy *et al.*, 2011; Guo *et al.*, 2011; Lock *et al.*, 2011; Alves *et al.*, 2015), but also promoting related processes such as angiogenesis and invasiveness (Shields 2000, Downward 2003). Our group has recently found that mutated KRAS induces autophagy in colorectal cancer cells through ERK activation and that autophagy induction significantly leads to tumour survival in starvation conditions (Alves *et al.*, 2015). Importantly, RAS protein are frequently

found mutated in cancer cells (approximately 20% of all cancers). KRAS is the most frequently mutated among all RAS proteins, accounting for about 85% of the total. KRAS mutations are particularly frequent in CRC (colorectal carcinoma), where they are found in around 30 to 50% of the cases (Malumbres and Barbacid, 2003). All the mutations of RAS proteins disrupt the proper endogenous GTP-ase activity of the protein, leading to the accumulation of the activated GTP-bound form of RAS and therefore to a constant pro-survival signalling in the cells. *KRAS* mutations are single nucleotide point mutations occurring mostly on codon 12 and 13 of exon 2. They all are substitution of a glycine with another amino acid, the most frequent of which is an aspartate (32.5%), followed by a valine (22.5%) (Andreyev *et al.*, 2001; Normanno *et al.*, 2009; Prior *et al.*, 2012). Mutation are found, even if less frequently, also in the other main RAS proteins, HRAS and NRAS. Every member of RAS family has its specific functions, pattern of expression and subcellular localization (Malumbres and Barbacid, 2003; Wennerberg *et al.*, 2005; Omerovic *et al.*, 2007; Castellano and Santos, 2011).

Importantly, KRAS has been found to interact with gal-3 in the cytoplasm. The interaction does not occur through gal-3 CRD and it only happens with activated KRAS (Elad-Sfadia *et al.*, 2004; Shalom-Feuerstein *et al.*, 2005). The interaction has been found to be specific, since gal-3 does not interact with any other RAS protein and KRAS does not interact with any other galectin (Ashery *et al.*, 2006). It has been hypothesized that gal-3 may render KRAS less sensitive to GAPs and therefore maintain KRAS in a state of perpetual activation, increasing the pro-growth signalling transmitted by KRAS through membrane Epidermal Growth Factors. Indeed, it has been noticed that the interaction between gal-3 and KRAS enhances PI3K activity and RAF-1 activation (Elad-Sfadia *et al.*, 2004). Moreover, when KRAS interacts with gal-3, the cell starts using preferentially KRAS at the expenses of the other RAS isoforms present and when KRAS is preferred over NRAS, the anti-apoptotic effect of gal-3 in increased (Shalom-Feuerstein *et al.*, 2005). Many of the effect due to the interaction between gal-3 and KRAS are caused by the fact that gal-3 acts as a scaffold protein for KRAS, maintaining KRAS in a proper orientation, relevant to regulate its activity (Abankwa *et al.*, 2010), and in organized nanoclusters at the cell membrane, which allow the access to its multiple effectors (Ashery *et al.*, 2006; Shalom-Feuerstein *et al.*, 2008; Bhagatji *et al.*, 2010). In addition, it seems that the

expression of gal-3 increases not only the activation, but also the expression of KRAS (Levy *et al.*, 2010). Since KRAS has an important role in promoting cell proliferation, autophagy and inhibition of apoptosis and since gal-3 appears to enhance KRAS activity through different mechanisms, it is conceivable to expect that this interaction could favour cancer progression. It has been proved that gal-3 interaction with KRAS enhances thyroid cancer progression, increasing KRAS signalling and thus proliferation (Levy *et al.*, 2010). In addition, gal-3 has been found overexpressed in pancreatic cancer and its increased amount augments the signalling of KRAS, influencing proliferation, adhesion and anchorage-independent growth (Song *et al.*, 2012). Gal-3 expression has an impact also on migration of colorectal cancer cells. Gal-3 overexpression increased migration, while it happened the opposite when gal-3 was downregulated. This effect was found to be mediated by RAS/RAF/ERK/MEK pathway. Indeed the knock-down of gal-3 decreased phosphorylated RAF and ERK1/2 expression, while its overexpression increased the level of the two proteins. Moreover, the silencing of *KRAS* decreased the migration rate, especially in cells overexpressing gal-3, confirming the hypothesis of a relationship between KRAS, gal-3 and tumorigenicity (Wu *et al.*, 2013). In conclusion, an increase expression or availability of cytoplasmic gal-3 confer to the cells tumorigenic properties, such as uncontrolled growth and downregulated apoptosis, that the cells also acquire upon mutation of the *KRAS* oncogene. This is why it is particularly important to better understand the interaction between the gal-3 and KRAS.

p16^{INK4a} (known as well as CDKN2A) is a well-established tumour suppressor protein, which acts as inhibitor of the cyclin-dependent kinases (CDK) 4 and 6, competing with cyclins-D (D1, D2 and D3) for their binding. The formation of the complex made of CDK and cyclin allows cell cycle progression and, in the specific case of these complexes, the transition of the G1/S restriction point and the beginning of DNA replication. CDK-cyclin complexes phosphorylate the Retinoblastoma (Rb) protein. The hyper-phosphorylation of Rb relieves p16^{INK4a} from its inhibitory role on E2F transcription-factor family, leading to the transcription of genes necessary for S phase (Reed, 1997; Sherr and Roberts, 1999; Coleman *et al.*, 2004; Bertoli *et al.*, 2013). By binding to CDK4 and CDK6, p16^{INK4a} inhibits the proper formation of the complexes and therefore the entry in S phase, having therefore a role in stopping cell cycle

progression. Not surprisingly, inactivation of $p16^{INK4a}$ are among the most frequently found aberrations in cancer (Hirama and Koeffler, 1995; Romagosa *et al.*, 2011). Moreover, $p16^{INK4a}$ has been implicated in senescence, being its expression and/or protein amount higher in senescent cells, and it seems to be involved in a cell cycle arrest with the same phenotypical features of senescence (Ruas and Peters, 1998; Rocco and Sidransky, 2001; Canepa *et al.*, 2007; Li *et al.*, 2011; LaPak and Burd, 2014).

Considering their respective roles, it is clear that the functions of RAS proteins and $p16^{INK4a}$ are antagonistic. Indeed, while $p16^{INK4a}$ is a strong inhibitor of CDK4 and 6 and cell cycle progression, it has been proved that RAS proteins are mediators of cell cycle progression through the activation of cyclins, among others. RAS proteins promote the transcription of cyclin D1 through the RAF/ERK/MEK pathway and PI3K activation; they increase its stability by inhibiting the kinases that cause its ubiquitination and consequent degradation; through PI3K activation, they induce cyclin D1 mRNA translation and they facilitate the assembly with CDK4 and CDK6. RAS pathway further helps cyclin D1 activity by reducing the level of CDK-cyclins complexes inhibitors such as p27 (Mittnacht *et al.*, 1997; Peeper *et al.*, 1997; Pruitt and Der, 2001; Coleman *et al.*, 2004). However, little is known about a possible direct relation between RAS proteins, in particular KRAS, and $p16^{INK4a}$. In the middle 90´s Serrano *et al.* published several works highlighting an indirect relationship between HRAS and $p16^{INK4a}$. They proved that the introduction of a plasmid encoding $p16^{INK4a}$ to cells transformed with $HRAS^{G12V}$ and c-Myc decreased the tumorigenicity of the cells (Serrano *et al.*, 1995). On the other way around, when *HRAS* was transfected into $p16^{INK4a-/-}$ fibroblasts, the tumorigenicity of the cells greatly increased, while no effect was observed when using $p16^{INK4+/+}$ or $p16^{INK4+/-}$ cells (Serrano *et al.*, 1996). Moreover, they showed that cells lacking functional $p16^{INK4a}$ do not undergo senescence (Serrano *et al.*, 1996) and that RAS-caused cell cycle arrest, phenotypically indistinguishable from senescence, is mediated by $p16^{INK4a}$. Indeed cells capable to block cell cycle progression through RAS over-activity are growing continuously only when $p16^{INK4a}$ is inactivated (Serrano *et al.*, 1997). Oncogenic $HRAS^{G12V}$ was proved to increase $p16^{INK4a}$ expression in late papilloma and the balanced activity of RAS pathway and $p16^{INK4a}$ induced senescence (Yamakoshi *et al.*, 2009). A reciprocal regulation

of the two proteins was discovered in the case of KRAS. In primary pancreatic duct epithelial cells (PDEC) it was noticed that KRAS^{wt} was able to increase p16^{INK4a} expression, inducing senescence and preventing cancer formation, while KRAS^{G12D} repressed almost completely p16^{INK4a} expression (Lee and Bar-Sagi, 2010). On the other side, also p16^{INK4a} has been shown to affect oncogenic KRAS (KRAS^{G12V}). Introduction of active p16^{INK4a} in cells in which p16^{INK4a} was not working restored its function and lowered selectively KRAS protein level by decreasing protein stability (Rabien *et al.*, 2012). Moreover, the simultaneous expression at normal level of p16^{INK4a} and KRAS^{G12V} caused the cell to became resistant to anoikis and highly clonogenic, while both these features did not occur in cells expressing p16^{INK4a} only, proving that the suppression of oncogenic KRAS is necessary for p16^{INK4a} to exert its anti-proliferative function (Rabien *et al.*, 2012). Interestingly, a relationship between p16^{INK4a} and gal-3 seems to exist as well. Upon p16^{INK4a} expression, a clear decreased in gal-3 level was observed both *in vitro* and *in vivo*, in particular p16^{INK4a} diminished the cell-surface expression of gal-3 and its consequent cell-surface presentation (Sanchez-Ruderisch *et al.*, 2010).

Due to the proven interaction between gal-3/KRAS/p16^{INK4a} and the importance of these interactions for several physiological features of cells, here we aimed at further investigate the mutual relationship and regulation of gal-3, KRAS and p16^{INKa} using colorectal cancer cells as a model. As previously stated, KRAS mutations are particularly relevant for this type of cancer, but no deep investigation has been made about a possible regulation of mutated KRAS by gal-3 (positive) and p16^{INK4a} (negative). Even less was known about the interaction between gal-3 and p16^{INK4a}.

With this work, we proved that the three proteins physically interact *in vitro* and co-localize frequently in cell culture. Moreover, the silencing of *KRAS*, *LGALS3* or both has an effect on the expression of all the three proteins and on cell survival and proliferation. This indicate a possible mutual regulation at the protein level and highlights the importance of KRAS and gal-3 for colorectal cancer cells survival.

MATERIALS AND METHODS

Cell lines and culture conditions

We used CRC-derived cell line SW480 (ATCC-CCL-228) (Ahmed *et al.*, 2013) and the non-cancerous colon NCM460 cell line (Moyer *et al.*, 1996). NCM460 cells were previously transfected with a plasmid harboring either FLAG-tagged wild-type KRAS ($KRAS^{wt}$) or mutated KRAS ($KRAS^{G12V}$) (Alves *et al.*, 2015). Both cell lines were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin. All cell lines were cultured in a humidified incubator with 5% CO_2 at 37°C. The medium was renewed twice or three times a week, depending on the growing conditions, and the cells were subcultures every week by detachment with 0.05% trypsin. The cells were used for the assays when they reached 80-90% confluence.

Galectin-3 and KRAS silencing by RNA interference

SW480 cells were transiently transfected with siRNA in order to obtain the silencing of gal-3, KRAS and the two proteins together. The target sequences used were 5'-CACGGTGAAGCCCAATGCAAA-3' (Hs_LGALS3_3) and 5'-AAGGAGAATTTAATAAAGATA-3' (Hs_KRAS2_8) (Qiagen), in the concentration of 50nM and 150nM, respectively. AllStars negative control siRNA (Qiagen) (5'-AATTCTCCGAACGTGTCACGT-3') was used as scramble control. The transfections were performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer instructions. Briefly, OptiMEM (reduced serum medium) and Lipofectamine 2000 were mixed in the desired ratio. After 5 minutes of incubation at room temperature, the siRNA was added and further incubated for 20 minutes. The siRNA mixture was then transferred into the appropriate culture plate. SW480 cells at the density of $4x10^5$ cells/ml, suspended in RPMI 1640 supplemented only with 10% FBS, were added on top of the siRNA mixture and shaken carefully. The cells were incubated for 24 h, considered the time 0 of the assay, after whicd the medium was replaced with completed RPMI 1640. The effects of the proteins transient silencing were analyzed after 48 h of incubation in completed RPMI 1640.

Western blot analysis

The total protein content was extracted from cells at the end of the siRNA experiments or when they reached 85-90% confluence, in the case of different experiments. The cells were rinsed repetitively with PBS 1X (phosphate buffer saline) and detached by 0.05% trypsin. The so obtained pellet was rinsed twice in cold PBS 1X. RIPA (radioimmunoprecipitation assay) buffer supplemented with 20 mM NaF, 20 mM Na_3VO_4, 1 mM PMSF and 1:100 proteases inhibitor cocktail (Sigma-Aldrich) was added to the cell pellet, subsequently incubated on ice for 1 h, vortexed every 10 minutes. After centrifugation, the protein content of the supernatant was quantified using the protein Bradford Assay (Bio-Rad), according to the manufacturer instructions. The volume of protein extracts containing 50 µg of proteins was mixed with Laemmli Buffer 5x (Laemmli, 1970) and dH_2O to a final volume of 20 µl. The samples were run in a 15% polyacrylamide gel until complete separation. The proteins were then blotted on a PVDF (polyvinylidene difluoride) membrane, treated with the appropriate primary and secondary antibody and revealed by chemiluminescence (ECL Bio-Rad). The antibodies used were: anti-galectin-3 (Santa Cruz Biotechnology), anti-$p16^{INK4a}$ (Proteintech), anti-KRAS and anti-β-actin (Sigma-Aldrich) as primary; anti-goat (Santa Cruz Biotechnology), anti-rabbit and anti-mouse (Sigma-Aldrich) as secondary. Densiometry analysis was performed using the free software ImageJ (http://imagej.nih.gov/ij/) and protein expression level were normalized to the level of β-actin.

Cell proliferation and viability determination

After RNA interference (RNAi) experiments, both cells attached to the culture plate and from the supernatant medium were used to determine proliferation and viability. To determine proliferation, cells were counted using a Neubauer chamber. The cell concentration (n°cells/ml) was normalized to the control cells that was considered 100%. To determine viability, the cell suspension was mixed with a 0.4% trypan blue dye (Sigma-Aldrich) solution in a 1:1 ratio. Stained (unviable) and unstained (viable) cells were counted separately in a Neubauer chamber. Viability was calculated as percentage of unstained cells/total number of cells.

Cell cycle analysis

Cell cycle analysis was performed on cells subjected to RNAi experiments. Cells were harvest from both the plate surface and the supernatant medium and washed three times in cold PBS 1x. They were fixed in 70% ethanol, adding it drop by drop while vortexing, and then they were incubated at 4°C for at least 24 h. The cell pellet was washed twice in dH_2O and resuspended in a solution containing 50 µg/ml PI (propidium iodide; Sigma-Aldrich) and 9 µg/ml RNAase A (NZYTech) in PBS 1x. The samples were incubated in the dark, for 1h, at 37°C and analyzed using an Epics® XL™ (Beckman Coulter) flow cytometer at 488nm/600nm excitation/emission. In the flow cytometer 20.000 to 30.000 cells were examined per sample. Data were analyzed using the freeware Flowing Software 2. Image were obtained using FlowJo software.

Annexin V/PI assay

A total amount of $2.5x10^4$ cells was collected from both suspended and attached cells after RNAi experiment and washed twice in binding buffer 1x (BD Biosciences, San Jose, CA, USA). Annexin V (AV)/PI assay was performed as described previously (Alves *et al.*, 2015). The dye amount was adjusted to 3 µl AV-fluorescein isothiocyanate (BD Biosciences, San Jose, CA, USA) and 1 µl PI 1 mg/ml. 20.000 to 30.000 cells were examined per sample. Data were analyzed using the freeware Flowing Software 2.

Physical interaction analysis by immunoprecipitation assay

Protein extracts for co-immunoprecipitation experiments were obtained as in the above section (western blot analysis). A volume corresponding to 250 to 500 µg of protein was used. Immunoprecipitation of the proteins with the appropriate antibodies was done using Dynabeads® Protein G (LifeTechnologies), following the manufacturer's instructions. Anti-galectin-3 (Santa Cruz Biotechnology), anti-p16^{INK4a} (Proteintech) and anti-KRAS (Sigma-Aldrich) were used as antibodies. The immunoprecipitated proteins were eluted in 60 µl of Leammli buffer 2x (Laemmli, 1970). 20 µl of the eluted immunoprecipitation were load in each well of a 12% polyacrylamide gel, to test co-immunoprecipitation with KRAS of gal-3.

Western blot analysis was performed as described in the previous section, using anti-KRAS (Sigma-Aldrich) and anti-gal-3 (Santa Cruz Biotechnology) as primary antibodies and anti-mouse (Sigma-Aldrich) and anti-goat (Santa Cruz Biotechnology) as secondary antibodies, respectively.

Immunofluorescence assay

Protein localization in the cells was assessed by immunofluorescence. NCM460 transfected with either Flag-tagged wild type KRAS ($KRAS^{wt}$) or mutated KRAS ($KRAS^{G12V}$) were grown in normal condition on microscope glass slide up to 60-70% confluence and fixed in paraformaldehyde (PFA). Briefly, cells culture was gently washed 3 times in PBS 1x. For fixation with PFA, a solution of 2% PFA was added to the cells and incubated for 12 minutes at room temperature. For all the following step, washing and incubation solutions were made in PBS 1x. Fixed cells were washed three times and permeabilized with 0.7% Triton X-100 for 7 minutes. They were blocked in 10% FBS, incubated sequentially with two primary antibodies (the first one overnight at 4°C and the second for 1 h and 30 minutes, at room temperature) and, after three washes, with the secondary antibodies. In the case of co-immunofluorescence of gal-3 and $p16^{INK4a}$, also secondary antibodies were added sequentially (1 h of incubation with the first, followed by three washes and 1 h of incubation with the second). Finally, DAPI (4', 6-diamidino-2-phenylindole) was added for 5 minutes and cells were visualized in an Olympus FV1000 laser scanning microscope with the appropriate filter settings. The images obtained were analyzed using Olympus Fluoview software. Antibodies combinations used are listed in **table 1**.

Table 1: Primary and secondary antibodies used in the immunofluorescence assay in this study.

proteins	primary antibody	secondary antibody (ThermoFischer Scientific)
galectin-3 + FlagKRAS	Anti-galectin-3 1:100 (Santa Cruz Biotechnology)	Donkey anti-goat Alexa Fluor® 488
	Anti-Flag 1:100 (Sigma-Aldrich)	Chicken anti-mouse Alexa Fluor® 594
galectin-3 + p16^{INK4a}	Anti-galectin-3 1:100 (Santa Cruz Biotechnology)	Donkey anti-goat Alexa Fluor® 488
	Anti-p16^{INK4a} 1:50 (Santa Cruz Biotechnology)	Donkey anti-rabbit Alexa Fluor® 546
FlagKRAS + p16^{INK4a}	Anti-Flag 1:100 (Sigma-Aldrich)	Chicken anti-mouse Alexa Fluor® 594
	Anti-p16^{INK4a} 1:50 (Santa Cruz Biotechnology)	Goat anti-rabbit Alexa Fluor® 488

Analysis of the effect of galectins inhibitors on cell growth by Sulforhodamine B assay

SW480 cells were plated in 24 well plate at a density of 5x10^4 cells/ml, 0.5 ml/well. After 24 h of incubation in normal conditions, RPMI 1640 completed medium was replaced with medium supplemented with one of the two galectin inhibitors: MBS4Flac ($C_{21}H_{27}F_4NO_{12}$) or MBS5Flac ($C_{20}H_{24}F_5NO_{11}$), kindly provided by Prof. Oscarsson S. and collaborators, at the concentrations of 1 µM, 10 µM, 100 µM and 1000 µM. Negative control of cells consisted in cells treated with the maximum amount of buffer in which the inhibitors were dissolved (PBS 1x). Every condition was performed in duplicate for each experience. To determine the effect of the galectin inhibitors on proliferation after 48 h treatment, sulforhodamine B (SRB) assay was performed as in Vichai (Vichai and Kirtikara, 2006), with modifications. Cells were fixed in 1% v/v acetic acid in methanol at-20°C for at least 2 h instead of using 10% TCA (trichloroacetic acid) at 4°C. After dye solubilization, an aliquot of 100 µl was transferred into a 96 well plate and the optical density was measured at 540 nm. After calculating the average of the duplicates, proliferation was calculated as percentage compared to the negative control (cells cultured only with buffer), considered as 100% proliferation.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software. When multiple columns were compared, One-way Anova analysis was used. Results were considered statistically significant when p-value ≤0.05.

Results

Silencing of galectin-3 and/or KRAS shows a reciprocal regulation of galectin-3/KRAS/p16^{INK4a} expression

In order to understand the functions and reciprocal regulation of gal-3, KRAS and p16^{INK4a}, the expression of gal-3 and KRAS was downregulated by RNA interference. *LGALS3* and *KRAS* were silenced both alone and in combination, to understand the cellular effect of silencing both proteins. SW480 cell line was subjected to RNAi protocol using a scramble siRNA as control and siRNA for *LGALS3*, *KRAS* or both. The level of the three proteins was measured by western blot after each silencing experiment. The level of the silenced proteins decreased by approximately 75% in the single silencing for KRAS and gal-3. When the double silencing was performed, the efficiency of *KRAS* siRNA was proved to be lower compared to the single silencing (55% silencing), while *LGALS3* silencing remained unaltered (about 75%) **(Fig. 1A, B)**. We could observe that *LGALS3* and/or *KRAS* silencing affected the expression of both proteins underwent changes. Indeed, in the case of siRNA of *LGALS3*, KRAS expression increased by approximately 30%, while when *KRAS* was silenced, gal-3 level decreased by about 20% taking into consideration actin loading control levels. However, the decrease of gal-3 level upon *KRAS* silencing was very variable among different replicates, ranging from -60% to the same level as the control (100%), therefore the robustness of the statistical analysis was not as significant as KRAS level increase upon *LGALS3* silencing. This result suggests a strong correlation between gal-3 and KRAS expression regulation. The level of p16^{INK4a} did not significantly change when only one protein was silenced, even though it is possible to observe a tendency toward reduced expression, being more pronounced in the double silencing. We showed that there is a correlation between p16^{INK4a}, KRAS and gal-3 proteins expression. Our results show that this regulation may work as a feedback loop. When *LGALS3* and/or *KRAS* was silenced, p16^{INK4a} expression seems to decrease and the maximum effect was observed when both proteins were silenced at the same time. This result is in accordance with the hypothesis that p16^{INK4a} may act as inhibitor of gal-3 and KRAS and thus if the levels of gal-3 and KRAS are decreased, a lower amount of p16^{INK4a} would be necessary.

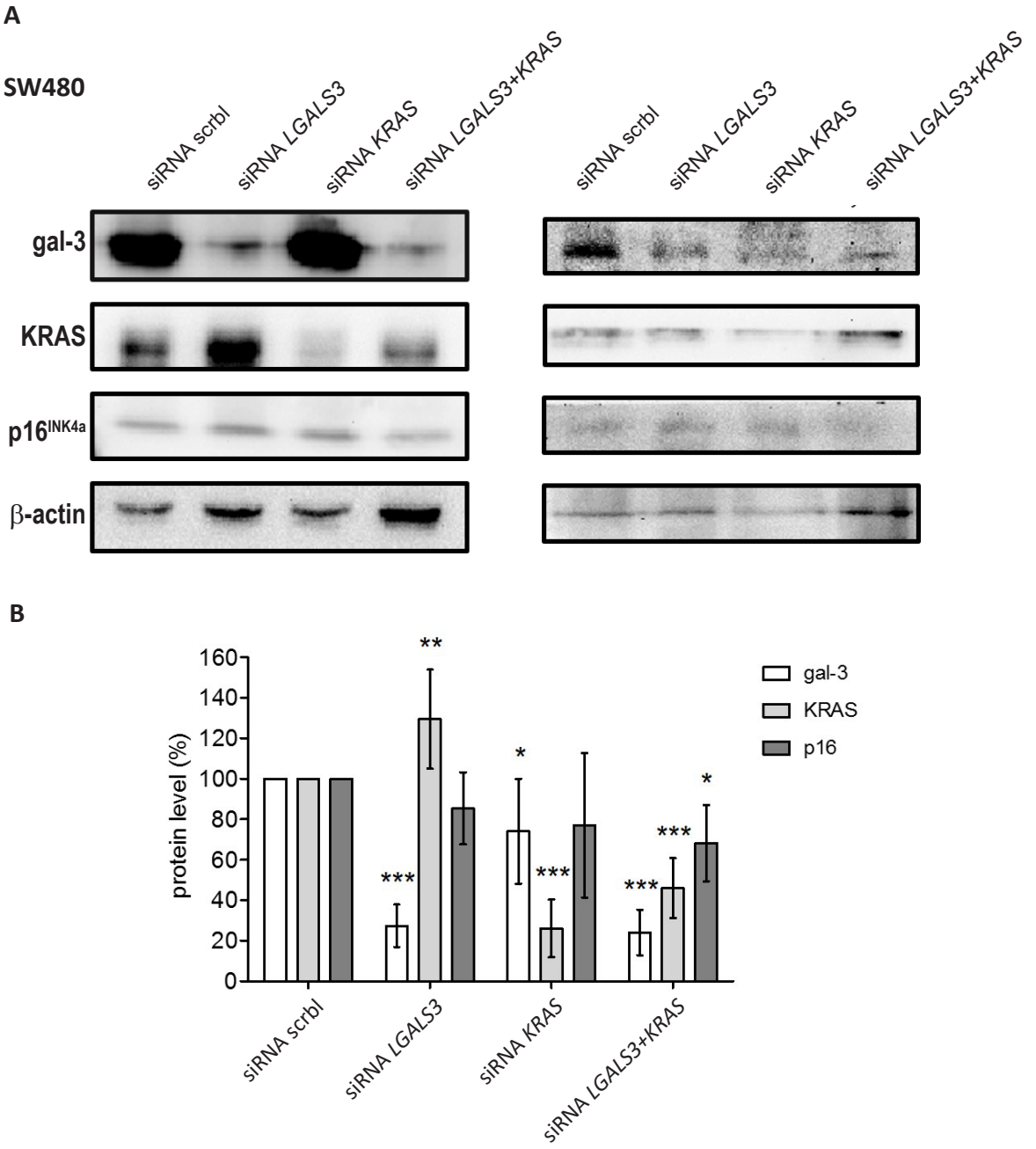


Figure 1: *Proteins level after siRNA experiments in SW480 cells*. The protein expression of KRAS and gal-3 was downregulated by siRNA both alone and in conjunction. The level of KRAS, gal-3 and p16^{INK4a} were measured by Western Blot analysis after every silencing experiments and compared to the control protein β-actin, as shown in two representative experiments of at least 5 independent experiments **(A)**. The bands intensity was measured using ImageJ software. The level of protein expression compared to β-actin level was normalized using the values of the protein level in cells subjected to scramble siRNA, which represents 100% of protein expression **(B)**. Values are average of 4 to 6 independent experiments ±SD. Statistically significant differences between the level of the protein in the siRNA scramble sample and the samples in which *KRAS* and/or *LGALS3* are silenced are shown: * (p-value ≤ 0.05), ** (p-value ≤ 0.01) and *** (p-value ≤ 0.001).

Galectin-3 and/or KRAS inhibition affects cells number and viability to a different extent

When performing the RNAi experiment, an identical number of cells was plated for each condition and, after 24 h of growth together with the silencing solution, the cells were allowed to proliferate in normal medium during 48 h. At the end of this period, the number of cells was counted to estimate the proliferation ability when the different proteins were silenced. The lowest number of cells, indicating the lowest proliferation capability, was obtained for the cells in which *KRAS* was silenced. The inhibition of gal-3 caused also a decrease in the number of cells, but not as evident as the one induced by *KRAS* silencing (44±2.9% for gal-3 and 56±18.5% for KRAS). Interestingly, the simultaneous silencing of *LGALS3* and *KRAS* did not cause a sum up effect on proliferation, but it led to a slight increase in the number of cells compared to the single silencing, since the reduction observed was only of 35±9.0% **(Fig. 2A)**.

The reduced number of cells is most likely caused by a decreased viability or an increased apoptosis or cell cycle arrest. The cells were therefore tested for several parameters that help estimate viability and apoptosis. A first viability test was performed using trypan blue exclusion assay. When the cells subjected to protein silencing were compared with the siRNA scramble control, a slight decrease in the percentage of viable cells was notice for all the samples **(Fig. 2B)**. As expected, the most significant decrease was observed for cells with lowered KRAS expression (15±7.5% reduction). In the cells subjected to *LGALS3* silencing, the loss of viability accounted for around 10%, while the double silencing caused a reversion of the effect caused by decreased KRAS or gal-3 level, triggering only a 5% decrease.

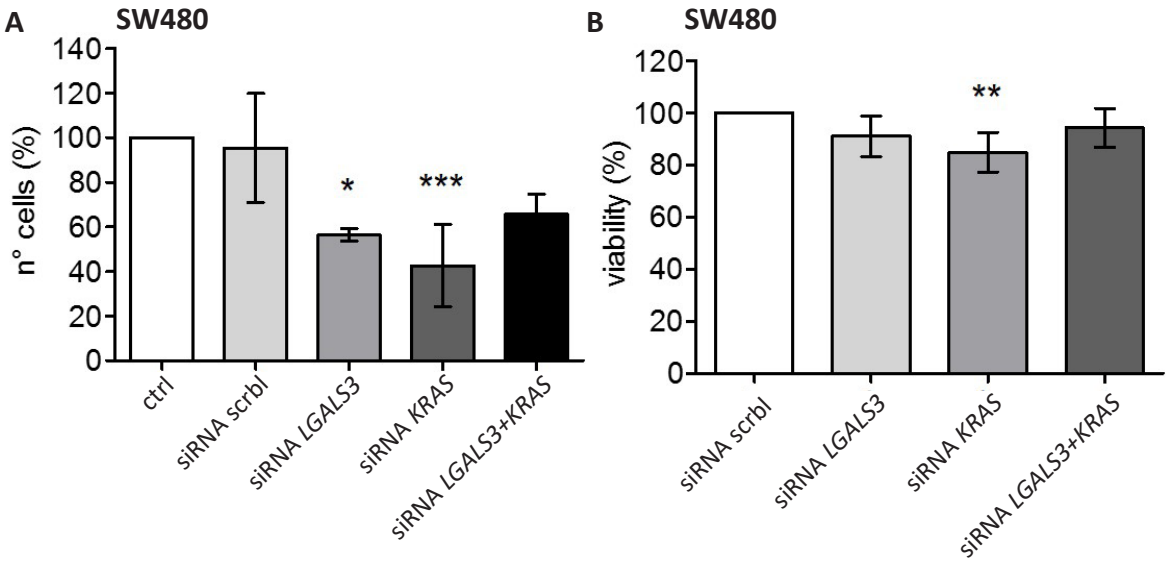


Figure 2: *Number of cells in cultures undergone siRNA for LGALS3 and/or KRAS.* SW480 cell cultures were subjected to RNAi protein downregulation for 24 h and the number of cells in each culture was counted with a Neubauer chamber after the recovery period of 48 h. The number of cells obtained in each samples was normalized with the number of cells of the control (no siRNA), which was considered 100% of cells (**A**). Values are an average of 3 to 5 independent experiments ±SD. Statistically significant difference between the siRNA scramble and the silenced proteins samples: * (p-value ≤ 0.05) and *** (p-value ≤ 0.001). *Viability of cells after silencing of LGALS3 and/or KRAS*. Cells undergone siRNA treatment were evaluated for viability using trypan blue exclusion assay after 48 h of recovery period. Cells in solution were mixed 1:1 with trypan blue dye and counted with a Neubauer chamber. Blue stained cells were considered unviable. Viability percentage was calculated as (n° viable cells/tot n° cells) x 100. The values were normalized to the viability percentage of siRNA scramble, which was considered 100% viable (**B**). The bars represent the average of 7 independent experiments ±SD. Statistically significant differences between the viability in the siRNA scramble sample and the samples in which *KRAS* and/or *LGALS3* are silenced are shown: ** (p-value ≤ 0.01).

Galectin-3 and/or KRAS inhibition effect in cell apoptosis

In order to determine more accurately the causes of the loss of viability and the possible induction of apoptosis, Annexin V/PI staining was performed on the cells after 48 h of recovery in complete medium. The results partially confirmed the ones obtained with trypan blue exclusion assay. At a first analysis, a similar pattern was observed comparing the trypan blue exclusion assay results and the percentage of apoptotic/late apoptotic/necrotic positive cells (AV+ PI+/-) obtained with the Annexin V/PI assay **(Fig. 3A)**. There was indeed an increase

in the unviable percentage of cells when one of the proteins were silenced, although not statistical significant. In particular, the highest percentage of dead cells was triggered by the silencing of *KRAS* (19±9.0% AV+ PI+/-), followed by the single silencing of *LGALS3* (13.5±1.3% AV+ PI+/-) in comparison with the scramble control (7.8±0.8 AV+ PI+/-). Similarly, to the trypan blue exclusion assay results, the simultaneous silencing of the two proteins reverted the effect caused by the single silencing. Indeed the percentage of death was 11±4.2% (AV+ PI+/-). It was further analyzed whether the loss of viability was to ascribe to early induction of apoptosis or late apoptosis/necrosis. The percentage of unviable cells (Annexin V positive) was therefore divided into apoptotic (AV+/PI-) and late apoptotic/necrotic cells (AV+/PI+) based on the PI staining. In all the samples analyzed, the percentage of cells that stained AV+/PI+ (late apoptotic/necrotic) was higher than the percentage of (AV+/PI-) **(Fig. 3B)**.

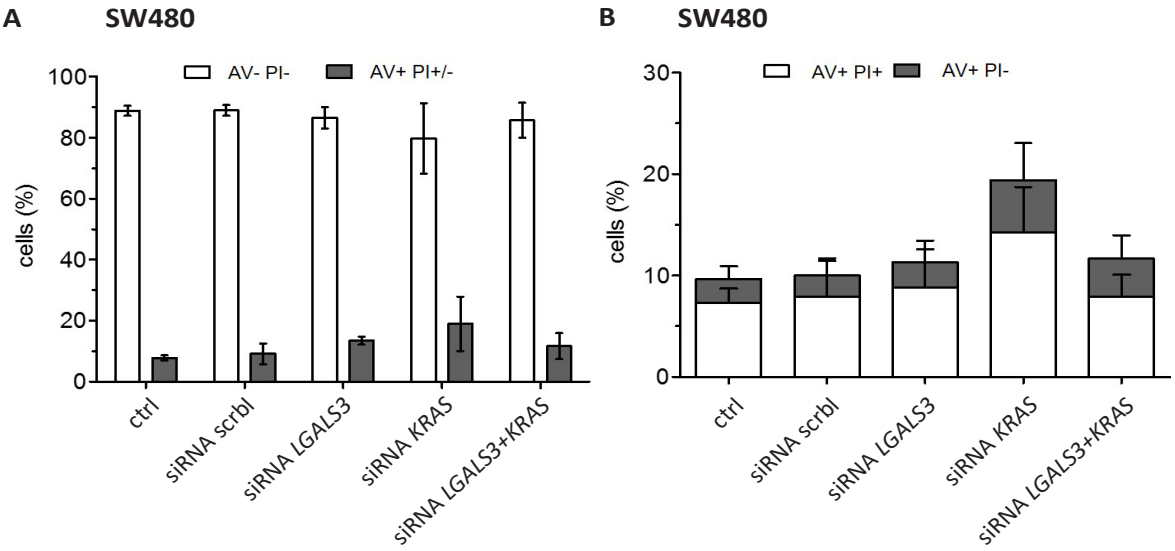


Figure 3: *Cell death analysis* by *Annexin V/PI*. After siRNA experiment, SW480 cells were evaluated for AV/PI staining by cytometry. Bars represent the percentage of viable (AV-/PI-) and unviable cells (AV+/PI±) **(A)**. Unviable cells were further analyzed to determine if the loss of viability was due to apoptosis (PI-) or necrosis/late apoptosis (PI+) **(B)**. Values are an average ± SD of 3 to 4 independent experiments. None of the p-value calculated for the differences between controls and treated samples was statistically significant at 95% level of confidence.

Galectin-3 and/or KRAS silencing influences the cell cycle

The cell cycle of the cell population was analyzed, in order to understand if the decreased proliferation of samples in which *KRAS* or *LGALS3* was silenced was due to block of the cell cycle at any checkpoint.

In *KRAS* silenced cells there was a statistically significant decrease in the percentage of cells in S phase compared to the cells subjected to scramble RNA interference **(Fig. 4A, B)**. This modification in the distribution of the cells throughout the cell cycle may account for a lower proliferation rate, and therefore for the lower number of cells. Indeed, the cells may be blocked at the G1-S checkpoint, delaying a correct DNA replication. Moreover, the *KRAS* silenced cells presented a higher percentage of sub-G1 cells (12.3±9.0%) compared to both the negative control and the siRNA scramble control, confirming the increased induction of cell death observed by trypan blue and Annexin V/PI analysis. The changes in the cell cycle of the other cell populations (siRNA *LGALS3* and siRNA *LGALS3*+*KRAS*) were not as evident. However, a pattern similar to the one obtained with the previous experiments was observed. The percentage of cells in sub-G1 for the population of siRNA *LGALS3* was slightly higher than the siRNA scramble (7.6±3.5% vs. 5.0±1.5%) and in the cells subjected to double silencing it was lower than in cells silenced with *KRAS* and *LGALS3* alone (6.5±2.2%). The number of cells in S phase did not change significantly from the siRNA scramble control in any of the two populations (siRNA *LGALS3* and siRNA *LGALS3*+*KRAS*), highlighting the severity of *KRAS* single silencing effect on cell behavior compared to the other two conditions tested.

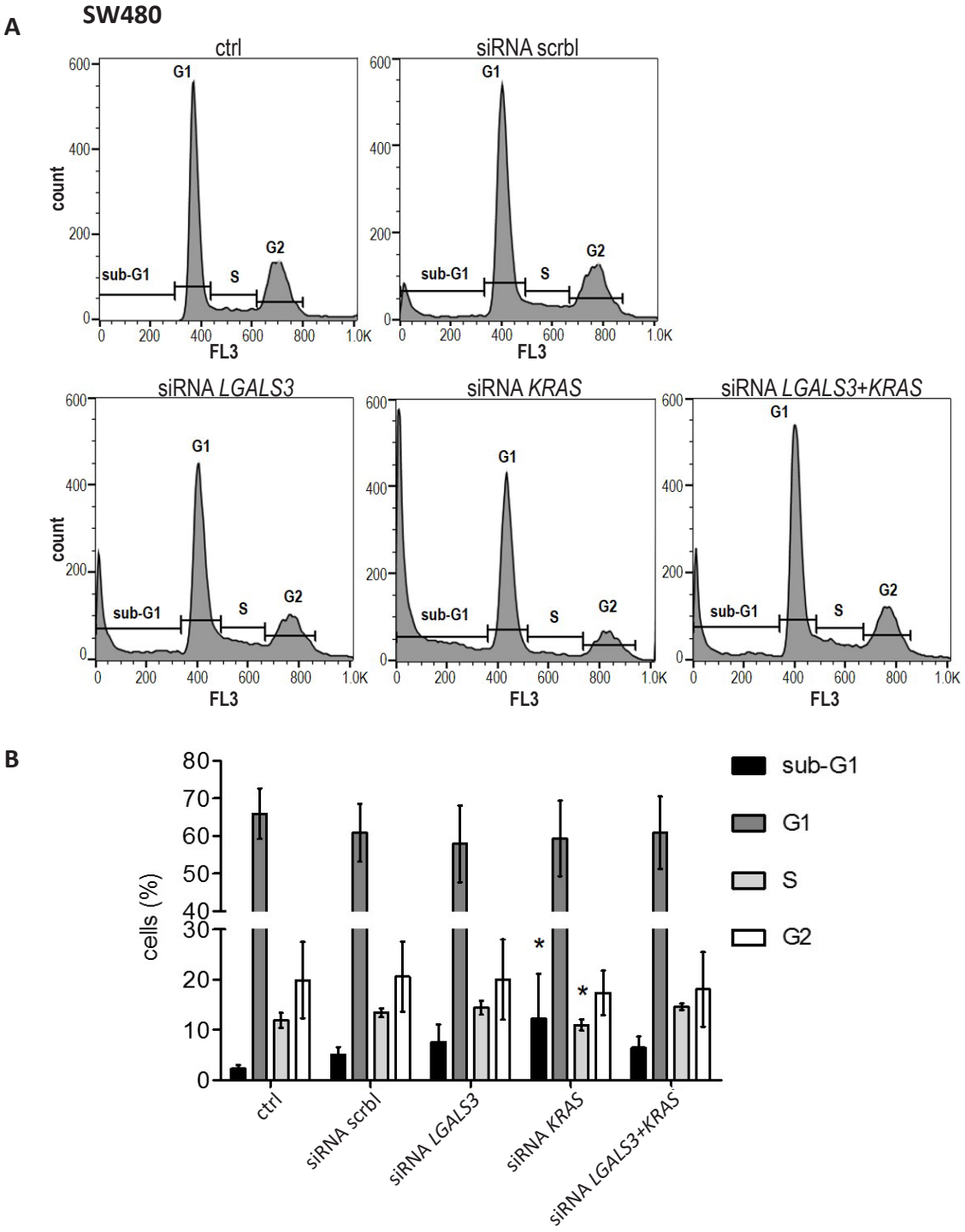


Figure 4: *Cell cycle analysis*. The distribution throughout the cell cycle phase of the SW480 cells subjected to siRNA was evaluated by PI staining measured by cytometer. The cells were subdivided into four different categories according to their DNA content: sub-G1, representing debris or apoptotic cells, G1, S and G2. A representative image of the cell cycle in the differently treated samples shows the different distribution in the cycle phases **(A)**. The percentage of cells in each phase was measured and the average ±SD of 5 independent experiments was plotted **(B)**. Statistically significant differences between the cells undergone scramble siRNA and *LGALS3* and/or *KRAS* siRNA are shown with * (p-value ≤ 0.05).

Galectin-3, KRAS and p16^{INK4a} interact physically in vitro and localize in the same cell compartment

Since the previous results strongly indicate a reciprocal regulation and a connection between the three proteins in analysis, it was evaluated if they have a physical interaction. A co-immunoprecipitation assay was therefore performed. The three proteins were immunoprecipitated with their specific antibody and subjected to western blot analysis using anti-KRAS **(Fig. 5A)** and anti-gal-3 antibodies **(Fig. 5B)**. It was shown that both KRAS and gal-3 interact *in vitro* with each other and with p16^{INK4a}.

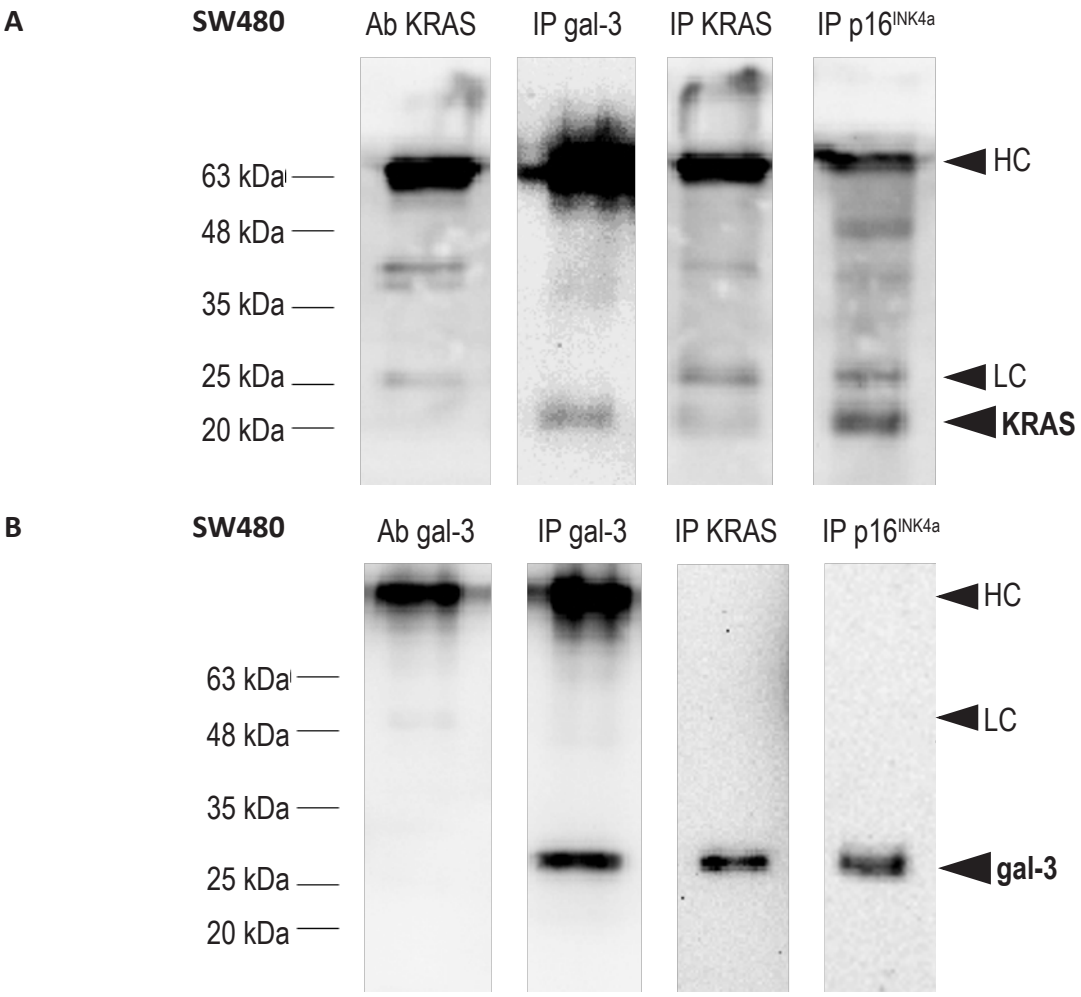
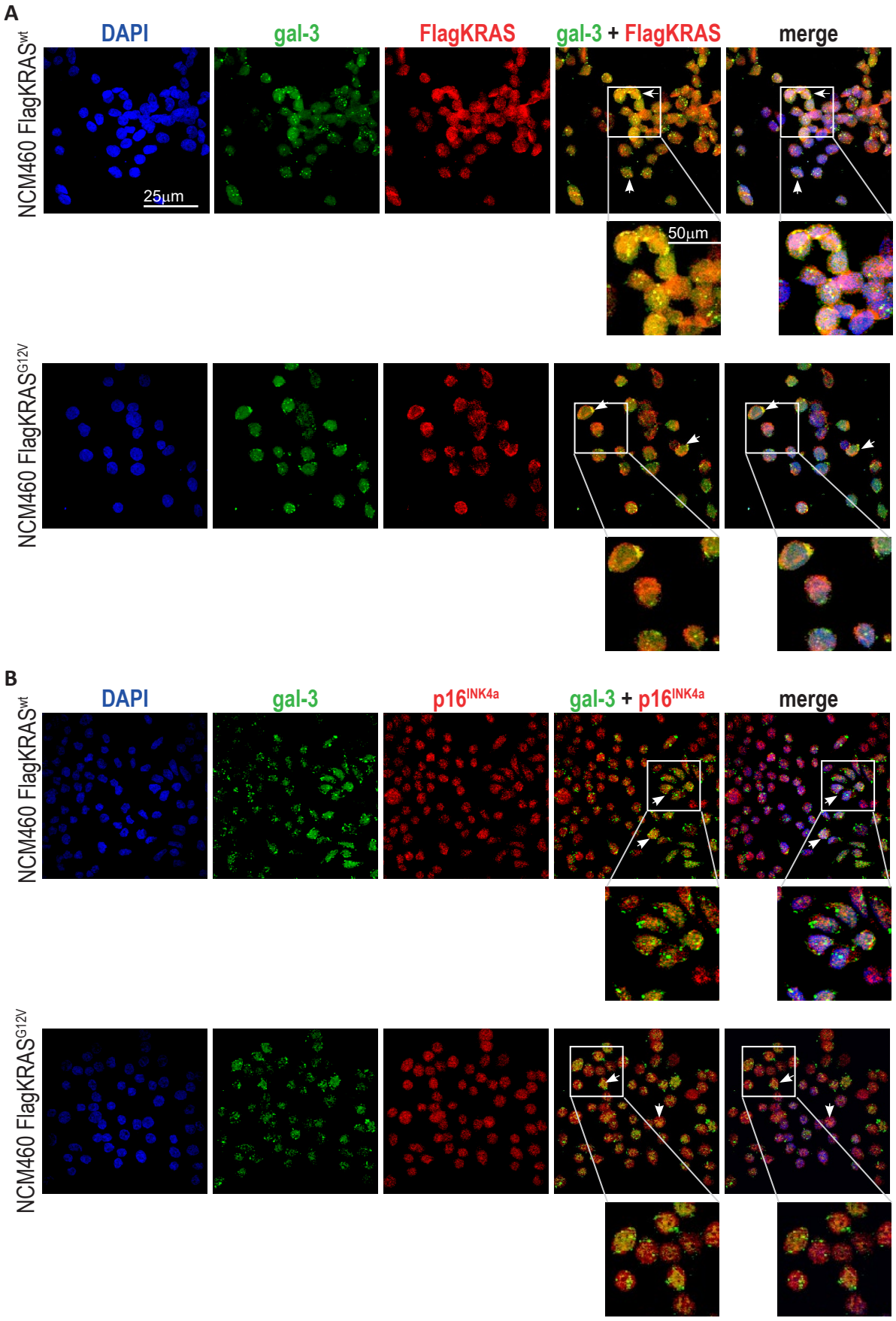


Figure 5: *Co-immunoprecipitation.* Protein extracts from SW480 were immunoprecipitated using anti-KRAS, anti-gal-3 and anti-p16^{INK4a} antibodies. The elutes obtained were used to perform Western Blot and proteins co-immunoprecipitation was detected using anti-KRAS (**A**) and anti-galectin-3 (**B**) antibodies. The elutes were not run in the same gel, thus the lanes were separated. The images show a representative result of two independent experiments.

In order to determine if this result has a significance in the co-localization of the proteins in the cells, the localization of the proteins was determined by immunofluorescence. First, the localization of each protein was evaluated alone. The cell line used for these experiments were NCM460 transfected with either Flag-tagged KRAS^{wt} or KRAS^{G12V}. Gal-3 had a ubiquitous localization throughout the cell and it was often found grouped, forming large agglomerates and not uniformly spread. These agglomerates may represent clusters or vesicles containing gal-3. KRAS was mainly found in the cytoplasm, but in some cases it seemed to localize in the nucleus as well. In the case of cells transfected with KRAS^{G12V}, a higher amount of KRAS protein was concentrated in the plasma membrane area compared to KRAS^{wt}. p16^{INK4a} was mainly found in the nucleus, uniformly spread, however, smaller amount of p16^{INK4a} was also localized in the cytoplasm. Due to the heterogeneous localization of the proteins in different cells and even inside the same cell, the evaluation of the co-localization was not always clear. KRAS and gal-3 signals were found overlapping in some of the cells in analysis. The overlapping was more intense in the case of cells transfected with KRAS^{wt} compared to the one transfected with KRAS^{G12V}. The highest percentage of co-localization was observed in the nucleus, but a smaller amount was detected in the cytoplasm as well. In some cases, the evaluation was made more difficult by the clustering of gal-3 proteins **(Fig. 6A)**. The same happened when evaluating the co-localization of gal-3 and p16^{INK4a} and it was possible to detect some signals overlapping, especially inside the nucleus (**Fig. 6B)**. Due to the different localization of p16^{INK4a} and KRAS, these two proteins were not found to co-localize in the majority of the cells analyzed. Some minor overlapping was detected in the nucleus of cells transfected with KRAS^{wt} and, to a lesser extent, in the cytoplasm of cells transfected with KRAS^{G12V} **(Fig. 6C)**.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DAPI
gal-3
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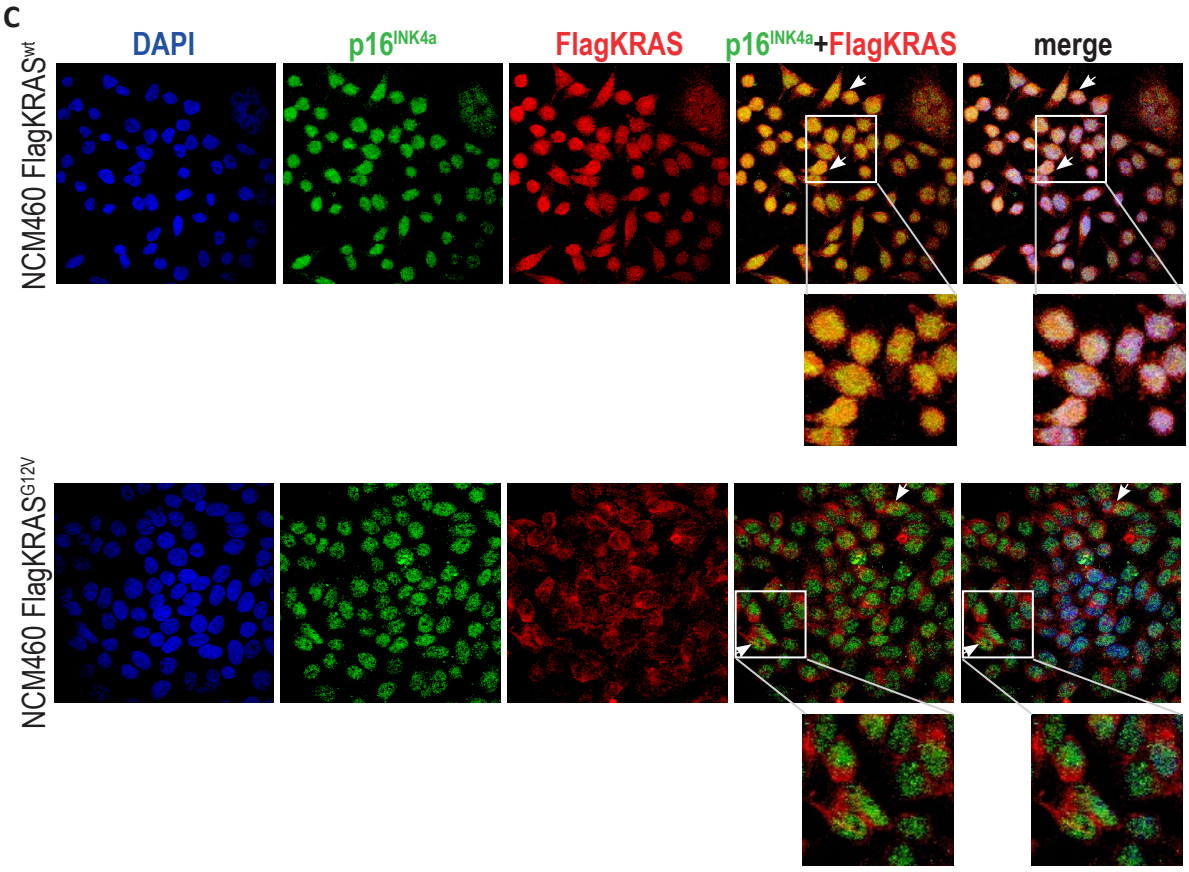

Figure 6: *Immunofluorescence*. NCM460 FlagKRAS^{wt} and NCM460 FlagKRAS^{G12V} PFA-fixed cells were treated simultaneously with two primary antibodies and two correspondent secondary antibodies with a florescent tag. Gal-3 (green) and FlagKRAS (red) co-localization is shown in (**A**), gal-3 (green) and p16^{INK4a} (red) in (**B**) and FlagKRAS (green) and p16^{INK4a} (red) in (**C**). Cell nuclei were stained by DAPI. The immunofluorescence photomicrographs were obtained using Olympus FV1000 laser scanning microscope with the appropriate filter settings and analyzed using the Olympus Fluoview software. All the images are represented at the same amplification (60x). A 200% enlargement is shown for each microphotograph.

Effect of newly synthetized galectins inhibitors in cell proliferation

In order to better understand the role of gal-3 in the cells, and use it as a possible target in CRC cells, two different newly synthesized galectins inhibitors were used and their effects on cell growth was evaluated by SRB assay. These inhibitors were never tested before in cell cultures and their effect on cell survival was not known. However, analog compounds were verified for binding capability to gal-3 and the IC_{50} value for binding inhibition of gal-3 to the appropriate substrate was estimated in the range of 1 to 10 μM (Sorme *et al.*, 2002). The

inhibitors were used at a final concentration between 1 and 1000 µM and incubated with the cells for 48 h. Both the inhibitors caused an effect on cell survival only at high concentrations. In particular, MBS4Flac reduced cell survival by 30% at 1000 µM **(Fig.7A)**, while MBS5Flac provoked a slight decrease (10%) when used at 100 µM **(Fig. 7B)**. Although we have not analyzed the levels or activity of gal-3, these results suggested that gal-3 inhibition in the cells using gal-3 inhibitors reduced only moderately cell survival, as we also observed for *LGALS3* siRNA. Moreover, the results showed that inhibitors with a good IC_{50} for chemical binding activity may not work *in vitro* in cells at comparable concentrations, thus we might have to test higher concentrations.

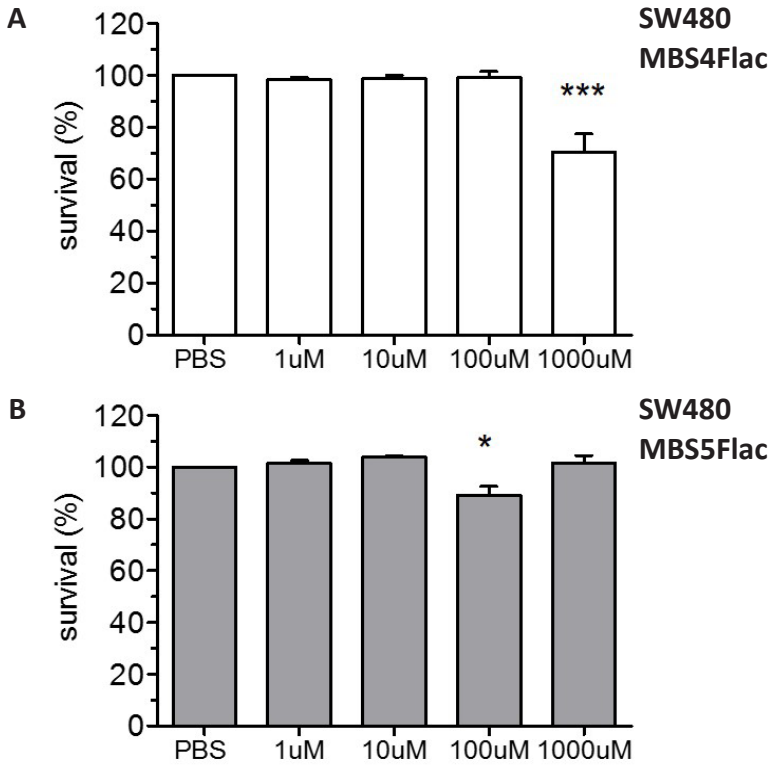

Figure 7: *Survival of cells treated with new galectins inhibitors*. Cells were treated for 48 h with two different galectins inhibitors, MBS4Flac and MBS5Flac and their survival was measured by sulphorhodamine B assay. The treatment was performed in parallel with a negative control composed by the buffer in which the inhibitors were dissolved (PBS 1x). The survival was calculated as percentage to the negative control, where the negative control constituted 100% of survival. Four different concentration of inhibitors were used. Bars represent the average of survival percentage after MBS4Flac **(A)** and MBS5Flac **(B)** treatment. Values are average ± SD of 3 independent experiments. Statistically significant differences between the negative control and the treated culture are shown: * (p-value ≤ 0.05) and *** (p-value ≤ 0.001).

Discussion

In this work we showed for the first time that gal-3, KRAS and p16^{INK4a} have a strong interaction in a colorectal cancer cell lines model, modulating reciprocally their protein levels. Moreover, we demonstrated that this association might be exerted by a physical binding, since we showed by co-immunoprecipitation assay that the three proteins physically interact and by immunofluorescence that they frequently co-localized in "normal" colon cells. A relationship between gal-3, KRAS and p16^{INK4a} has already been shown, even if in different models. In particular, there are several proves of interaction between RAS proteins and galectins (Elad-Sfadia *et al.*, 2004; Shalom-Feuerstein *et al.*, 2005; Ashery *et al.*, 2006; Shalom-Feuerstein *et al.*, 2008; Abankwa *et al.*, 2010; Bhagatji *et al.*, 2010; Levy *et al.*, 2010; Levy *et al.*, 2011; Song *et al.*, 2012; Wu *et al.*, 2013) and between RAS proteins and p16^{INK4a}(Serrano *et al.*, 1995; Serrano *et al.*, 1996; Serrano *et al.*, 1997; Lee and Bar-Sagi, 2010; Rabien *et al.*, 2012), but, as far as we are aware, not in the colorectal cancer model. Moreover, the axis regulation of these three proteins all together was never investigated in the colorectal cancer or in any other model. The interaction between KRAS and gal-3 is widely known. Gal-3 has been demonstrated to stabilize KRAS nanoclusters (Ashery *et al.*, 2006; Shalom-Feuerstein *et al.*, 2008; Abankwa *et al.*, 2010; Bhagatji *et al.*, 2010) and this interaction has been shown to promote cancer progression in different model, such as pancreatic (Song *et al.*, 2012), thyroid (Levy *et al.*, 2010), breast (Shalom-Feuerstein *et al.*, 2005; Shalom-Feuerstein *et al.*, 2008) and colorectal (Wu *et al.*, 2013) cancers. Moreover, gal-3 seems to be relevant to promote both the activity and the expression of KRAS (Levy *et al.*, 2010). Here we showed that KRAS and gal-3 regulate their reciprocal protein expression in colorectal cancer cells in an opposite manner. When the expression of KRAS was downregulated, gal-3 protein levels decreased as well, while when gal-3 was silenced, the levels of KRAS increased, suggesting a regulation of the two proteins. Indeed, if the role of gal-3 is stabilizing KRAS nanoclusters, we could hypothesize that, when low levels of KRAS are present, the cell may need less gal-3 to stabilize KRAS. Therefore, there should be a feedback loop mechanism that signals the amount of gal-3 needed to stabilize the amount of KRAS present at the membrane nanoclusters. Data

present in the literature suggest that a regulatory mechanism of reciprocal protein expression between gal-3 and KRAS exists. Several evidences reported that cell lines that express a high level of KRAS always express high level of gal-3 (Elad-Sfadia *et al.*, 2004; Shalom-Feuerstein *et al.*, 2005; Shalom-Feuerstein *et al.*, 2008; Levy *et al.*, 2010), whereas downregulation of gal-3 caused a reduced amount of active, but not a decrease level of KRAS protein (Song *et al.*, 2012). On the other hand, if less gal-3 is available to stabilize KRAS nanoclusters, it is plausible to suggest that a higher amount of KRAS might be necessary to mediate KRAS signalling. It is indeed known that the efficiency of KRAS signalling and the access to its downstream effectors is related to its proper orientation on the plasma membrane and to KRAS organization in nanoclusters (Omerovic *et al.*, 2007; Bhagatji *et al.*, 2010). Therefore, KRAS signalling is not as efficient when gal-3 is not at the optimal level and to compensate for it, KRAS expression may be upregulated. RAS proteins and $p16^{INK4a}$ are involved in the regulation of cell cycle in an opposite manner (Mittnacht *et al.*, 1997; Peeper *et al.*, 1997; Pruitt and Der, 2001; Coleman *et al.*, 2004). A direct antagonistic relationship between $p16^{INK4a}$ and HRAS was proved by Serrano and collaborators both in tumour progression and senescence induction. Indeed, they demonstrated that HRAS effect on cancer progression is greatly increased in the absence of $p16^{INK4a}$ (Serrano *et al.*, 1995; Serrano *et al.*, 1996) and that senescence caused by over-activity of HRAS is necessarily mediated by $p16^{INK4a}$ (Serrano *et al.*, 1997). KRAS was shown to decrease $p16^{INK4a}$ protein levels (Lee and Bar-Sagi, 2010) and $p16^{INK4a}$ was found to decrease KRAS protein functionality by reducing the protein level. Indeed, when functional $p16^{INK4a}$ was reinstated in cells with mutant $p16^{INK4a}$, KRAS protein levels were reduced, without alterations in the transcription rate or in the mRNA stability, suggesting an inhibitory mechanism of $p16^{INK4a}$ that acts at the protein level (Rabien *et al.*, 2012). When we silenced *KRAS* or *LGALS3*, we observed a slight decreased in $p16^{INK4a}$ protein levels, that reached the maximum level when both KRAS and gal-3 were downregulated at the same time. This could be explained if we consider the antagonistic roles of KRAS/gal-3 and $p16^{INK4a}$. When both *LGALS3* and *KRAS* are silenced, the pro-survival signal that they transduce is probably lower compared to normal level, therefore the tumour suppressor and inhibitor $p16^{INK4a}$ is not needed and its level might be reduced in order to maintain a balanced

equilibrium between cell cycle progression and blockage in the cancer cell.

To further confirm the reciprocal regulation of the three proteins, we aimed to uncover if they could physically interact by performing co-immunoprecipitation assay for gal-3 or KRAS. We showed that KRAS and gal-3 can co-immunoprecipitate with each other and they can both co-immunoprecipitate with p16^{INK4a}. These results are in accordance to our previous results and suggest that the reciprocal protein level regulation might be explained by a physical interaction between these proteins. This may be a sensing mechanism to detect gal-3, KRAS and p16^{INK4a} reciprocal protein levels and to signal them to the cells, in order to maintain the balance between oncogenes and tumour suppressor genes, by adjusting the levels of proteins in case of physiological variations from the normal state. However, co-immunoprecipitation assay only gives information about the possible biochemical interaction of proteins. The intracellular localization of the three proteins is known to be slightly different in most of the cases and this may raise some doubts about the actual interaction of the proteins in the cell. KRAS is a cytoplasmic protein that needs to translocate in the inner part of the plasma membrane in order to be activated. RAS proteins associations with the membranes of intracellular organelles, such as ER and Golgi, have also been found (Plowman and Hancock, 2005; Omerovic *et al.*, 2007; Prior and Hancock, 2012). However, RAS proteins have occasionally been found also in other localization, such as mitochondria (Bivona *et al.*, 2006; Wolfman *et al.*, 2006) and nucleus (Wurzer *et al.*, 2001; Birchenall-Roberts *et al.*, 2006; Jeong *et al.*, 2006; Fuentes-Calvo *et al.*, 2010). Gal-3 is mostly expressed in the cytoplasm and nucleus (Wang *et al.*, 2004; Haudek *et al.*, 2010), but it is expected to localize in the inner side of plasma membrane when interacting with KRAS. p16^{INK4a} main activity is performed in the nucleus, but it is hypothesized to found its partners, the CDKs, in the cytoplasm before they can form an active complex (Guan *et al.*, 1996; Reynisdottir and Massague, 1997). Considering the reported localization of the proteins, we hypothesised if KRAS and gal-3 co-localize at the inner leaflet of the plasma membrane and if p16^{INK4a} and gal-3 co-localize in the nucleus. We observed co-localization of gal-3 and KRAS in the cytoplasm, with gal-3 organized probably in multimers, since the fluorescent signal was grouped into distinct dots, but, surprisingly, both proteins were found in the nucleus as well and often co-

localizing. In the case of $p16^{INK4a}$, the co-localization with gal-3 happened less frequently and in most occasions in the nucleus. Interestingly, also KRAS and $p16^{INK4a}$ seemed to co-localize in the nucleus, especially in cells with $KRAS^{wt}$. The most difficult event to interpret is the co-localization of KRAS and $p16^{INK4a}$. Cells transfected with oncogenic $KRAS^{G12V}$ presented a much higher amount of plasma membrane associated KRAS, suggesting a constantly active state of mutated KRAS, therefore allowing a less frequent co-localization with $p16^{INK4a}$ in the nucleus. Taken all together, the results show that the three proteins seemed to have a shared localization in the nucleus, especially when expressing $KRAS^{wt}$, where at least a portion of KRAS is not active and therefore free to be in localizations other than the inner plasma membrane. This somehow unusual localization of KRAS may be caused by the Flag-construct plasmid used to overexpress KRAS in our "normal" colon model cells. Indeed, we used cells transfected with either $KRAS^{wt}$ or $KRAS^{G12V}$, whose protein products are overexpressed in cells that already harbour endogenous wild type KRAS. The total amount of KRAS protein resulted in excess compared to physiological levels and this may be the reason why a percentage of the protein was found in less usual cellular compartments, such as the nuclei. On the other hand, the three proteins may actually interact at some level in the nucleus and this interaction m ay regulate their reciprocal protein expression levels. The fact that the proteins are able to physically interact provide support for this hypothesis. It remained to be unclosed if this regulation happens directly at the protein level, or rather at the translational or transcriptional level.

We also confirmed the effect that KRAS and gal-3 have on cancer progression. Indeed, their silencing decreases cell proliferation and slightly increases cell death. However, we demonstrated that the double silencing of the two proteins does not sum up the effects of the single silencing and not only it does not significantly increase cell death, but also seems to revert the effect of the silencing of *KRAS* alone.

We further analysed the cellular effects of *KRAS* and/or *LGALS3* silencing on cancer cell. The notion that KRAS, gal-3 and $p16^{INK4a}$ seem to be so finely regulated in colorectal cancer cells may be due to their relevance for cancer cell progression. As expected, in all the assays performed, which measured cell viability and cell death induction, the silencing of *KRAS*

always caused the most dramatic effects, while the silencing of *LGALS3* induced only mild decrease in viability. This is probably due to the fact that KRAS has the most central role in cell proliferation (Downward, 2003; Rajalingam *et al.*, 2007), cell cycle progression (Pruitt and Der, 2001; Coleman *et al.*, 2004), autophagy regulation (Furuta *et al.*, 2004; Elgendy *et al.*, 2011; Alves *et al.*, 2015) and escape from death (Downward, 1998; Cox and Der, 2003), while gal-3 has an accessory role in relationship to KRAS (Shalom-Feuerstein *et al.*, 2005; Ashery *et al.*, 2006; Shalom-Feuerstein *et al.*, 2008; Abankwa *et al.*, 2010). Interestingly, the double silencing of the two proteins never triggered a sum of effects. In most of the assays performed, the double silencing actually caused the effects of KRAS silencing to be reverted. Taking into consideration the fine regulation of the two proteins, it may be hypothesized that the cell might use a different pro-survival pathway and bypass the KRAS/gal-3/p16^{INK4a} axis regulation when KRAS and gal-3 pro-survival activity is below a certain threshold. This may not happen when only one protein is silenced, because the cells try to compensate modulating the other proteins levels and because the lack of functionality is not enough to severely compromise the cell balance.

The effects observed in all the assays, cell proliferation, viability and cell death induction, were never as severe as expected considering the pivotal roles of the proteins silenced. This was somehow surprising, but it can be explained by several factors. First of all, the silencing of the proteins never reached 100% of effectiveness and varied between experiments. The residual level of proteins was probably enough to provide a survival mechanism and the lack of a percentage of protein, both KRAS and gal-3, only caused a mild effect. Second, this may be a further prove of the accurate mechanism of regulation that the cells have to maintain their survival. They compensate the loss of one protein by up- or downregulating the others, thus keeping the balance constant. In case both proteins were loss, the cells probably bypass their pathway, choosing a different way to maintain its viability. The decrease in number of cells when KRAS or gal-3 were silenced was more evident compared to loss of viability in the same conditions, detected in a statistically significant way only by trypan blue assay and not by Annexin V/PI. It seems that the silencing of *KRAS* or *LGALS3* stopped the replication of the cells at an earlier stage during the recovery time, but did not altered significantly the

viability of the cells that were able to proliferate and grow. Moreover, the different results obtained with the two viability assays may indicate that the loss of viability encountered is mainly due to late apoptosis or necrosis. In our cellular model the lack of KRAS most likely triggers blockage in proliferation, evidenced by a decreased cell number after 48 h. The reduced number of cells that we observed could be caused by either a massive arrest at G1/S restriction point due to the lack of KRAS or to an early death. Cell cycle analysis showed that a slight decrease in the number of cell in S phase occurred when *KRAS* was silenced, but was not accompanied by a significant increase in the G1 phase. However, a higher percentage of cells were detected in sub-G1, indicating a higher number of cells debris associated with cell death. These results indicate that the reduction in cell number at 48 h was most probably due to an early induction of apoptosis and not to the arrest of the cell cycle. It seems conceivable that the cells die earlier than 48 h, having the time to be almost completely degraded. A support for this hypothesis is the fact that much less cells are detected at 48 h, with no significant increase of apoptosis, and that the unviable cells found at 48 h mostly are in the late phases of death process. In order to be able to confirm this hypothesis, further work would be necessary, focused on shorter timing after protein silencing induction.

Finally, here we showed that the usage of new galectins inhibitors in cancer cells may be a starting point or a co-adjuvant, but it does not seem to be enough for effective cell death induction, at least at low concentrations. Recently it has been proven that a gal-1 inhibitor used together with the more common anti-cancer agent rapamycin ablated almost completely the HRAS-driven tumour growth (Michael *et al.*, 2016). Taking into consideration these findings and the similarity between gal-1/HRAS and gal-3/KRAS interactions (Paz *et al.*, 2001; Elad-Sfadia *et al.*, 2002), we tested for the first time novel gal-3 inhibitors in a cell culture context. We observed that gal-3 has an effect on cell proliferation, even if not as significant as the one caused by KRAS. However, we may hypothesise that the effectiveness of galectin inhibitors may be higher than siRNA silencing, since we observed a significant, even if not massive, reduction of cell viability when cells are treated with galectins inhibitors, but not when gal-3 is silenced by siRNA. Very few is known about these inhibitors, since they have never been tested on cell cultures. Similar inhibitors were only tested in *in vitro*

assays, to determine the binding specificity and affinity (Sorme *et al.*, 2002; Sorme *et al.*, 2005). We therefore performed a preliminary assay to test the possible cytotoxicity of these compounds. We used several concentrations, in order to determine the most effective one. The results of the viability assay show a slight decrease in viability only when using the highest concentrations, in the order of 100-1000 µM, in contrast with the established binding IC_{50} value that ranges in the order of 1-10 µM (Sorme *et al.*, 2002). The low efficacy of these compounds may be due to several reasons. First of all, as we observed in our cellular model, the lack of gal-3 did not cause dramatic changes in cell proliferation and viability. Furthermore, the inhibitors used were not strictly specific for gal-3, thus they may bind to other galectins as well, decreasing the specific effect on gal-3. Finally, we have to consider that there are significant differences between a cell model context, in which multiple partners interact and relate to a changing environment, and a binding assay in which only gal-3 and substrate are present. However, this result must not discourage, as it shows for the first time that gal-3 has the potential to inhibit, at least partially, cell proliferation, with the consistent advantage to be dissolved in a non-toxic buffer for normal cells. They may be further developed to optimize binding ability and concentration and they might be thought as co-adjuvant in stopping cancer cell proliferation. We suggest the improvement of the galectin inhibitors here tested for the first time, and the optimization of their concentration, to reach a better level of cell proliferation inhibition, in order to study their usage as co-adjuvant in the treatment of colorectal carcinoma.

In conclusion, with this work we provide a first insight into a novel axis regulation in colorectal cancer cell, specifically involving gal-3, KRAS and p16^{INK4a} (**Fig. 8**). These three proteins seem to regulate their reciprocal protein levels to maintain the equilibrium between oncogenes and tumour suppressor genes, in order to ensure cell survival. We hypothesize that the reciprocal regulation between them may occur by direct physical interaction, since gal-3, KRAS and p16^{INK4a} were found to co-localize and to physically interact by co-immunoprecipitation. We further proved the pivotal role of KRAS and gal-3 for cell survival, since their silencing caused a decreased in cell number and an increased in cell death. Interestingly, we showed that the simultaneous silencing of the two proteins does not augment the pro-apoptotic effect of

the single silencing, but, contrarily, it seems to slightly revert it. We therefore suggest that when both proteins are silenced, the cell might prefer an alternative pro-survival pathway, which allows to bypass KRAS/gal-3/p16^{INK4a} axis to ensure proliferation. We showed that the silencing or inhibition of these two proteins may not be enough to stop dramatically cell proliferation and induce complete cell death, probably because of the fine modulation mechanism of the level of the proteins that ensure the proper balance between pro- and anti-survival signals and, in case of failure of this mechanism, a second system that does not rely exclusively on the gal-3/KRAS/p16^{INK4a} axis will act.

Therefore, this study suggests that many factors must be taken into account when considering the development of new treatment strategies for CRC. In particular, this work evidences that it is often not enough to focus on a single protein, but that it is necessary to consider signalling pathways, and the cell, as a whole. Moreover, this research gives strong indications on the fact that the simultaneous inhibition of proteins involved in cancer progression might not always be a good strategy, since the cell may develop resistance by feedback loops in response to the protein downregulation, thus bypassing the inhibited proliferation pathway by choosing/activating a different one.

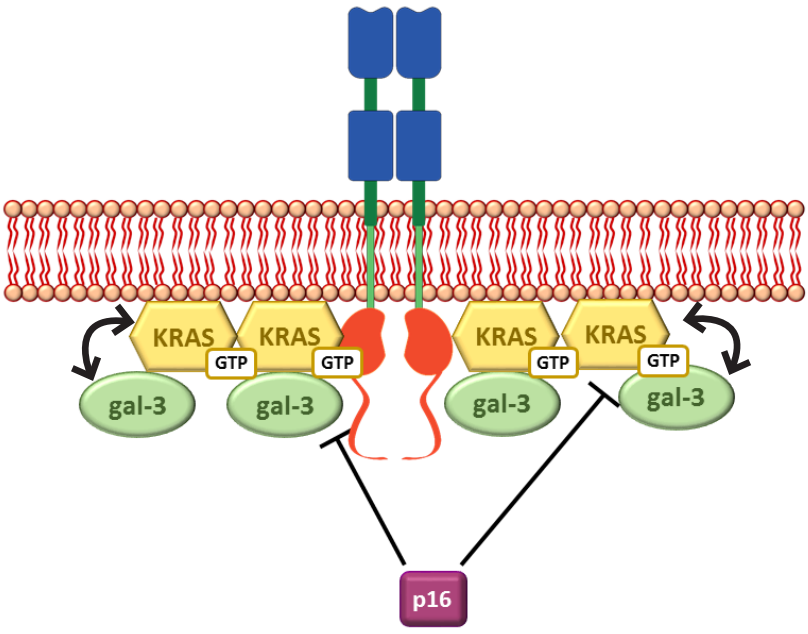

Figure 8: *proposed model of KRAS/galectin-3/p16^{INK4a} axis regulation in CRC.* The hypothesized model of KRAS/gal-3/p16^{INK4a} axis regulation envisages a reciprocal regulation of protein level and stabilization of position between KRAS and gal-3, which are in turn inhibited by p16^{INK4a}.

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Final Conclusion and Future Perspectives

The work hereby developed encompasses some of the goals and objectives defined within the Glycopharm ITN network. This included diverse expertise (on structural chemistry, pharmacology, biomedicine and molecular biology) to assess several aspects of the biology of galectins, in particular galectin-3. The central aim of this work was to build a set of yeast strains that could be further used as a platform for pharmacological drug screening. The knowledge in regard to galectins in yeasts was almost inexistent, since these organisms do not naturally express galectins, so the work had to begin from zero. The effects of exogenously added galectin-3 on yeast were assessed and compared with the effects of expressing and accumulating gal-3 intracellularly. These were further compared with the effects caused by other galectins representing other structural groups. This was done using in parallel two very distinct yeast models, *S. cerevisiae* and *C. albicans*, as well as mutants defective in the yeast Ras pathway, known to govern cell growth and proliferation in animal cells, with the specific contribution of gal-3. Concurrently, human KRAS was identically expressed in yeast, as a step towards the future co-expression of gal-3 and KRAS. Furthermore, the relation between gal-3, KRAS and $p16^{INK4a}$, fundamental proteins in the oncogenic processes, was assessed using colorectal carcinoma cells as model. All taken, the work answered to some major questions, as presented below, while providing the yeast platform of six yeast strains humanized through the expression of either human gal-3 or KRAS.

One comment must go to the methodologies used in the present work. Galectins are not available in very high amounts (neither from personal or commercial origin). Therefore, the use of these proteins to perform regular yeast protocols demanded for their extreme miniaturization, up to the microliter scale. Being aware that downsizing a protocol does not necessarily ensures its success, and though this is not described in the thesis, the effectiveness and reproducibility of each protocol was tested before using galectins. Moreover, the cell surface microarrays had never been attempted before with yeasts whole cells. They were created and optimized for bacteria use. The optimization of the arrays protocols for yeasts was also done within the scope of the thesis. The success of these methodologies was the foundation for all the work presented.

Does yeast recognize and react to the external presence of human galectins? Are the effects caused by exogenous galectins mediated by galectin binding to yeast cells?

Various galectins have a well-established role in recognizing glycans on the surface of several different microorganisms, including viruses, bacteria and parasites. The recognition by galectins of specific glycans present on the cell surface can trigger different cellular responses, such as the onset of the immune defence by the host, the cell adhesion and fusion by the invading organism and the direct clearance of the pathogen (Sato *et al.*, 2009; Vasta, 2009; Vasta *et al.*, 2012; Baum *et al.*, 2014). Among the various microorganisms investigated, pathogenic fungi such as *Candida albicans* never had a prominent role. Very few studies report the ability of galectins to bind to the surface of this pathogenic yeast. In particular, only gal-3 was identified to bind to *C. albicans* β-1,2-linked oligomannosides, causing morphological changes and mortality (Fradin *et al.*, 2000; Jouault *et al.*, 2006; Kohatsu *et al.*, 2006). Since galectins bind with highest affinity to galactosides, which are supposedly absent from yeast cell wall, it was difficult to hypothesize recognition in spite of the reported phenotypes.

In this work, the analysis of galectins binding to yeasts was expanded, exposing yeast to at least one galectin from each structural subgroup (gal-3, the only member of the *chimera* type group, gal-4 from the *tandem repeat* group, and gal-7 and gal-1 from the *proto-type* group), and comparing their effects on the pathogenic model yeast *C. albicans* and on the non-pathogenic model yeast *S. cerevisiae*. Additionally, the binding ability of the same galectins to the surface of the yeast cells was investigated, in order to confirm that direct binding of the galectins to the yeast cell surface occurs.

Three galectins were able to cause intracellular effects on yeasts and to bind to their surface, gal-3, gal-4 and gal-7. Gal-1 did not trigger any recognizable response in either *C. albicans* or *S. cerevisiae*, nor did it bind to yeast cell surface. This difference suggests that each galectin behaves differently and specifically in regard to binding and subsequent effects. Concurrently, the three galectins showed different binding pattern and induced

different responses in each yeast species. Moreover, in general both binding and cellular effects were more evident in *S. cerevisiae* than in *C. albicans*, in opposition to the only two previous reports available (Jouault *et al.*, 2006; Kohatsu *et al.*, 2006).This could be due to the different genetic backgrounds of *S. cerevisiae* and *C. albicans,* as well as to the very different experimental conditions and physiological state of the cells described by those authors. Both strongly impact in the molecular composition of the cell wall and membrane.

Gal-3, gal-4 and gal-7 caused a specific combination of effects in *S. cerevisiae*. Gal-3 induced a stress response characterized by cell enlargement, DNA alterations and ROS accumulation that ultimately led to decreased viability. Gal-4 and gal-7 caused similar effects, namely ROS accumulation and plasma membrane rupture. The three galectins bound to the *S. cerevisiae* concentrated cell wall and membrane fractions, though with different intensity. Different binding ability could implicate the activation of different responses from the cells, *i.e*. different signalling pathways, or at least different intensity of response/pathway activation.Gal-4 and gal-7 showed binding to yeast whole cells. This could mean that gal-4 and gal-7 could find more binding sites/ligands on yeast surface and therefore yielding a more significant binding, detectable also when ligand samples were less concentrated, as when using whole cells. The different binding ability can additionally be caused by different ligands typology, since galectins are sensitive to the organization of glycans presentation on the cell surface (Gabius *et al.*, 2011; Andre *et al.*, 2015) and clusters of glycans are differently recognized by different galectins. Moreover, each type of galectin has a different ability to multimerize, depending on the number of CRDs and linker peptides, and that leads to different affinity for the ligands and the consequently a different duration of the binding (Gabius *et al.*, 2011; Andre *et al.*, 2015). All these factors contribute to make both the galectins binding to the yeast cells and the effects caused in the cell extremely differentiated and specific.

Intracellular components also greatly contribute to determine the final response of the cell to exogenous galectins Namely, the correspondent response signalling pathways must be fully functional. The outcome of signalling greatly depends on the integrity and molecular composition of the cell outer structures, membrane and wall, with inevitable consequences in recognition by extracellular effectors. That is the case of deleting the *RAS2* isogene in

S. cerevisiae. The *Δras2* mutant was the yeast strain showing the biggest binding ability for all the galectins, either using sub-cellular fractions or yeast whole cells. However, the exposure of this mutant to exogenous gal-3. Other galectins were not tested with *Δras2* did not yield any significant effect. This means that the interruption of the Ras pathway causes rearrangements of the yeast cell surface that improve the cell affinity for galectins, but, on the other side, that the Ras2 protein is needed to activate the specific stress response induced by gal-3. As mentioned above, the cell surface changes are not necessarily in composition, they can simply be glycans rearrangements. Finally, it cannot be excluded the hypothesis that in the *Δras2* mutant gal-3 might activate a different pathway, not causing a stress response identical to the correspondent wild type strain.

In conclusion, the present work showed that galectins cause a response in yeast cells, more evident in *S. cerevisiae* than in *C. albicans*, and that this response is galectin-specific. Concomitantly, galectins were shown to bind to the yeast cell surface, and also in this case the binding was galectin-specific. Therefore, it was hypothesized that yeast response to galectins may be directly mediated by the galectins binding to the yeast cell surface. Moreover, the necessity of having functional intracellular Ras/cAMP/PKA signalling pathway to mediate gal-3 response in *S. cerevisiae* was showed. This could work by providing the necessary proteins to activate intracellularly the response, or by modulating the yeast surface composition.

Future perspectives

Further work is needed to better understand the effects of galectins on yeasts, namely how the binding occurs. In this regard, the next step could be to further purify the yeast cell surface components, in order to identify the specific ligand of each galectin. As mentioned, yeast cell wall does not present galactosides (Klis *et al.*, 2006; Free, 2013). After showing that microarray technology is suitable for yeast printing, this new tool could be used for that purpose. Additionally, using various other lectins with different and well-established specificity, it could contribute to determine the composition and glycan presentation of yeast cells. This could be particularly useful to discover the differences between pathogenic and

non-pathogenic yeasts, helping in the identification of factors that could be significant for pathogenesis. In addition, future work should be done to better understand the intracellular response of yeast to each galectin. Specifically, it would be very informative to measure the activity of Ras/cAMP/PKA pathway by determining the intracellular concentrations of cAMP. Testing other mutants of the Ras and other pathways for the response and binding to gal-3 or other galectins would be useful to further establish if gal-3 causes an hyper- or hypo-activation of the pathway and if other pathways may be involved.

Does the presence of yeast Ras proteins influence the effects of endogenous galectin-3 on yeast?

Similarly to the results obtained when analysing on yeast the effects and binding of exogenous galectins , the functionality of the *S. cerevisiae* Ras pathway influenced the response to the endogenously expressed human gal-3.

Human gal-3 and KRAS were separately expressed in *S. cerevisiae* and basic features of yeast life, such as growth rate, chronological life span and stress response, were analysed. These features, in addition to be useful to determine the impact of human proteins on yeast viability, can also be easily used to test other proteins or compounds that can revert the identified phenotypes, therefore inhibiting the human proteins. This is the conceptual basis behind the construction of the referred platform. Importantly, the human proteins were expressed in wild type strains and in *RAS* pathway deletion mutants, *Δras1* and *Δras2*. When gal-3 was expressed in yeast, the presence or absence of endogenous yeast Ras proteins greatly influenced the outcome of the experiments. Gal-3 expression did not affect growth rate in either *RAS* mutants, but affected growth rate in the wt strain. The other way around, gal-3 only affected CLS in *RAS* mutants but not in the wt strain. Gal-3 has been described in human cells as a partner and regulator of KRAS, mainly directing and stabilizing its membrane specific localization in nanoclusters (Elad-Sfadia *et al.*, 2004; Ashery *et al.*, 2006; Shalom-Feuerstein *et al.*, 2008; Bhagatji *et al.*, 2010). The interaction between KRAS and gal-3 drives cell proliferation and apoptosis escape in different types of cancers, including pancreatic,

colorectal, thyroid and breast cancers (Shalom-Feuerstein *et al.*, 2005; Shalom-Feuerstein *et al.*, 2008; Levy *et al.*, 2011; Song *et al.*, 2012; Wu *et al.*, 2013), having therefore an important clinical role. The results obtained in the present work suggest that in yeasts, similarly to human cells, the endogenous Ras proteins, in particular Ras2, might cooperate with gal-3 in conditioning the survival/death processes.

This work showed for the first time that galectins can be successfully expressed in yeast, even though no galectins orthologue is present in yeast (Kasai and Hirabayashi, 1996; Dodd and Drickamer, 2001), and showed also that the presence of yeast Ras proteins determines the outcome of the results when gal-3 is expressed or simply allowed to interact with yeast extracellularly. This instils hope in the applicability and usefulness of the yeast platform to test compounds able to inhibit gal-3/KRAS interaction.

The further cloning of KRAS in yeast wt and RAS pathway mutants resulted in the absence of complementation of either RAS gene deletion, but showed a hyper-activation of the pathway caused by the extra RAS allele in the transformed wt strain, which implies that the human protein is actually contributing to the pathway flux. These strains are part of the above-mentioned platform and will be controls for the further construction of the yeast strain expressing both human gal-3 and KRAS.

Future perspectives

The logical consequence of the results obtained with this work is to obtain the fully operational platform, expressing both human cDNAs and amenable to simple phenotyping. Presently, the phenotypes that can be used to assess independently the effects of either gal-3 or KRAS in yeast may not be the ideal ones to assess the interaction between the two proteins. The platform will provide a useful tool to test chemical compounds or other proteins able to revert the associated phenotypes, and thereby able to inhibit gal-3 and/or KRAS and the consequent cell proliferation in human cells.

Do the results obtained using S. cerevisiae *as a model translate in higher eukaryotes?*

Our findings in yeast about the relevance of gal-3/Ras relationship for cell behaviour, especially viability and proliferation rate, confirm previous reports on the importance of gal-3/KRAS interaction for the progression of various types of cancers (Shalom-Feuerstein *et al.*, 2005; Shalom-Feuerstein *et al.*, 2008; Levy *et al.*, 2011; Song *et al.*, 2012; Wu *et al.*, 2013; Seguin *et al.*, 2014) and give firmer ground to our findings in colorectal cancer cells.

Similarly to what happened in yeast, the level of KRAS protein in CRC influenced cell behaviour, specifically viability and growth, and, importantly, modulated as well gal-3 protein levels. We indeed identified a reciprocal regulation axis among gal-3, KRAS and a third protein, the tumour suppressor $p16^{INK4a}$. Gal-3 and KRAS seem to modulate their reciprocal protein level, together with the one of $p16^{INK4a}$, adjusting them to the needs of the cell. Namely, when KRAS is silenced, also gal-3 level decreases, while the opposite happens when gal-3 silenced, with KRAS being overexpressed. When both proteins are silenced, also p16INK4a level diminishes. Our hypothesis is that the protein levels adapt to the cancer cell proliferation needs. If few KRAS is available, less gal-3 is necessary to stabilize KRAS nanoclusters, but when low gal-3 is present, a higher amount of KRAS is needed, because when KRAS is not organized in nanoclusters by gal-3 is less functional (Tian *et al.*, 2007). When neither protein is present, less amount of their inhibitor $p16^{INK4a}$ is necessary to maintain the equilibrium between gal-3 and KRAS activation and inhibition.

With our work in human cells, we confirmed the well-established relationship between KRAS and gal-3, with the support of the data obtained in yeast, and we discovered a new partner in this axis regulation, $p16^{INK4a}$. Indeed, in addition to modulating their protein levels, the three protein physically interact and co-localize in cells.

Future perspectives

Further work is needed to confirm the KRAS/gal-3/$p16^{INK4a}$ axis regulation in colorectal cancer, mainly by showing a similar mechanism in other CRC cell lines.

In addition, the effects of KRAS and/or gal-3 silencing on cell proliferation and death should be further investigated. Other important cellular processes in which gal-3 and KRAS are involved, both independently and when interacting, namely cell adhesion and motility, should be analysed when the proteins are silenced.

Important informations could derive by experiments in which the third member of the axis regulation, $p16^{INK4a}$, is silenced, in order to determine if its downregulation causes similar effects on the level of the other two proteins and opposite effects on viability and death, since its putative role is to inhibit gal-3 and KRAS.

Finally, CRC cell line could be a useful model to test the gal-3 and/or KRAS inhibiting compounds that could be identified in the *S. cerevisiae* platform. In CRC cell line we confirmed the importance of both gal-3 and KRAS for proper proliferation and death escape, and we provide important information supporting the notion that these proteins interact and regulate each other. Therefore, the idea would be to first screen a high number of compound using the *S. cerevisiae* platform and then test the best performing ones in CRC cell lines, in order to identify useful drug capable to stop or slow down cancer progression.

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