Universidade do Minho
Escola de Ciências

Marta Sofia Aguiar Carvalho de Oliveira Teixeira

The role of vacuolar membrane proteins in acetic acid-induced cell death

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outubro de 2019

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration. I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

ABSTRACT

Saccharomyces cerevisiae has been one of the most widely used model organism for understanding the molecular mechanisms underlying apoptosis. Apoptosis is a form of regulated cell death that can be triggered by a wide variety of external or internal stimuli, such as acetic acid (AA). This acid triggers, in the yeast, a cascade of intracellular apoptotic-like events, both at mitochondria and vacuole level. A similar process occurs during acetate-induced apoptosis in colorectal cancer (CRC) cells. Although not much is known about the role of vacuole/lysosome, their membrane permeabilization (VMP/LMP) appears to be crucial in the regulated cell death process triggered by AA/acetate. Herein, we aimed to assess the involvement of different vacuolar membrane proteins in AA-induced apoptosis, as well as to evaluate their putative role in VMP and translocation of the protease Pep4p from the vacuole to the cytosol. To this end, a functional genetic approach based on a set of mutants lacking the vacuolar membrane proteins Csc1p, Pep3p, Vma4p, Vma16p, Vtc4p and Zrt3p, together with biochemical and analytical techniques, were used. We found that absence of Zrt3p, Vtc4p, Csc1p and Vma4p render cells more resistant to AA. The resistant phenotype of *csc1*Δ and *vtc4*Δ mutants was associated with a delayed VMP and release of Pep4p to the cytosol, as previously shown for the *zrt3*Δ mutant. Altogether, these results indicate that these three proteins or their associated cellular functions, such as their contribution to the intracellular levels of zinc, calcium and polyphosphate, determine cell survival in response to AA. Particularly, and corroborating the involvement of zinc, we found that zinc availability influences survival of cells undergoing AA-induced cell death, reducing or enhancing cell survival under zinc limitation or supplementation, respectively. The levels of intracellular calcium also appear to play a role in AA-induced cell death, as suggested by Ca^{2+} lower levels in *csc1*Δ cells after AA treatment. While deletion of *VMA16* does not affect cell survival in response to AA*, PEP3* and *VMA4* deletions do affect. However, further studies are required to characterize the phenotypes of these deletion mutants. In summary, this study allowed to unveil molecular components/cellular processes involved in AA-induced cell death, contributing to the elucidation of the underlying mechanisms and their modulation towards the improvement of yeast industrial strains and the design of a non-conventional therapy for CRC.

Keywords: acetic acid, apoptosis, Pep4p, *Saccharomyces cerevisiae*, vacuolar membrane permeabilization, vacuole

RESUMO

A levedura *Saccharomyces cerevisiae* tem sido um dos organismos modelo mais utilizados para elucidar os mecanismos subjacentes à apoptose. A apoptose é uma forma de morte celular regulada, que pode ser desencadeada por vários estímulos, como por exemplo o ácido acético (AA). Este ácido desencadeia, na levedura, uma cascata de eventos intracelulares a nível mitocondrial e vacuolar. Um processo semelhante ocorre na apoptose induzida por acetato em células do cancro colorretal (CCR). Embora pouco se saiba sobre o papel da permeabilização seletiva da membrana do vacúolo/lisossoma (PMV/PML), esta parece ser crucial na morte celular induzida por AA/acetato. Neste trabalho, pretendemos avaliar o envolvimento de diferentes proteínas da membrana vacuolar na apoptose induzida por AA, bem como avaliar o seu papel na PMV e na translocação da protease Pep4p do vacúolo para o citosol. Para tal, foi utilizada uma abordagem de genética funcional baseada na utilização de mutantes deficientes nas proteínas Zrt3p, Pep3p, Csc1p, Vtc4p, Vma4p e Vma16p, assim como métodos bioquímicos e analíticos. A ausência das proteínas Zrt3p, Vtc4p, Csc1p e Vma4p torna as células mais resistentes ao AA. O fenótipo de resistência dos mutantes *csc1*Δ e *vtc4*Δ foi também associado a um atraso na PMV e na libertação da Pep4p para o citosol, tal como demonstrado anteriormente para o mutante *zrt3*Δ. Estes resultados indicam que estas proteínas, ou as suas funções celulares, como a regulação dos níveis intracelulares de zinco, cálcio e polifosfato, determinam a sobrevivência celular em resposta ao AA. Relativamente ao zinco, descobrimos que a sua disponibilidade influencia a sobrevivência celular em resposta ao AA, reduzindo-a/aumentando-a em situações de limitação/suplementação de zinco, respetivamente. Os níveis de Ca^{2+} intracelulares parecem também desempenhar um papel na morte celular, uma vez que se observam baixos níveis de Ca^{2+} no mutante *csc1*Δ após tratamento com AA. A deleção do *VMA16* não afeta a sobrevivência celular em resposta ao AA, no entanto a deleção do *PEP3* e *VMA4* afeta. Contudo, são necessários mais estudos para caracterizar os fenótipos destes mutantes. Em suma, identificamos proteínas/processos celulares envolvidos na morte celular induzida por AA, contribuindo para a elucidação dos mecanismos subjacentes e para a sua modulação com vista ao melhoramento de estirpes industriais de leveduras e ao desenvolvimento de terapias não convencionais para o CCR.

Palavras-chave: ácido acético, apoptose, Pep4p, permeabilização da membrana vacuolar, *Saccharomyces cerevisiae,* vacuolo

SCIENTIFIC OUTPUT

Panel communications

1. Marta Teixeira, Joana Terra-Matos, Cátia S. Pereira, Susana R. Chaves, Maria João Sousa, Hernâni Gerós, Manuela Côrte-Real (2019) "The impact of zinc in yeast cell death induced by acetic acid". V Simpósio PDBMA, 10/10, Braga, Portugal.

2. Joana Terra-Matos*, Marta Teixeira*, Cátia S. Pereira, Susana R. Chaves, Maria João Sousa, Hernâni Gerós, Manuela Côrte-Real (2019) "The role of zinc in acetic acid-induced cell death in yeast"." XII Encontro Nacional de Estudantes de Bioquímica", 13-15/4, Aveiro, Portugal.

3. Joana Terra-Matos, Marta Teixeira, Cátia S. Pereira, Susana R. Chaves, Maria João Sousa, Hernâni Gerós, Manuela Côrte-Real (2019) "Uncovering the role of vacuolar membrane proteins on Pep4p translocation/vacuolar membrane permeabilization during acetic acid-induced cell death". "I Jornadas da Ciência",11-15/3, Braga, Portugal.

4. Joana Terra-Matos, Cátia S. Pereira, Marta Teixeira, Susana R. Chaves, Maria João Sousa, Hernâni Gerós, Manuela Côrte-Real (2018) "Uncovering the role of vacuolar membrane proteins in acetic acid-induced cell death". IV Simpósio PDBMA, 25/10, Braga, Portugal.

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LIST OF ABBREVIATIONS

AA- Acetic Acid

AAC- ADP/ATP carrier

ABC- ATP Binding Cassette

ACD- Accidental Cell Death

AcLi- Lithium Acetate

ADP- Adenosine Diphosphate

Aif1p- Apoptosis Inducing Factor-1

ANT- Adenine Nucleotide Carrier

Apaf1- Apoptotic Protease Activating Factor- 1

ATP- Adenosine Triphosphate

Bak- Bcl-2 homologous antagonist/killer

Bax- Bcl-2 Associated protein-X

Bcl-2- B-cell Lymphoma 2

Bcl-X_L- B-cell lymphoma-extra-large

Bid- BH3-interacting death domain agonist

CAP- Cellular Adapter Proteins

c.f.u.- colony forming units

CMAC- 7-amino-4-cloromethylcoumarin

CoA- Acetyl-Coenzyme A

COX- Cytochrome Oxidase

CORVET- class C core vacuole/endosome tethering

cyt *c*- cytochrome *c*

DD- Death Domain

DED- Death Effector Domains

DHE- Dihydroethidium

DISC- Death Inducing Signalling Complex

DNA- Deoxyribonucleic Acid

DR- Death Receptors

FS- Forward Scatter

HOPS- Homotypic Fusion and Vacuole Protein Sorting

LMP- Lysosomal Membrane Permeabilization

MOMP- Mitochondrial Outer Membrane Permeabilization

NBT- Nitro Blue Tetrazolium

OMM- Outer Mitochondrial Membrane

PCD- Programmed Cell Death

PolyP- Polyphosphate

RCD- Regulated Cell Death

ROS- Reactive Oxygen Species

TNFr- Tumor Necrosis Factor receptor

SNARE- Soluble N-ethylmaleimide-sensitive Factor Attachment Receptor

SS- Side Scatter

ssDNA- single stranded DNA

VDAC- Voltage-dependent Anion Channel

VMP- Vacuolar Membrane Permeabilization

VTC- Vacuolar Transporter Chaperone

YEPD- Yeast Extract Peptone Dextrose

CHAPTER I - INTRODUCTION

I.1. Yeast: one cell, multiple applications

Yeast is an unicellular, asexual eukaryote belonging to the Fungi kingdom. Over the centuries, in the human's daily life, yeasts have been used and manipulated in various activities. Since the 19th century, yeast plays crucial roles in the production of bread and beer (Heitmann *et al.*, 2018). However, besides the food industry, its use is currently widespread in different fields such as for the production of natural compounds, enzymes, fuels, and pharmaceutical compounds, including the production of vaccines and probiotics **(Fig.1)** (Paulino *et al.*, 2017).

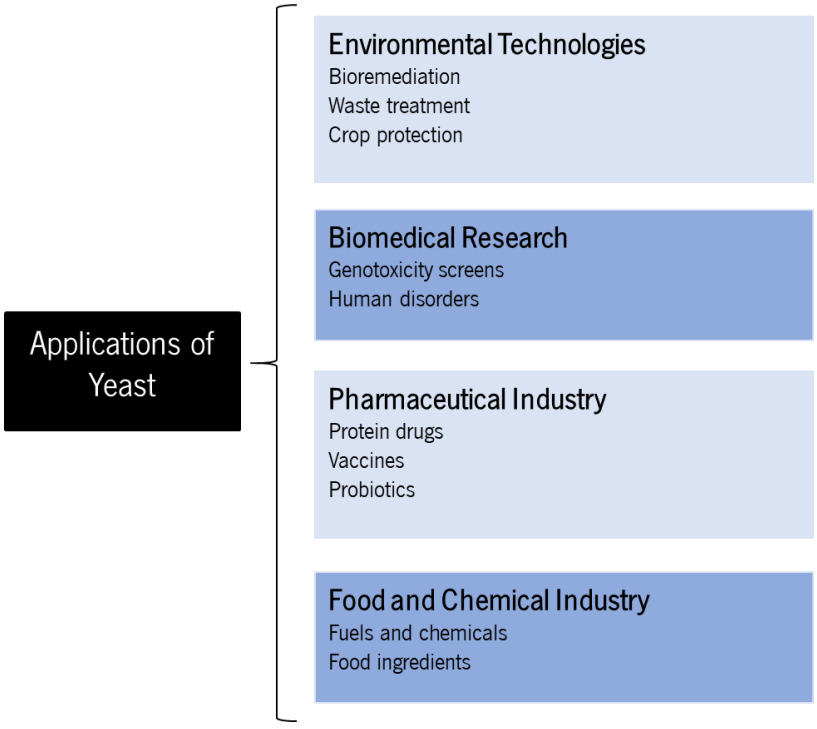


Figure 1 - The importance of yeast in humans' daily life. Yeast is used in various industries as well as in the biomedical field. Yeast is used in the production of fuels, chemicals, food ingredients and pharmaceuticals, being therefore often referred as a "cell factory". In addition, yeast has been shown to play a relevant role in the development of new technologies (adapted from Mustacchi *et al.*, 2006).

Saccharomyces cerevisiae is a non-pathogenic organism with low culture costs, rapid growth (Eisenberg *et al.*, 2010), and genetically tractable (Carmona-Gutierrez *et al.*, 2010). The genome of this model organism was the first eukaryotic genome to be sequenced (Goffeau *et al.*, 1996), being involved in many research topics, namely in studies of cellular aging (Petranovic & Nielsen, 2008; Zadrag-Tecza *et al.*, 2018), cell physiology (Petranovic & Nielsen, 2008), genetic regulation (Costa & Moradas-Ferreira, 2001), protein folding (Coughlan & Brodsky, 2003), oxidative stress (Costa & Moradas-Ferreira, 2001),

autophagy (Długońska, 2017), necrosis (Eisenberg *et al.*, 2010) and regulated cell death (Carmona-Gutierrez *et al.*, 2010; Ludovico & Côrte-Real, 2017; Samanfar *et al.*, 2017). Furthermore, since these cell processes are highly conserved in higher eukaryotes and their deregulation is associated with several diseases, it is not surprising that yeast studies have enabled the elucidation of the mechanisms associated with several disorders, thus contributing to the development of new therapeutic strategies (Ludovico *et al.*, 2003; Ludovico & Côrte-Real, 2017).

Taking into account that yeasts can undergo a regulated cell death (RCD) process, in the sense that it can be pharmacologically or genetically regulated, understanding the mechanisms behind yeast cell death will greatly impact both the biotechnological and biomedical fields.

This thesis will address some aspects of RCD, specifically the apoptotic-like cell death process triggered by acetic acid (AA) and the involvement of the yeast vacuole in this process. Different features of this organelle such as its morphology, dynamics and cellular functions, as well as its membrane proteome will also be emphasized. At the vacuolar proteome level, the functions of the zinc transporter Zrt3p, the chaperone vacuolar transport complex Vtc4p, the stress-gated calcium channel Csc1p, the subunits of V-ATPase Vma4p and Vma16p and the protease Pep3 will be explored in more detail.

I.2. Accidental *versus* regulated cell death

The homeostasis of each organism depends on a dynamic balance between cell death and the mechanisms of cell renewal (Berghe *et al.*, 2014; Krogan *et al.*, 2006). In unicellular and multicellular organisms there are numerous reactions and metabolic processes under a strict control, which ensure their viability, preventing or correcting any errors that may occur throughout the life cycle of each cell. Tendentially, in multicellular organisms, when cells are no longer needed or are damaged, they eventually engage into cell death in order to ensure that defects are not transmitted to their offspring and do not affect the neighbouring cells. In some cases, such as in unicellular organisms like yeasts, the death of the older cells can function as sustenance for younger cells.

Cell death can occur through different processes depending on the trigger/dose. When exposed to adverse environmental conditions such as high temperatures or high pressures, accidental death (ACD), a passive and unregulated process is triggered (Lorenzo Galluzzi *et al.*, 2018). However, in addition to this process, cells can trigger a RCD process, which occurs in situations where there is a failure in response to internal or external stress. RCD includes programmed cell death (PCD), a process that occurs in strictly physiological situations such as cell development and aging. Besides PCD, there are other types of RCD such as autophagy associated with cell death, apoptosis, ferroptosis, necroptosis, pyroptosis and

mitotic catastrophe. Initially, necrosis was characterized as an ACD process and, therefore, considered a passive and disorganized form of cell death (Galluzzi *et al.*, 2012; Kanduc *et al.*, 2002; Krogan *et al.*, 2006). However, more recently it has also emerged as an alternative form of RCD whose activation is controlled – the so-called regulated necrosis **(Fig. 2)** (Edinger & Thompson, 2004)**.** In the following paragraphs, two of the three most studied types of RCD will be briefly described: autophagy associated with cell death and regulated necrosis. In contrast, apoptosis will be discussed in more detail as it is the focus of this thesis. Since cell death studies were originally conducted in higher eukaryotic cells, data from studies with both mammalian cells and yeast cells are discussed.

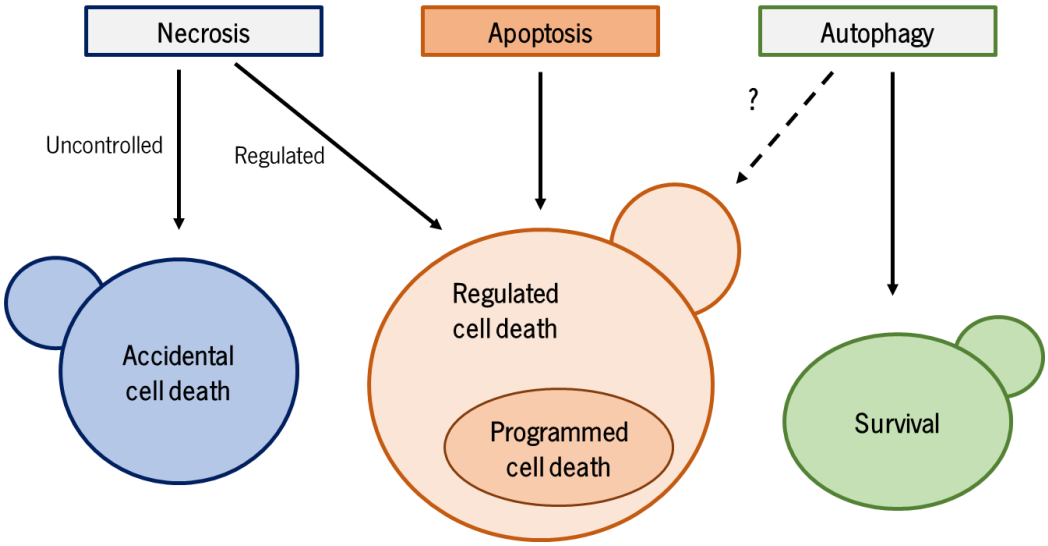


Figure 2 - Different types of yeast cell death. In yeast cells, cell death can occur by different processes. Under adverse environmental conditions, accidental cell death (ACD) is predominantly triggered. However, when cells are subjected to moderate stress conditions, regulated cell death (RCD) occurs. Programmed cell death (PCD), which is triggered in physiological conditions, is included in RCD. Autophagy plays a dual role, being involved in either cell death or survival (adapted from Carmona-Gutierrez *et al.*, 2018).

Although currently the definition of necrosis is still controversial, most scenarios characterized as necrotic are not considered ACD. Indeed, the fact that genetic manipulation can inhibit or stimulate the process, it confers to necrosis the essential characteristics of a RCD process. When necrosis occurs, the cell undergoes several alterations such as organelle swelling, loss of intracellular content resulting from plasma membrane disruption, random deoxyribonucleic acid (DNA) degradation, and energy impairment (Eisenberg *et al.*, 2010; Zong & Thompson, 2006). Compounds such as H_2O_2, AA and heavy metals (e.g. Cu^{2+} and Mn^{2+}) are known to induce necrosis when used at high concentrations (Falcone & Mazzoni, 2016; Galluzzi *et al.*, 2012). Regulated necrosis is a type of regulated cell death with necrotic characteristics,

such as intracellular disorganisation and loss of plasma membrane integrity. It is an event orchestrated by a genetically controlled machinery that can be inhibited by specific pharmacological or genetic interventions (Falcone & Mazzoni, 2016).

Yeasts are constantly subjected to different conditions, which forces them to respond rapidly to intracellular and extracellular changes by altering the anabolic and catabolic pathways (Codogno & Meijer, 2005; Suzuki & Ohsumi, 2007). Autophagy is considered a cell survival process being induced in response to nutrient deprivation and other physiological and pathological stimuli. It allows to restore the cellular homeostasis, balancing the synthesis and degradation of macromolecules and organelles (Długońska, 2017; Jin & Klionsky, 2015). When autophagy occurs, cell organelles and proteins are degraded and recycled in the vacuole (yeast) or lysosome (mammalian cells) (Codogno & Meijer, 2005; Debnath *et al.*, 2005; Długońska, 2017; Tsukada, 1993). Autophagy in yeast is a multi-step process that involves the formation of new vesicles and the action of different enzymes and specific machinery, from which the autophagy-related proteins (ATG proteins) stand out. Although still slightly controversial, several authors have shown that autophagy may be involved in cell death, with autophagy-dependent cell death being a form of RCD, which depends on the cellular machinery of autophagy and its components to accomplish cell demise (Yonekawa & Thorburn, 2014).

I.2.1. Apoptosis

The apoptotic process is not restricted to multicellular organisms, as it also occurs in unicellular organisms such as *S. cerevisiae* Apoptosis is the most well characterized process of RCD and can be initiated by a wide range of internal stimuli such as DNA damage, or external stimuli such as H_2O_2 (Madeo *et al.*, 1999) and AA (Ludovico *et al.*, 2001). Initially, the physiological role of apoptosis in unicellular organisms was questioned since it made no sense for these organisms to commit suicide. However, yeasts cells are individuals of a population and their elimination can be beneficial to the whole population. This theory is, in fact, now accepted, and yeast cell death is considered an altruistic process, resulting in nutrient saving that will be used by the younger cells or best fitted cells, thus contributing to increased viability and reproductive success of the population (Büttner *et al.*, 2006).

Apoptosis is an energy-dependent organized process, and unlike necrosis, plasma membrane integrity is assured avoiding the release of intracellular contents (Eisenberg *et al.*, 2010). Therefore, in contrast to necrosis, cellular changes involved in apoptosis occur without inflammation processes (Kroemer & Dallaporta, 1998). In mammals, the onset of apoptosis may be influenced by the adenosine triphosphate (ATP) levels and the activation of caspases. In mammalian cells, apoptosis is mainly divided

into two phases, the first comprises the formation of apoptotic bodies and the second their degradation by phagocytic cells. Apoptotic cells exhibit typical morphological characteristics, the so-called apoptotic markers, such as nuclear chromatin condensation, decreased cell and nucleus volume, phosphatidylserine externalization, DNA degradation, cytochrome *c* (cyt *c*) release and accumulation of reactive oxygen species (ROS). As these morphological and biochemical changes occur, cytoplasm blebs form from the plasma membrane that detaches and forms apoptotic bodies. The content of these apoptotic bodies is variable and often consists in condensed nuclear chromatin or cytoplasmic elements **(Fig.3)** (Carmona-Gutierrez *et al.*, 2010; Falcone & Mazzoni, 2016; Kerr *et al.*, 1972; Wu *et al.*, 2014).

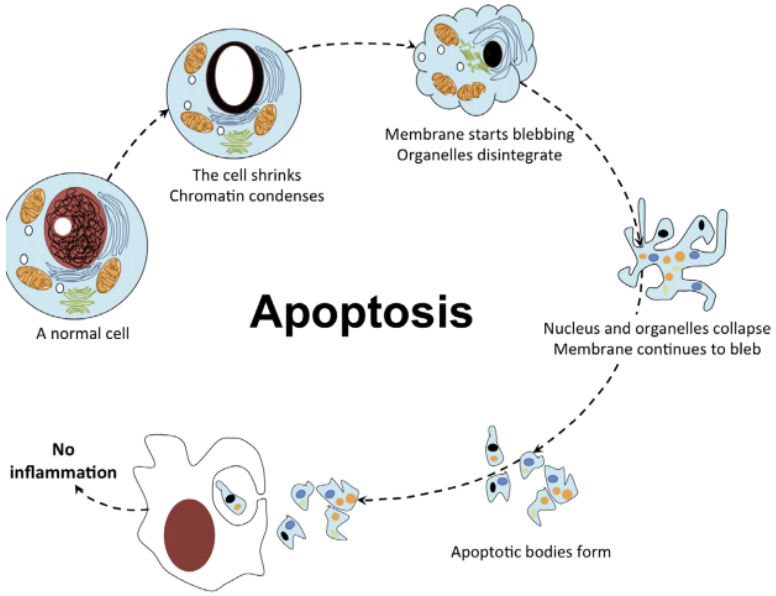


Figure 3 - Morphological events that occur in the apoptotic process of multicellular organisms. Apoptosis is a process of regulated cell death characterized by maintenance of plasma membrane integrity and absence of inflammation. In this process, typical nuclear cellular events are triggered such as chromatin condensation and nuclear/DNA fragmentation, while other organelles essentially maintain their integrity in a initial phase (adapted from Abou-Ghali and Stiban 2015).

Although there are many similarities between apoptosis in yeast and mammals, important proteins in the process such as the transcription factors of the p53 family and most proteins of the Bcl-2 (B-Cell Lymphoma 2) family are only present in mammalian cells (Carmona-Gutierrez *et al.*, 2010; Youle & Strasser, 2008). In mammalian cells, permeabilization of the outer mitochondrial membrane and subsequent release of cyt *c* is controlled by Bcl-2 proteins family and has been reported as a crucial event for cell death. The Bcl-2 protein family plays a major role in the apoptotic process and can promote or prevent it. Although in yeast there are no orthologs of these proteins, they maintain an identical activity and behaviour, when they are expressed in yeast (Polčic *et al.*, 2015). Thus, the absence of Bcl-2 family

orthologs favours the use of yeast in the study of the function of each Bcl-2 family members in apoptosis without the interference of the other members (Pereira *et al.*, 2008).

In apoptotic cell death, most of the observed changes are triggered by caspases. These proteases contain a cysteine residue at the active site, critical for their proteolytic activity, which exhibits a high affinity for aspartate, cleaving the substrates after these residues (Parrish *et al.*, 2013; Riedl & Shi, 2004; Wu *et al.*, 2014). Caspases play a crucial role in translating apoptotic signals and are divided into two broad classes: initiating caspases and effector caspases, based on their position in the apoptotic signalling cascade (Bao & Shi, 2007; Palai & Mishra, 2015; Parrish *et al.*, 2013; Riedl & Shi, 2004).

I 2.1.1. Two distinct pathways to apoptosis

Two main apoptotic pathways are currently described: the extrinsic (cytoplasmic) pathway and the intrinsic (mitochondrial) pathway (Elmore, 2007).

The extrinsic pathway **(Fig. 4, right panel)** is primarily mediated by the binding of extracellular ligands to specific cell death membrane receptors (DR), which belong to the tumour necrosis factor receptor (TNFr) superfamily. In addition to the ligand-interacting domain, the DR contains a homologous intracellular (cytoplasmic) sequence called the death domain (DD). Signal transmission begins with the interaction of specific ligands with receptors such as FasR, TNF-α-R1 and DR4 rich in cysteine residues, allowing ligand recognition. For instance when the ligand binds to the FasR, death-inducing signalling complex (DISC), is formed upon trimerization of FasR and recruitment of DD associated with cellular adapter proteins (CAP) and pro-caspase-8. CAP besides DD have death effector domains (DED) which interact with the DED of pro-caspase-8, which forms dimers. Pro-caspase-8 is then activated by proteolytic cleavage through a mechanism known as proximity induction. Activation of initiator caspases results in the subsequent activation of effector pro-caspases, such as caspase-3, which execute the apoptotic cell death (Daniel *et al.*, 2001; Elmore, 2007; Hengartner, 2000; Kiraz *et al.*, 2016; Lawen, 2003; Youle & Strasser, 2008).

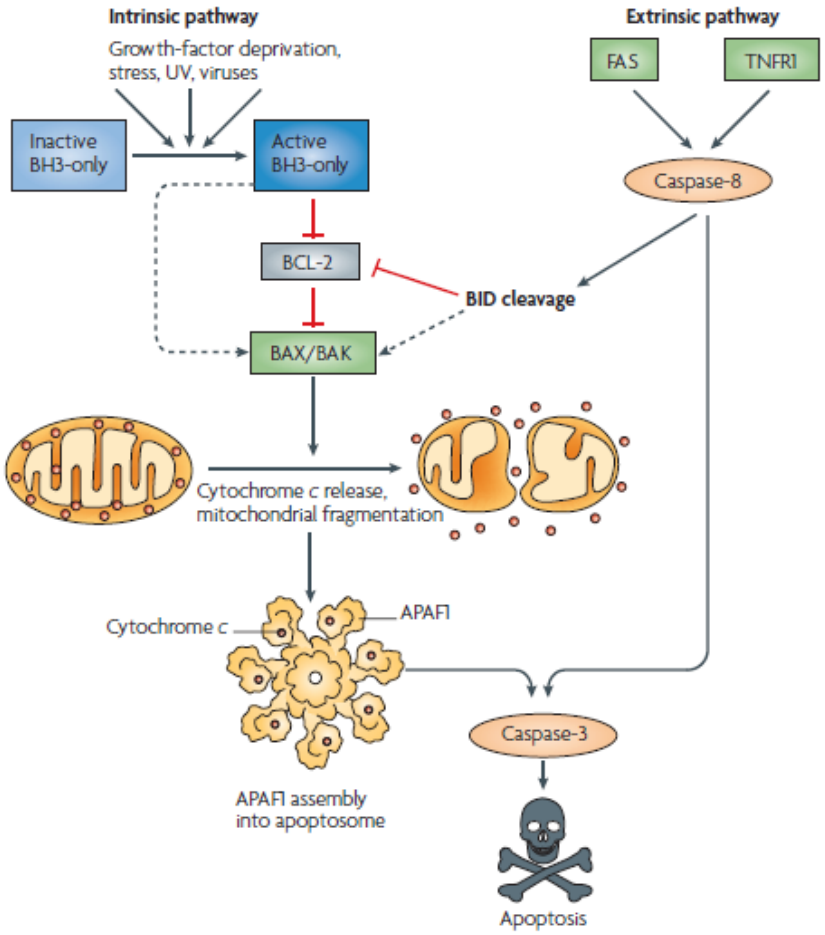


Figure 4 - Schematic representation of the intrinsic and extrinsic apoptotic pathways. Apoptosis can occur by two different pathways: the extrinsic pathway that is induced through death receptors such as FasR and TNF-α-R1 (right); or the intrinsic pathway that is triggered for example by deprivation of growth factors, transcriptional signals or stress conditions (left). In the extrinsic pathway, caspase-8 is activated, which leads to caspase-3 activation and cell death. In the intrinsic pathway, the inactivation of some members of the Bcl-2 family activates the pro-apoptotic proteins BAX and BAK, which in turn stimulates apoptosis by promoting mitochondrial outer membrane permeabilization. This leads to cyt *c* release, activation of Apaf-1, apoptosome formation, activation of caspases 9 and 3, and finally cell death. The Bcl-2 family plays an important role in intrinsic pathway regulation and can modulate the extrinsic pathway as well (adapted from Youle and Strasser, 2008).

The intrinsic or mitochondrial pathway **(Fig. 4, left panel)** are activated by different stimuli such as oxidative stress, cytotoxic drug treatment or DNA damage. All the process is associated with a centralized control mechanism at the mitochondrial level. Permeabilization of the outer mitochondrial membrane (OMM) is one, if not the most crucial point, for apoptosis to occur intrinsically, and the exact molecular components/mechanisms involved are still elusive. Subsequently, there is the release of cyt *c* and other pro-apoptotic factors from the mitochondrial intermembrane space to the cytosol. Cyt *c* binds

then to the inactive Apoptotic Protease Activation Factor-1 (Apaf-1) in the presence of ATP (Mazzoni & Falcone, 2008; Wang, 2001). The binding of cyt *c* to Apaf-1 leads to its activation and the formation of a complex called "apoptosome", which recruits and activates pro-caspase-9. After activation, caspase-9 activates effector caspases, such as caspase-3, leading to the activation of a cascade of events that causes several morphological changes characteristic of apoptosis and the formation of apoptotic bodies (Bao & Shi, 2007; Reubold *et al.*, 2011; Wang, 2001; Yuan & Akey, 2013).

In mammals, the key function of Bcl-2 family is to regulate cell death by controlling the integrity of the MOMP and the release of pro-apoptotic factors from the mitochondrial intermembrane compartment to the cytosol. This family is divided into two subfamilies: the anti-apoptotic members, which include Bcl-2 and Bcl-X_L (B-cell lymphoma-extra-large); and pro-apoptotic members, which include Bax (Bcl-2 Associated protein-X), Bak (Bcl-2 homologous antagonist/killer), and proteins that only have the BH-3 domain (Borner, 2003; Frohlich & Madeo, 2000; Youle & Strasser, 2008). The anti-apoptotic proteins are located at the MOM (Bcl-x_L) or at intracellular membranes (Bcl-2) and have the function to protect the cell from apoptosis (Ludovico *et al.*, 2001; Polčic *et al.*, 2015). On the other hand, pro-apoptotic proteins are essential for mitochondrial outer membrane permeabilization (MOMP) and for the release of cyt *c* from mitochondria to cytosol, which triggers the intrinsic apoptotic pathway (Polčic *et al.*, 2015; Youle & Strasser, 2008).

Although the extrinsic and intrinsic pathways are triggered by different stimuli and have different mechanisms, both have in common a final pathway that begins with caspase-3 cleavage (Elmore, 2007). Indeed, the cleavage of Bid (BH3-interacting death domain agonist) by caspase-8 establish a crosstalk between both pathways **(Fig. 4)** (Carmona-Gutierrez *et al.*, 2018).

I.3. Acetic acid as an apoptotic cell death inducer in yeast

AA is a weak monocarboxylic acid that can be formed as a by-product of the alcoholic fermentation process carried by yeasts, in particular by *S. cerevisiae* (Arneborg *et al.*, 1995; Sousa *et al.*, 2013; Toit & Lambrechts, 2002). The amount of acetic acid produced depends on the fermentation conditions. For instance, the amount of acetic acid produced is much higher in fermentation of grape must than of malt must for the production of wine and beer, respectively (Guldfeldt & Arneborg, 1998). Although this compound is a by-product of fermentation, when it is present above certain concentrations, it becomes cytotoxic (Arneborg *et al.*, 1995; Giannattasio *et al.*, 2005; Pampulha & Loureiro-Dias, 2000).

In glucose-repressed cells, AA is not metabolized by yeast and enters the cell in its undissociated form by simple diffusion through the plasma membrane only in situations where extracellular pH is lower

that the intracellular pH (Ludovico *et al.*, 2001; Pampulha & Loureiro-Dias, 1989). When inside the cell, it dissociates, which increases the concentration of acetate and protons, and consequently leads to intracellular acidification. This intracellular acid accumulation affects various cellular processes. Under high glucose growth conditions, most *Saccharomyces* spp. are unable to metabolize acetate (Casal *et al.*, 1996; Guldfeldt & Arneborg, 1998; Ludovico *et al.*, 2001). Accordingly, several works with *S. cerevisiae* report that AA decreases cell viability (Pinto *et al.*, 1989) and triggers an apoptotic-like regulated cell death process (Ludovico *et al.,* 2001; Ludovico *et al.*, 2002). Therefore, understanding this process is the first step towards the development of strategies to modulate it (Jan *et al.*, 2002; Marques *et al.*, 2013). On the other hand, it was also found that acetate triggers apoptosis in cancer cells, thus reinforcing the importance of studies aiming to uncover the mechanisms underlying AA/acetate-induced cell death (Ferro *et al.*, 2016).

Indeed, many studies have been done under conditions where glucose is used as a carbon source, and it has been observed that, at AA concentrations between 20 and 120 mM (pH=3), yeasts dye exhibiting apoptotic markers such as chromatin condensation, DNA cleavage and exposure of phosphatidylserine on surface of the plasma membrane (Ludovico *et al.*, 2001) and release of cyt *c* (Ludovico *et al.,*2002). In cultures low in fermentable sugars, AA is metabolized to acetyl-coenzyme A (CoA), which enters the Krebs and glyoxylate cycles to fulfil the needs for energy and biosynthetic metabolites (Flores *et al.*, 2000).

Mitochondria are dynamic organelles responsible for producing most of the cellular ATP through oxidative phosphorylation, being involved in lipid and phospholipid metabolism (Braun, 2012). Furthermore, this organelle contributes to various responses to cell stress, including cell death (Braun, 2012; Giannattasio *et al.*, 2005; Sousa *et al.*, 2013). As in mammals, mitochondria also play an essential role in AA-induced cell death in yeast (Ludovico *et al.*, 2002; Pereira *et al.*, 2008; Pereira *et al.*, 2007; Guaragnella *et al.*, 2012). In fact, it has been found that AA induces ROS accumulation at mitochondrial level (Ludovico *et al.*, 2002; Giannattasio *et al.*, 2005; Pereira *et al.*, 2008), hyperpolarization followed by loss of mitochondrial membrane potential, decreased activity of cytochrome oxidase (COX) (Ludovico *et al.*, 2002), alterations in the level of mitochondrial respiration and changes in mitochondrial structure and function (Ludovico *et al.*, 2003), namely reduction in the number of cristae and decrease mitochondrial volume (Sousa *et al.*, 2011), as well as release of pro-apoptotic factors such as cyt *c*, Aif1p (Apoptosis Inducing Factor-1) and Nuc1p (the yeast orthologs of mammalian AIF and EndoG, respectively) in yeast (Büttner *et al.*, 2007; Wissing *et al.*, 2004). Moreover, strains deficient in mitochondrial DNA (Rho^0) or mitochondrial ATPase (*atp10*Δ) were shown to be resistant to the cytotoxic

effect of AA associated with no release of cyt *c*. These data support the hypothesis that mitochondria are essential in AA-induced apoptosis. Additionally, studies have also showed the involvement of mitochondrial membrane transporters in AA-induced apoptosis. The adenine nucleotide transporter (ANT) is a protein present in the inner mitochondrial membrane responsible for the exchange of adenosine diphosphate (ADP)/ATP. In yeast, the absence of ADP/ATP carrier (AAC) proteins (*aac1/2/3*Δ), the yeast ANT orthologous, was found to increase cell resistance to AA. In contrast, when the *POR1* gene, which encodes the mitochondrial voltage-dependent anion channel, VDAC, is removed, there is an increase in apoptosis triggered by AA (Pereira *et al.*, 2007; Sousa *et al.*, 2011; Trindade *et al.*, 2016). In addition to these proteins, the mitochondrial protein Ysp2p is also involved in AA-induced cell death, and its deletion confers resistance to the RCD process (Sokolov *et al.*, 2006).

Besides mitochondria, studies have recently reported the role of the vacuole in AA-induced regulated cell death as it will be discussed in the next section.

I.3.1. What is the role of the vacuole in acetic acid-induced apoptosis?

Lysosomes/vacuoles and cathepsins/vacuolar proteases have also been shown to be involved in the regulation of acetate/AA-induced apoptosis in mammals/yeast as reported by our group and others (Guicciardi *et al.*, 2004; Pereira *et al.*, 2010; Schauer *et al.*, 2009; Marques *et al.*, 2013)

In yeast, AA is known to induce a vacuolar membrane permeabilization (VMP) associated with the release of the vacuolar protease proteinase A (Pep4p) from the vacuole to the cytosol **(Fig. 5)**. Interestingly, depending on the genetic background, Pep4p plays a role in protection or execution of AA-induced apoptosis. (Pereira *et al.*, 2010). In fact, BY4741 cells lacking *PEP4* become more resistant to AA, while in the strain W303-1A, the deletion of this protein confers higher susceptibility to AA treatment (Pereira *et al.*, 2015). Pep4p overexpressing W303-1A cells become more resistant to AA treatment, which is in line with the protective role of this protein reported in this strain. In this same strain, although Pep4p deficient cells are more predisposed to undergo apoptosis, mitochondrial degradation is retarded during the process of AA-induced cell death. Cells overexpressing this protease exhibit increased survival and mitochondrial degradation in response to AA. These facts suggest that the removal of damaged mitochondria is associated with the protective role of Pep4p in AA-induced cell death (Pereira *et al.*, 2010; Sousa *et al.*, 2013).

Although the involvement of the vacuole and Pep4p in mitochondrial degradation is not very clear, it was shown to be independent of autophagy and, apparently, also involves non-vacuolar proteins Pereira *et al.*, 2010. In fact, AAC deficient cells, which are not deficient in Pep4p release, exhibit a reduction in

mitochondrial degradation in response to AA treatment. This reinforces the idea that the susceptibility of cells to AA by *PEP4* deletion is dependent on AAC proteins likely due to the role in mitochondrial permeabilization (Pereira *et al.*, 2015; Sousa *et al.*, 2011). Moreover, as autophagy is not activated in cells subjected to AA-induced apoptosis, VMP and the ensuing release of Pep4p may serve as an alternative process to mitochondrial degradation. Inhibition of autophagy associated with AA-induced cytosolic acidification may thus favour Pep4p activity upon release into the cytosol. Therefore, the fact that mitochondrial AAC proteins and vacuolar Pep4p present in yeast interfere with mitochondrial degradation points to complex regulation and dynamic interactions between mitochondria and the vacuole in this process of regulated cell death **(Fig. 5)** (Pereira *et al.*, 2010; Sousa *et al.*, 2011).

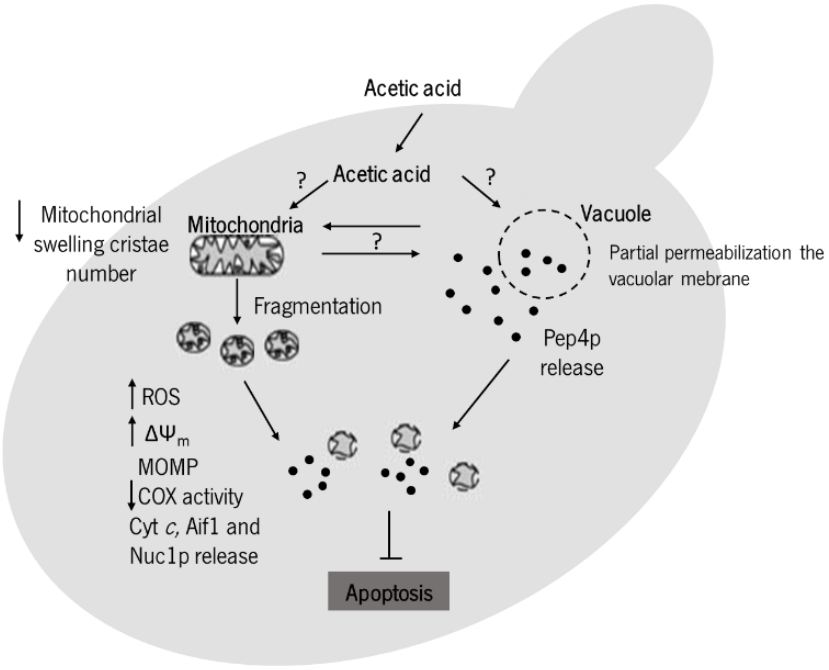


Figure 5 - Interaction of mitochondria and yeast vacuole in acetic acid-induced cell death. In response to AA treatment, the vacuolar and mitochondrial membranes undergo permeabilization, which leads to the release of cyt *c* and Aif1p from the mitochondria, and Pep4p from the vacuole, to the cytosol (adapted from Sousa *et al.,* 2011).

In mammalian cells, in response to a variety of apoptotic signals, lysosomal membrane permeabilization (LMP) seems to be associated with mitochondrial membrane permeabilization (Marques *et al.*, 2013; Pereira *et al.*, 2010; Sousa *et al.*, 2011). Depending on the stimulus, LMP can have an important function in activating the apoptotic signalling cascade or may occur at a later stage of the apoptotic process. The hallmark of lysosomal permeabilization is the partial loss of membrane integrity and consequent release of cathepsins to the cytosol such as cathepsin D (CatD), the mammalian ortholog

of Pep4p. CatD is a lysosomal aspartic protease found in most mammalian cells. Like other cathepsins, it is involved in many physiological processes such as apoptosis. Cathepsins promote apoptosis by degradation of anti-apoptotic Bcl-2 homologues or Bid (Repnik *et al.*, 2014; Sousa *et al.*, 2011; Yu *et al.*, 2016). CatD protease is significantly up-regulated in colorectal carcinoma cells but it is expressed at low levels in normal tissues. In studies with colorectal cancer cells undergoing acetate-induced apoptosis, CatD was shown to be released to the cytosol (Oliveira *et al.*, 2015), in a process identical to the release of Pep4p in yeast cells treated with AA (Sousa *et al.*, 2011).

Thus, the vacuole appears to play a crucial role in AA-induced apoptosis in yeast, as does the lysosome in acetate-induced apoptosis in colorectal cancer cells. Carrying out studies that allow a better understanding of the role of these organelles and the mechanisms underlying their selective permeabilization will be an asset to understand the process of cell death triggered by these stimuli as well as its modulation.

I.4. The yeast vacuole: a multifunctional organelle

The vacuole is the largest compartment and acidic organelle of yeast cells, occupying up to one quarter of the total intracellular volume with a well-defined lipid and protein composition (Rodrigues *et al.*, 2013). This organelle has distinct characteristics playing a crucial role in protein degradation, ion and metabolite storage, renewal of certain organelles (mitochondria and peroxisomes), detoxication and cell death processes, such as apoptosis. Furthermore, it is responsible for responding to nutrient deprivation and osmotic/ionic stress (Guicciardi *et al.*, 2004; Klionsky *et al.*, 1990; Li & Kane, 2010). Since the yeast vacuole has many similarities and physiological characteristics to the mammalian lysosomes, yeasts have also been used as a model to study processes involving lysosomes (Rodrigues *et al.*, 2013).

The yeast vacuole has a protein-rich content, but not all of them play a direct role in this organelle. The vacuole proteome is mostly made up of proteases, hydrolases and some proteins related to autophagy, vacuole fusion and transport **(Fig. 6).** Accordingly, the vacuole is one of the major systems of cell protein degradation. Vacuolar proteases such as Pep4p (proteinase A) , carboxypeptidase Y-deficient protein 3 (Pep3), proteinase B (Prb1p) and carboxypeptidase S (Csp1p) have been used as model proteins in traffic studies . Usually, these vacuolar proteases are non-specific and are involved in the degradation of different substrates. Initially, these proteases are transported to the vacuole in its inactivated state, and there, at the acidic pH, they become activated through a complex cascade (Hazel *et al.*, 1996; Li & Kane, 2010).

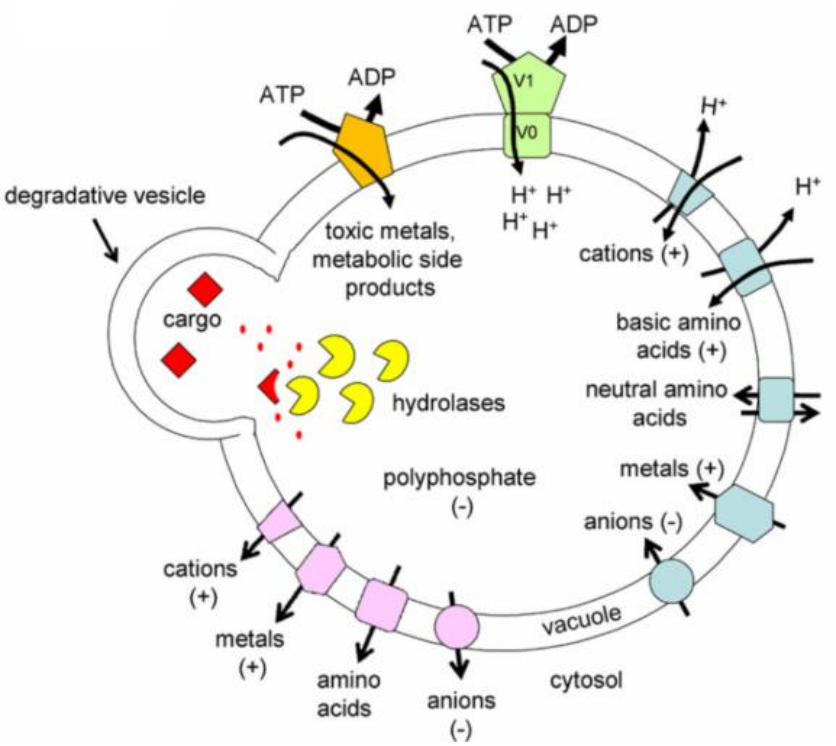


Figure 6 - Overview of the protein content of the yeast vacuole. The yeast vacuole is rich in hydrolases, proteinases and it has different membrane transporters responsible for uptake of amino acids, ions and metals. The combined activity of these carriers is essential for ion homeostasis and amino acid recycling (adapted from Li and Kane 2009).

The vacuole is the main storage compartment for amino acids, phosphate, calcium and other metal ions (Klionsky *et al.*, 1990). This organelle contains high concentrations of phosphate and stores it in the form of polyphosphate, serving as a cytosolic phosphate buffer in phosphate-deficient situations (Carroll & Shea, 2002; Thomas & Shea, 2005). The vacuole also regulates free ionized calcium levels, allowing intracellular calcium accumulation due to the H^+/Ca^{2+} antiport (Vcx1p) and a Ca^{2+}-ATPase that drives calcium uptake (Miseta *et al.*, 1999). Vacuoles are also essential in nutrient limitation conditions, where the autophagy process aforementioned is triggered to provide nutrients that ensure cell viability (Kim & Klionsky, 2000; Klionsky *et al.*, 2003). Additionally, there are two ATP binding cassette (ABC) transporters, Ycf1p and Bpt1p, present in the vacuolar membrane responsible for cell detoxification. These two pumps are responsible for transporting substrates such as by-products resulting from adenine metabolism and toxic metals, such as arsenite and cadmium. Although both have similar specificities, the regulatory mechanisms of each one are unique (Sharma *et al.*, 2002).

The morphology of the vacuole depends on the growth phase changing in response to intracellular and extracellular stimuli. Throughout the exponential phase, the yeast cells contain intermediate size

fragmented vacuoles. During the stationary phase or in situations of glucose deprivation they fuse, resulting in a large vacuole. When under osmotic stress, vacuoles can adjust their amount and shape to improve the release/uptake of ions or water. Hypotonic media promote the union of vacuoles, while hypertonic conditions induce rapid fragmentation (Li & Kane, 2010; Michaillat & Mayer, 2013). For this reason, it is essential that the vacuolar membrane has the capacity to undergo fission and fusion in order to preserve cellular homeostasis (Baars *et al.*, 2007; Peplowska & Ungermann, 2005; Wickner, 2002). The fusion occurs essentially in four phases (priming, tethering, docking and fusion), where many proteins such as vacuolar transport chaperone complex (VTC) and lipids are involved (Ostrowicz *et al.*, 2008; Qiu, 2012). As for the vacuolar fission process, coating proteins such as COPI, COPII and clathrin are involved (Li & Kane, 2010; Peplowska & Ungermann, 2005). Interestingly, in yeasts, the V-H^+-ATPase proton pump in addition to regulating intracellular pH is necessary in vacuolar fusion and fission processes (Baars *et al.*, 2007).

I.4.1. The vacuolar membrane proteome

Most studies focus only on vacuolar lumen proteins and hence knowledge about vacuolar transport proteins is still very limited, and not usually identified and characterized by classical genetics (Wiederhold *et al.*, 2009). One of the few examples is the study by Wiederhold *et al.*, which aimed to identify the proteins involved in catalysis of solute transport across the *S. cerevisiae* vacuolar membrane, where 69 vacuolar proteins were identified, from which only 27 proteins are present in the vacuolar membrane. Among these proteins, 18 vacuolar transport proteins are well characterized, such as the zinc transporter Zrc1p and the neutral amino acid transporters Avt1p and Avt3p. From the total vacuolar transport proteins, 10 were also located in other subcellular compartments such as cytosol, mitochondria and plasma membrane. All of them are involved in the fusion of the vacuolar membrane belonging to the homotypic fusion and vacuole protein sorting complex (HOPS). Besides these, Ncr1p has also been identified as a vacuolar carrier with an important role in fusion. Vtc1p, Vtc4p and Cot1p proteins were also found in the pure fraction of the vacuolar membrane, which indicates that they are present at the membrane. This fraction lacked the Zrt3p transporter as expected since the cells were grown in complete rich medium and expression of this transporter is only increased in Zn^{2+} deficient conditions. Also, Pep3p, Pep5p, Vps16p, Vps33p, Vps41p and Vam6p proteins, subunits of the SNARE(Soluble N-ethylmaleimide-sensitive Factor Attachment Receptor) complex and of the HOPS, were also identified in the vacuolar membrane as well as different components of Torc1p and Torc2p. Additional proteins present in the vacuolar membrane are the Nhx1p, which is responsible for Na^+ transport; V-H^+-ATPase that is involved

in the generation of the pH gradient; the carrier Ycf1p, involved in detoxification processes; and Csc1p (calcium permeable stress-gated cation channel) involved in calcium transporter (Kellermayer *et al.*, 2003; Mielniczki-Pereira *et al.*, 2011; Miseta *et al.*, 1999; Wiederhold *et al.*, 2009). Additionally, five proteins belonging to the fusion, transport and targeting functional group of vacuoles (Vps30p, Vps62p, Rsn1p, Sec17p and Akr2p), which had previously not been localized to any specific organelle, were categorized in this study as vacuolar membrane proteins. All carriers described in this topic together with the remaining vacuolar membrane proteins are crucial to cell homeostasis.

As the Zrt3p, Vt4cp, Csc1p, Vma4p, Vma16p and Pep3p proteins were the focus of this thesis, their features and functions will be covered in more detail in the following topics.

I.4.1.1. Vacuolar membrane proteins involved in the regulation of acetic acid-induced cell death in yeast

An interesting study identified the genes involved in the positive and negative regulation of AA-induced RCD. To this end, the authors used *S. cerevisiae* cells grown in YPD medium with 400 mM AA and performed a functional analysis of a collection of mutants deleted in all non-essential yeast genes (the EUROSCARF collection). Yeast mutants with higher resistance or greater sensitivity to AA-induced cell death were identified by comparison with the wild type strain (BY4741). Focusing on the mutants lacking vacuolar membrane proteins, most of them exhibited an increased resistance to AA, as shown in **Table 1**, which indicates that these genes somehow mediate this process. In contrast, other genes when deleted confered sensitivity to AA and therefore play a protective role. Overall, of the cellular components, vacuole stands out as an important organelle in AA-induced RCD, with major relevance for the proteolytic functions and transport across the vacuolar membrane of amino acids and cations, namely protons, calcium, manganese, copper and iron (Kawano-Kawada *et al.*, 2018; Sousa *et al.*, 2013).

Table 1 - Effect of acetic acid on mutant strains deleted in genes encoding proteins present at the vacuolar membrane. The table shows the phenotype of cells deleted in the depicted genes ("Genes" column) treated with 400 mM AA. The phenotypes presented are represented as: "+", indicative of increased cell resistance to AA-induced RCD; and "-", indicative of increased cell sensitivity to AA-induced RCD. Adapted from Sousa *et al.*, 2013.

Gene	Locus	Protein	Phenotype
AMS1	*YGL156W*	Alpha-mannosidase	+
COT1	*YOR316C*	Cobalt uptake protein COT1	+
PEP4	*YPL154C*	Proteinase A	+
PHO8	*YDR481C*	Repressible alkaline phosphatase	+
PMC1	*YGL006W*	Calcium-transporting ATPase 2	+
VAC8	*YEL013W*	Vacuolar protein 8	+
VAM6	*YDL077C*	Vacuolar morphogenesis protein 6	+
VAM7	*YGL212W*	Vacuolar morphogenesis protein 7	+
VBA2	*YBR293W*	Vacuolar basic amino acid transporter 2	+
VBA4	*YDR119W*	Vacuolar basic amino acid transporter 4	+
VHC1	*YBR235W*	Vacuolar cation-chloride cotransporter 1	+
VMA6	*YLR447C*	V-type proton ATPase subunit d	+
VMA7	*YGR020C*	V-type proton ATPase subunit F	+
VMR1	*YHL035C*	ABC transporter ATP-binding protein/permease VMR1	+
VPS60	*YDR486C*	Vacuolar protein-sorting-associated protein 60	+
VPS62	*YGR141W*	Vacuolar protein sorting-associated protein 62	+
VTC1	*YER072W*	Vacuolar transporter chaperone 1	+
VTC4	*YJL012C*	Vacuolar transporter chaperone 4	+
YCF1	*YDR135C*	Metal resistance protein YCF1	+
PEP3	*YLR148W*	Carboxypeptidase Y-deficient protein 3	-
PEP5	*YMR231W*	E3 ubiquitin-protein ligase PEP5	-
VMA16	*YHR026W*	V-type proton ATPase subunit c	-
VMA3	*YEL027W*	V-type proton ATPase subunit c	-
VMA4	*YOR332W*	V-type proton ATPase subunit E	-
VMA5	*YKL080W*	V-type proton ATPase subunit C	-
VPS16	*YPL045W*	Vacuolar protein sorting-associated protein 16	-
VPS33	*YLR396C*	Vacuolar protein sorting-associated protein 33	-

I.5. Vacuolar membrane proteins studied in this thesis

Aiming to further understand the role of the vacuole in AA-induced cell death, we focused on the study of the vacuolar membrane proteins Zrt3p, Vtc4p, Csc1p, Pep3p, Vma4p and Vma16p that appear to have different roles in the response to lethal concentrations of AA.

I.5.1. The Zrt3p zinc transporter

There are several metal ions with a prominent role in the cell, the availability of many of them at cytosolic level is regulated by specific transporters. One of these metals is zinc. Zinc is an indispensable catalytic component of over 300 enzymes and is required for the function of more than 3% of the yeast proteome. In humans, approximately 90% of this total zinc is tightly bound to proteins. However, in excess, zinc can be toxic to cells. Zinc toxicity may be associated with its binding to inappropriate sites in proteins or co-factors (MacDiarmid *et al.*, 2002).

The vacuole plays a crucial role in zinc homeostasis, storing zinc for use in Zn^{2+}-deficient conditions or sequestering it when in excess, thereby allowing for the maintenance of intracellular zinc levels. The homeostasis of zinc is maintained by distinct transporters, such as Zrt1p, Zrt2p, Zrt3p, Zrc1p, Fet4p and Cot1p **(Fig. 7).** The genes *ZRT1*, *ZRT2* and *ZRT3* respond to intracellular zinc levels by transcriptional regulation mediated by the zinc responsive transcription factor ZAP1 (MacDiarmid *et al.*, 2000). The Zrt1p and Zrt2p are transporters on the plasma membrane, involved in zinc uptake from the extracellular environment. The yeast transporter Zrt3p, was reported to mediate the efflux of the stored zinc from the vacuole to the cytosol and, in yeast, its expression is upregulated under low zinc conditions (Devirgiliis *et al.*, 2004; MacDiarmid *et al.*, 2002; Simm *et al.*, 2007).

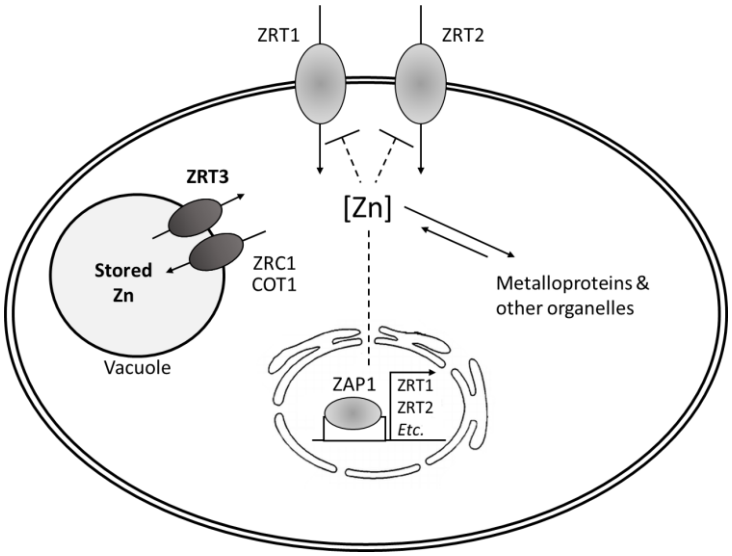


Figure 7 - Zinc transport and trafficking in the yeast. Zinc enters the cell via the Zrt1p and Zrt2p transporters and is transported into the vacuole by Zrc1p and Cot1p transporters. When zinc becomes limited, the Zrt3p transporter mobilizes zinc stores from the vacuole into the cytosol (adapted from MacDiarmid *et al.*, 2000).

Studies with a *S.cerevisiae* mutant strain lacking Zrt3p showed that these strains have high levels of intracellular zinc, suggesting inadequate mobilization of stored zinc, which prevents the cell from using zinc in zinc deficient conditions. Since the *zrt3*Δ mutant is viable, the *ZRT3* gene is not essential for yeast. However, its deletion results in low nuclear/cytoplasmic zinc pool in the yeast cell (MacDiarmid *et al.*, 2000).

The study carried by Wan *et al.* in *S. cerevisiae* demonstrated that zinc modulates cellular amino acid metabolism and redox balance, particularly biosynthesis of alanine and glutathione to exert its antioxidant effect (Wan *et al.*, 2015). In humans, Zn^{2+} deficiency leads to many defects, such as growth retardation, cognitive disorders, and infertility (Li *et al.*, 2015). Studies in others organisms, specifically in the yeast cells, have shown that zinc deficiency can increase intracellular levels of ROS (Eide, 2009). Although there are studies with the *zrt3*Δ mutant, the information about its phenotypes is still scarce.

I.5.2. The vacuolar transporter chaperone complex subunit Vtc4p

A large part of vacuolar proteins have membrane transport and vesicular traffic functions. The VTC complex are among them. Numerous studies indicate that the VTC complex is in the vacuolar membrane as well as in other cellular compartments. This complex consists of five subunits, Vtc1p, Vtc2p, Vtc3p, Vtc4p and Vtc5p, which are integral membrane proteins and contain three C-terminal transmembrane helices (Desfougères *et al.*, 2016; Muller *et al.*, 2003). Regarding the Vtc2p, Vtc3p and

Vtc4p subunits, they have a N-terminal with large hydrophilic domains exposed in the cytosol **(Fig. 8)** (Cohen *et al.*, 1999; Muller *et al.*, 2003; Ostrowicz *et al.*, 2008).

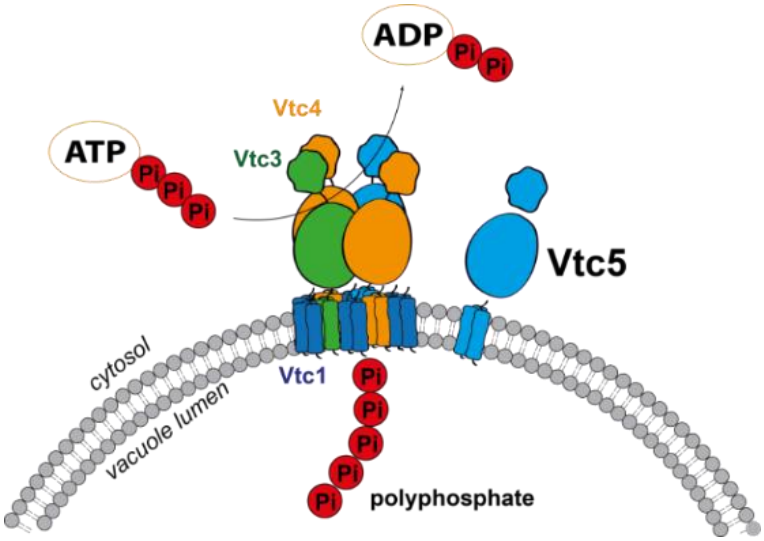


Figure 8 - VTC complex protein topology. The VTC complex consists of five proteins: Vtc1p, Vtc2p, Vtc3p, Vtc4p and Vtc5p, which are found in the vacuolar membrane (adapted from (Desfougères, 2016).

The VTC complex has an important role in several processes, namely vacuolar fusion and microautophagy (Muller *et al.*, 2003; Uttenweiler *et al.*, 2007). In addition, this complex is also involved in the regulation of V-ATPases, which are present in various organelles, namely in the vacuolar membrane (Muller *et al.*, 2002). In what regards the function of each subunit, it is known that all have distinct roles. The Vtc4p subunit, consisting of 721 amino acids (Muller *et al.*, 2003), is involved in the synthesis and transfer of polyphosphate (polyP) to the vacuole (Hothorn *et al.*, 2009b). This subunit regulates membrane traffic and acts on non-autophagic vacuolar fusion (Muller *et al.*, 2003), acting together with the Vtc1p subunit in the regulation and activation of vacuolar SNAREs (Qiu, 2012).

Studies reported by Bru *et al.* and Ogawa *et al.* suggested that mutants that do not express Vtc4p (*vtc4*Δ) are compromised in cell cycle progression, specifically in the transition from phase G1 to phase S. Moreover, in the mutant *vtc4*Δ, polyP accumulation no longer occurs, which suggest that Vtc4p is necessary for transport and consequent accumulation of polyP in the vacuole (Bru *et al.*, 2016; Ogawa *et al.*, 2000). In other studies, cells with catalytically inactive Vtc4p or lacking Vtc4p exhibit a large number of small vacuoles. By contrast, in the wild type strain essentially 1-2 large vacuoles are present. These observations suggest that in *vtc4*Δ cells there is a reduction in vacuolar fusion activity (Desfougères *et al.*, 2016). It is also known that *S. cerevisiae* can survive in extremely dry conditions. In an analysis of the most important genes in the desiccation response, Vtc4p was identified as one of most importance in this regulation (Ratnakumar *et al.*, 2011).

I.5.3. The calcium permeable channel Csc1p

The activation of cell death is regulated by a complex interaction that allows different signals to be decoded, among which calcium (Ca^{2+}) plays a significant role in this process. Increasing its intracellular concentration is considered a key event to trigger the process of apoptosis. Subsequently, other changes occur at the cellular level, such as increased ROS levels, mitochondrial depolarization and increased matrix volume, which allows the release of cyt *c* and other pro-apoptotic proteins (Demaurex & Distelhorst, 2003; Pinton *et al.*, 2008). In yeast, cytosolic concentrations of Ca^{2+} are controlled by its accumulation in organelles such as the vacuole, endoplasmic reticulum and Golgi complex (Cui *et al.*, 2009; Cunningham & Fink, 1994; Cyert & Philpott, 2013). However, when apoptotic stimuli occur, there is an increase in the cytosolic Ca^{2+} concentration followed by an increase in the mitochondrial cation concentration. For this to happen, the cation must be transferred from the organelles where it is stored or from the extracellular medium to the cytosol (Carraro & Bernardi, 2017).

Yeasts have several channels that allow the directed movement of cations, atoms or small molecules into or out of the cell, or between cells (Wiederhold *et al.*, 2009). Csc1p is a stress sensitive permeable cationic channel that is in endoplasmic reticulum, vacuolar membrane and plasma membrane, and allows calcium to enter or exit. This channel is thought to play an important role in detoxification (Liebert *et al.*, 2006) and autophagy. Furthermore, it is known that this channel is also associated with endocytic and vacuolar biosynthetic pathways, but its function is not yet well understood (Shirahama *et al.*, 1997).

In fact, little is known about the role of this calcium channel in yeast, however some studies have revealed countless similarities between yeast *CSC1* and *Arabidopsis thaliana ATCSC1*. AtCSC1 is a transmembrane multi-stranded protein that functions as a channel for various cations including Ca^{2+}, K^{+} and Na^{+}, and that can be activated by hyperosmotic shock. Csc1p homologous membrane topology is thought to be conserved in several species including *S. cerevisiae* and humans **(Fig. 9)** (Hou *et al.*, 2014).

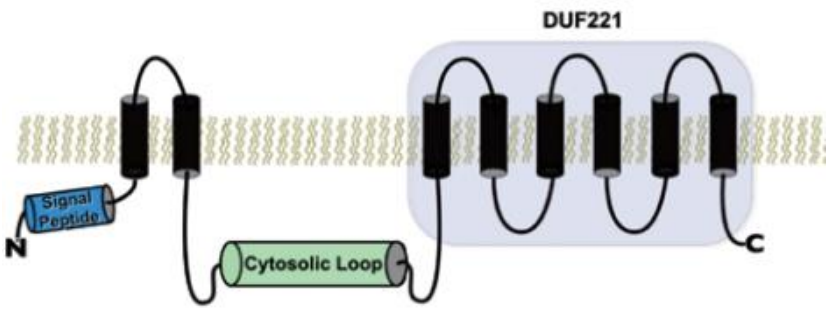


Figure 9 - Predicted transmembrane topology of AtCSC1. Csc1p is a channel present in the vacuolar membrane that shares the same membrane topology with the AtCSC1 homolog. Both N- and C-terminal of this transmembrane protein are located in the cytosol (adapted from Hou *et al.*, 2014).

The phenotypes exhibited when the *CSC1* gene is deleted in *S. cerevisiae* cells differ depending on the conditions to which they are exposed. In a study by Trotter *et al.*, the sensitivity to acrolein was studied in order to identify the protection mechanisms that eliminate reactive aldehydes in yeast. The cells were incubated with an acrolein concentration that allows normal growth of the wild type strain but prevents the growth of sensitive mutants. Under these conditions, no growth of *csc1*Δ mutants was observed, indicating that deletion of this gene makes cells hypersensitive to acrolein (Trotter *et al.*, 2006).

Although there are some works that address the phenotypes of *csc1*Δ mutant, the available information is still very scarce and, therefore, it would be pertinent to study and unveil possible roles of this carrier.

I.5.4. The Pep3p protein, a component of tethering complexes

As stated in previous topics, vacuolar fusion is divided into four phases. Various proteins are required for the tethering phase in vacuolar fusion. All the proteins of the HOPS and the CORVET (class C core vacuole/endosome tethering) complexes play a crucial role in vacuolar fusion. Pep3p is a component of these complexes that acts during the docking stage of vacuole fusion (Balderhaar & Ungermann, 2013).

It is known that the *PEP3* deletion causes deficiencies in Proteinase A (Pep4p), Proteinase B, carboxypeptidase Y (CpY), and alkaline phosphatase (ALP) activities, gross defects in vacuolar structure, and a variety of additional phenotypic abnormalities that may or may not be directly related to vacuolar functions (Preston *et al.*, 1991). This mutant is also defective in autophagy (Rieder & Emr, 1997). It was also reported by Ding *et al.* that overexpression of *PEP3* increases yeast tolerance to AA by shortening the lag phase. In this same study, they proposed that overexpression of *PEP3* may shift the balance to

fission events by interfering with the HOPS complex-mediated fusion reaction, alone or in concert with other non-HOPS component(s). Furthermore, overexpression of *PEP3* is associated with increased V-ATPase activity (Ding *et al.*, 2017).

I.5.5. The V-ATPase and its Vma4p and Vma16p subunits

In all eukaryotic cells, the main responsible for organelle acidification is the proton-translocating ATPase (V-ATPase) present in various organelles, including the vacuole/lysosome, Golgi apparatus and endosomes. V-ATPase is an electrogenic proton pump responsible for pumping cytosol H^+ into to the lumen of vacuole, and therefore for establishing and maintaining its acidic pH. This process is ATP-dependent, and also allows a positive transmembrane potential in this compartment. Besides its function in cellular pH homeostasis, the V-ATPase plays a core role in the regulation of vacuolar fusion and fission and in intracellular transport (Baars *et al.*, 2007; Parra *et al.*, 2014).

This multimeric protein complex is composed of 14 different subunits, which are organized into two macrodomains, V_1 and V_0. These domains have a different size, V_1 has a weight of 650 kDa while V_0 is smaller and has a weight of 260 kDa. The V_1 domain is a peripheral complex located on the cytoplasmatic side of membrane responsible for ATP hydrolysis. This domain in yeast is composed of eight different subunits (A, B, C, D, E, F, G, H). The V_0 domain is an integral membrane domain and it contains six different subunits (a, c, c´, c", d and e) (Aufschnaiter & Büttner, 2019) with a role in rotational catalysis and transport of protons across the membrane **(Fig. 10)** (Kane, 2006).

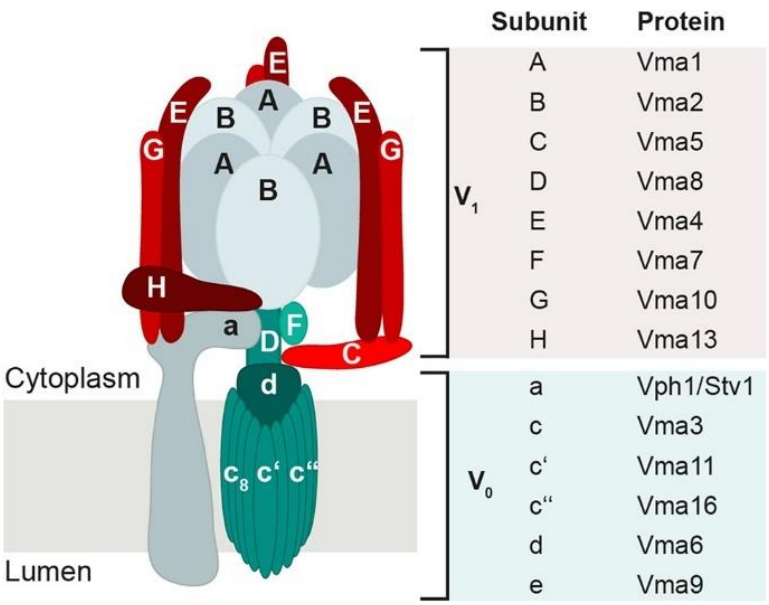


Figure 10 - Molecular structure of the V-ATPase. The V-ATPase is a multimeric protein complex, organized in distinct domains. It is composed of a peripheral domain (V_1), which is responsible for ATP hydrolysis; and an integral domain (V_0), involved in proton translocation across the membrane (adapted from Aufschnaiter & Büttner, 2019).

The hydrophilic polypeptide Vma4p is the subunit E of the V_1 domain of V-ATPase. This is a stator subunit and it has a relevant importance in the binding to the RAVE complex (Regulator of the ATPase of Vacuolar and Endosomal membranes). RAVE complex plays a role in the assembly and disassembly of the V-ATPase. This complex together with aldolase enzyme binds to this subunit and allows the assembly of V-ATPase (Lu *et al.*, 2001). This peripheral subunit appears to be an essential component of the V-ATPase "motor", since in its absence a complete failure to assemble the V-ATPase occurs. So, this subunit is critical for the assembly and activity of the V-ATPase (Ho *et al.*, 1993). The *VMA4* gene is not crucial for the survival of the yeast cells under physiological conditions, however, it is required under conditions of temperature stress (Foury, 1990). Yeast cells lacking Vma4p are more sensitivity to oxidative stress caused by H_2O_2 (Ando *et al.*, 2007). Also, when the pH is changed to alkaline, *vma4*Δ presents higher level of sensitivity (Serrano *et al.*, 2004). These evidences support the importance of this subunit for the normal function of cell, mostly under stress conditions.

The Vma16p is the subunit c" of the V_0 domain of V-ATPase. This is an integral protein with a weight of 23 kDa and has two carboxylates, one in helix 3 and the other in helix 5. This is a rotary subunit that enable the H^+ transport, and it is essential for enzyme activity. This subunit contain a glutamic acid residue important for its function, and inactivating mutations on this residue influences the assembly of the enzyme complex (Hirata *et al.*, 1997). Cells lacking Vmap16p have similar phenotypes to cells deleted

in Vma3p and Vma11p. The loss of one these subunits results in the failure to assemble the V_0 subcomplex (Graham *et al.*, 2000)

CHAPTER II – *RATIONALE* AND AIMS

Since many of the higher eukaryotic cellular processes are conserved in the yeast *Saccharomyces cerevisiae*, it has been exploited as a simple cell model system to understand the molecular mechanisms underlying regulated cell death processes triggered by different stimuli (Botstein & Fink, 1988). Several authors have reported that lethal doses of acetic acid (AA) can trigger regulated cell death in yeast (Ludovico *et al.*, 2001, 2003). This fact has attracted the interest of several research fields, such as biotechnology with the view of developing strategies to modulate regulated cell death and improve *S. cerevisiae* performance for industrial applications. During AA-induced apoptosis in *S. cerevisiae*, numerous changes occur in the cell. Several studies have shown that the vacuole plays an important role in this process by regulating the cytosolic levels of various ions, proteases and hydrolases (Mira *et al.*, 2010; Pereira *et al.*, 2010; Schauer *et al.*, 2009). It has also been shown that the vacuolar protease Pep4p, for example, is released from the vacuole into the cytosol during AA-induced apoptosis, playing a protective or executing role depending on the genetic background (Pereira *et al.*, 2010). Interestingly, a similar process occurs during acetate-induced apoptosis in colorectal cancer cells since CatD, the human ortholog of the yeast Pep4p, is released from lysosomes into the cytosol (Marques *et al.*, 2013). Thus, although little is known about their role, these organelles (vacuole in yeast and lysosome in mammalian cells) seem to be crucial in the regulated cell death process triggered by AA/acetate. Therefore, understanding the involvement of vacuolar proteins, particularly of vacuolar membrane proteins on the partial vacuolar/lysosomal membrane permeabilization and subsequent apoptotic cell death triggered by these stimuli will contribute to deeper understand these processes with potential impact on biotechnological/biomedical fields.

To achieve this goal, in this work, the putative involvement of different vacuolar proteins in this process was assessed, taking advantage of yeast functional genomic approaches. Specifically, we selected the mutant strains lacking distinct vacuolar membrane proteins namely Zrt3p, Vtc4p, Csc1p, Pep3p, Vma4p and Vma16p to:

- Characterize their phenotype in response to AA-induced apoptosis;
- Determine the involvement of those proteins or their associated cellular functions in the vacuolar membrane permeabilization and translocation of Pep4p from vacuole to cytosol.

To accomplish the objectives established for this study, classical biochemistry techniques, molecular biology and analytical methods, such as fluorescence microscopy and flow cytometry, were used.

CHAPTER III – MATERIALS AND METHODS

III.1. Yeast strains

All *S. cerevisiae* BY4741 strains used in this study are listed in **Table 2** along with their genotype. All strains were also used to perform cell survival assays. For flow cytometry assays, the wild type (wt) and *csc1*Δ strains were used. For fluorescence microscopy studies, the wt and the mutant strains *csc1*Δ and *vtc4*Δ were transformed with the plasmid pRS413-Pep4-mCherry to monitor the release of Pep4p.

Table 2 - *S. cerevisiae* strains used in this study.

Yeast Strains	Genotype	Source
BY4741	MATa; *ura3Δ0*; *leu2Δ0*; *his3Δ1*; *met*15Δ*0*	EUROSCARF
***zrt3*Δ**	BY4741 YKL175w::*kanMX4*	EUROSCARF
***vtc4*Δ**	BY4741 YJL012c::*kanMX4*	EUROSCARF
***csc1*Δ**	BY4741 YPR173c::*kanMX4*	EUROSCARF
***pep3*Δ**	BY4741 YLR148w::*KanMX4*	EUROSCARF
vma4Δ	BY4741 YOR332w::*kanMX4*	EUROSCARF
***vma16*Δ**	BY4741 YHR026W::*KanMX4*	EUROSCARF
BY 4741 pRS413-Pep4-mCherry	BY4741 pRS413-Pep4 -mCherry (HIS3)	Matos, 2018
BY 4741 *vtc4*Δ pRS413-Pep4-mCherry	*vtc4*Δ harboring pRS413-Pep4-mCherry (HIS3)	This study
BY 4741 *csc1*Δ pRS413-Pep4-mCherry	*csc1*Δ harboring pRS413-Pep4-mCherry (HIS3)	Matos, 2018

III.2. Growth conditions and treatments

Cells were grown in specific growth media under the same conditions and subjected to different treatments **(Fig. 11)**. All conditions associated with growth and treatments are described in the following sub-topics.

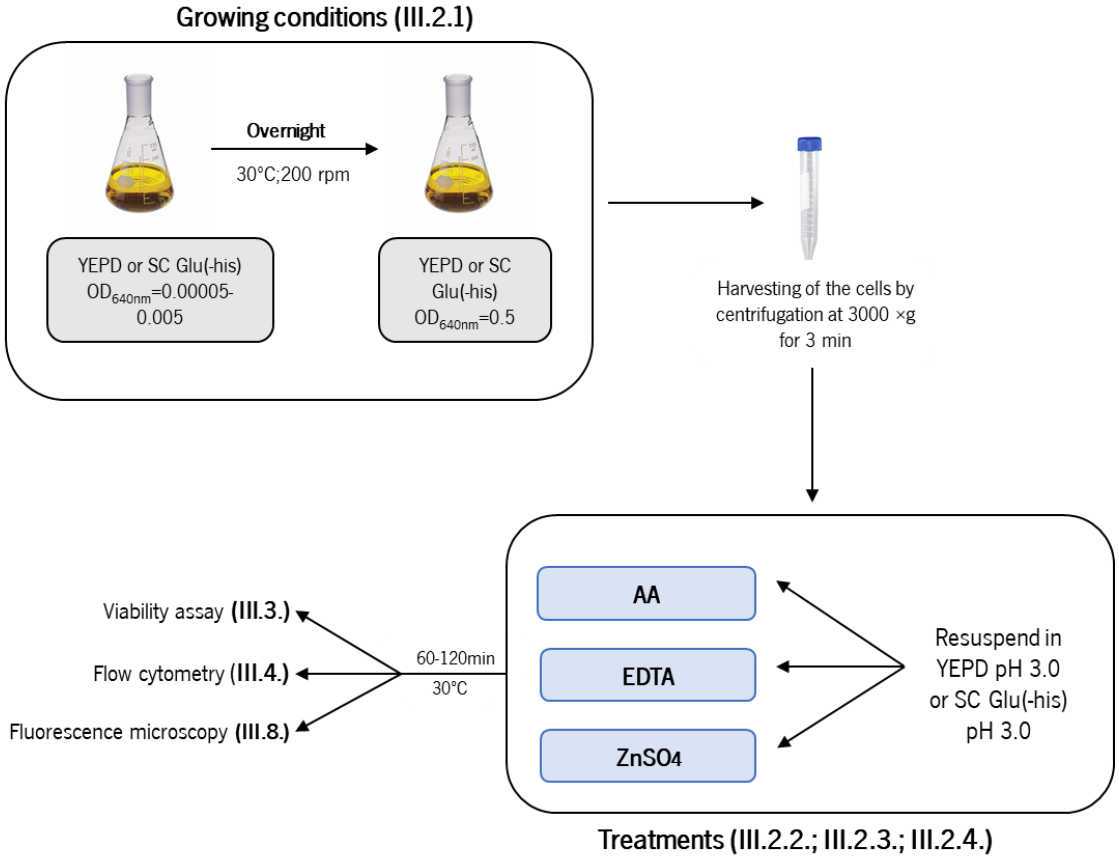


Figure 11- Schematic representation of the growth conditions used in this work. Cells were cultivated in an appropriate medium, incubated overnight with 200 rpm agitation at 30˚C until OD_{640nm} = 0.5. Each assay was started by collecting the cells by centrifugation at 3000 ×g for 3 min, followed by resuspension in the same medium at pH 3.0 .

III.2.1. Growth conditions

S. cerevisiae strains were grown on YEPD (Yeast Extract Peptone Dextrose) medium plates [1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose and 2% (w/v) agar] at 30°C for 2 days. Afterwards, cells were transferred to liquid YEPD with a cell density adjusted to an optical density at 640 nm (OD_{640nm}) of 0.0005-0.005 depending on the strain and incubated at 30°C overnight with shaking (200 rpm) until exponential phase (OD_{640nm}=0.5) is reached.

The strains that were transformed with pRS413 plasmid were selected and grown in synthetic complete medium without histidine [2% (w/v) glucose; 0.5% (w/v) ammonium sulphate; 0.7% (w/v) yeast nitrogen base w/o ammonium sulphate and w/o aminoacids; 0.2% (w/v) dropout mixture; 0.01% (w/v) uracil and tryptophan; 0.02% (w/v) leucine]. The dropout mix contains 1.44% (w/w) adenine, 5.76% (w/w) arginine, 5.76% (w/w) asparagine, 5.76% (w/w) aspartate, 5.76% (w/w) cysteine, 5.76% (w/w) glutamate, 5.76% (w/w) glutamine, 5.76% (w/w) glycine, 5.76% (w/w) isoleucine, 5.76% (w/w) lysine, 5.76% (w/w) methionine, 5.76% (w/w) phenylalanine, 5.76% (w/w) proline, 5.76% (w/w) serine, 5.76% (w/w) threonine, 5.76% (w/w) tyrosine, 5.76% (w/w) valine, 5.76% (w/w) myo-inositol, and 0.58% (w/w) para-aminobenzidine. All these strains were grown at 30°C with agitation at 200 rpm, until they reach the exponential phase (OD_{640nm}=0.5).

III.2.2 Acetic acid treatment

For acetic acid (AA) treatment, strains were cultured under the conditions described above (III.2.1.). When cells reached the exponential phase, they were collected and resuspended in the appropriate medium adjusted to pH 3.0 **(Fig. 11)**. This pH is important because, at this pH, the undissociated form of the acid predominates, which facilitates its entry into the cell by simple diffusion (Pereira *et al.*, 2010).

The treatment was performed by adding or not AA to the resuspended cells to obtain a final concentration of 75 mM or 100 mM (in the case of the *zrt3*Δ mutant assays). Then, the cells were incubated at 30°C with 200 rpm shaking for 120 min. The cells were then harvested before the addition of AA and after 60 and 120 min after treatment.

III.2.3. EDTA treatment

EDTA was used as a zinc chelating agent to restrict the free zinc available in the cell. In the zinc limitation assays, cells were cultured as described above (III.2.1). When the cells reached the exponential phase, pre-treatment with and without 1 mM EDTA (stock solution of 100 mM), was performed for 30 min at 30°C and 200 rpm. After harvesting and resuspending cells in the specific medium adjusted to pH 3.0, cells were treated with 1 mM EDTA, 75 mM AA or co-treated with 1 mM EDTA and 75 mM AA, followed by 90 min incubation at 30°C, 200 rpm. The time point 0 was obtained before the addition of AA and EDTA. For the negative control, cells were processed under the same conditions but were not incubated with EDTA and AA.

III.2.4. $ZnSO_4$ treatment

Zinc sulphate was added to the extracellular medium to increase the intracellular zinc levels. In the zinc supplementation assays, cells were cultured as described above (III.2.1). When the cells reached the exponential phase, they were collected and resuspended in the specific medium adjusted to pH 3.0. Subsequently, cells were treated with 10 mM $ZnSO_4$ (stock solution of 200 mM), 100 mM AA or co-treated with 10 mM zinc sulfate and 100 mM AA, followed by 60 min incubation at 30°C, 200 rpm. The time point 0 was obtained before the addition of AA and $ZnSO_4$. For negative control, cells were processed under the conditions but were not incubated with $ZnSO_4$ and AA.

III.3. Assessment of cell survival by colony forming units (c.f.u.) counting and spot assays

Cells were treated as aforementioned (III.2.2, III.2.3, III.2.4). After treatment, cell survival was evaluated at different timepoints by the standard plate count method taking 100 µl of the culture and diluting it in a total of 1 ml of sterile deionized water, followed by 10^{-4} serial dilutions. The 0 min sample was collected before any treatment. Five drops of 40 µl from each dilution were then plated on YEPD plates. These plates were incubated at 30°C for 2-3 days and cell viability was assessed by counting the colony forming units (c.f.u.). When the OD of the cell suspension did not increase along treatment, the percentage of cell survival was calculated from the ratio between the mean number of colonies at each time point and the mean number of colonies at time zero of the respective strain, multiplied by 100. When the OD changed along treatments, the percentage of cell survival was calculated from the ratio between the mean number of colonies of each condition and the mean number of expected colonies, estimated by considering the OD and respective number of colonies counted at time zero (see Fig. 22 on Supplementary Material for OD change along time). To perform the spot assays, 5 µl drops taken directly from the different cell cultures adjusted to the same ODs and 5 µl drops of each serial dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) were plated in YEPD plates.

III.4. Evaluation of superoxide anion accumulation and calcium intracellular levels in response to acetic acid treatment by flow cytometry

In order to assess the accumulation of the superoxide anion, the dihydroethidium (DHE) probe was used. In the absence of $O_2^{\cdot-}$, the probe exhibits blue fluorescence in the cytosol. However, in the presence of this anion, the probe exhibits red fluorescence due to its oxidation. In addition to the change

in fluorescence, the probe localization is also altered and visible in the nucleus and not in the cytosol, because it intercalates with the DNA (Dikalov & Harrison, 2014; Zhao *et al.*, 2003).

Cells were treated as described in III.2, collected by centrifugation at 3000 ×*g* for 3 min, washed with deionized water and resuspended in 500 µl of PBS 1× (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4). Cells were then stained with DHE at a final concentration of 5 µg/ml for 20 min at room temperature. After staining, the samples were analyzed by flow cytometry. The autofluorescence of the unstained cells in the red channel (FL4) was used to set the DHE-positive cells' gate.

In order to assess the levels of intracellular calcium, the Fluo4-AM probe was used. This probe is used in the esterified (AM, acetoxymethyl) form, which facilitates its diffusion through the cell membrane. Within the cell, the AM group is cleaved by intracellular esterases, releasing Fluo4 that binds specifically to free calcium ions. In this sense, in the presence of Ca^{2+}, the fluorescence of this probe greatly increases (Gee *et al.*, 2000).

Cells were treated as described above (III.2), and then stained with 5 µM Fluo4-AM for 90 min at 30°C in the dark. Subsequently, cells were washed with PBS 1× and analysed at the flow cytometer. As positive controls, BY4741 cells were treated with H_2O_2 and calcimycin. Regarding calcimycin experiments, cells were resuspended in 50 mM Tris, 2mM $CaCl_2$, pH 7.0 buffer and then treated with different concentrations of calcimycin (2 µM, 4 µM, 10 µM) for 30 minutes at 30°C. As for H_2O_2, cells were treated with 3 mM H_2O_2 for 60 min at 30°C. Next, all conditions were stained with 5 µM Fluo4-AM for 90 minutes at 30°C in the dark and subsequently washed with PBS 1×. After staining, the samples were analyzed by flow cytometry. Green mean fluorescence intensity (FL1) of unstained cells was considered the autofluorescence of each sample.

Flow cytometry analysis were performed using an EC800 Sony Biotechnology flow cytometer equipped with an argon-ion laser emitting a 488 nm beam at 50 mW. For evaluation of superoxide anion accumulation, yeast cell populations with high frequency and homogeneity were gated in a histogram of Side Scatter (SS) × Forward Scatter (FS), and then in a SS × FL-4 (660-700 nm) histogram for fluorescence analysis. Cells exhibiting higher FL4 fluorescence intensity comparing to the autofluorescence were gated and considered superoxide anion (DHE)- positive cells. In the case of calcium levels measurement, the approach was similar but, for the fluorescence analysis, the parameter used was the mean fluorescence intensity of the green channel. For each sample, about 30 000 events were evaluated and further analyzed using FlowJo® 7.6 software.

III.5. Analysis of catalase and superoxide dismutase activity

III.5.1. Preparation of protein extracts

To prepare total protein extracts, cells were grown and treated as described above. After treatment, cells were collected by centrifugation at 3000 ×*g* for 3 min and washed with 20 ml of H_2O. Next, cell lysis was carried out in a FASTprep equipment (5-10 × 30s cycles) using a cell suspension resuspended in lysis buffer (0.6 M Mannitol, 2 mM EGTA, 10 mM Tris, 1 protease inhibitor cocktail tablet without EDTA/10 ml, pH 6.8) and glass beads (1/3 volume). To ensure that the cycles required for cell disruption were performed, the lysis of the cells was followed under the optical microscope. Next, cell debris were removed by centrifugation at 13000 ×*g* for 10 min at 4˚C, and the supernatant (corresponding to the total protein extract) was collected.

III.5.2. Protein quantification

Protein concentration was determined by the Lowry method (Lowry *et al.*, 1951), using a Thermo Sprectronic Genesys 20 spectrophotometer. Briefly, 750 µl of solution A (2% NaK Tartrate, 1% $CuSO_4$, 0.1 M NaOH, 4% Na_2CO_3) were added to 10 µl of each sample. Next, 75 µl Folin-Ciocalteu reagent was added and samples were incubated for 15 min at 37°C. Absorbance at 750 nm was measured and protein concentration was estimated in relation to a standard curve built with 0.1 µg/µl - 10 µg/µl BSA.

III.5.3. Evaluation of superoxide dismutase activity by native gel electrophoresis

Protein extract samples (10, 20 or 60 µg) were separated by 15% non-denaturing polyacrylamide gel electrophoresis (native PAGE) to assess Sod1p activity. Electrophoresis was run for approximately 3 h at 20 mA per gel at 4˚C. In each assay, the samples were run in duplicate to further perform two staining methods: Coomassie blue and Nitro Blue Tetrazolium (NBT).

Sod1p activity was monitored using NBT method. To this end, one of the gels was stained using a solution containing 50 mM potassium phosphate (pH 7.8), 0.1 µl/ml TEMED, 0.13 mg/ml NBT, and 0.1 mg/ml riboflavin for 45 min in the dark. After 45 min of incubation in slow agitation, the gel was exposed to light for 2 min. Under light exposure, riboflavin is reduced, which leads to the production of superoxide radicals. Then, O_2^- is reduced by NBT to create insoluble blue formazan. Sod1p activity is seen as a colorless band in the blue background because Sod1p scavenges the superoxide, thus inhibiting the blue color formation (Weydert & Cullen, 2010).

In addition, the second gel was carefully transferred to a Coomassie staining solution (0.2% coomassie blue, 7.5% of acetic acid, 40% ethanol) for 20 min under stirring. Subsequently, the staining solution was removed, and the gel was washed with discoloration solution (10% of AA, 20% ethanol). After saturation of this solution, it was removed and a new one was added to the gel. Gel washing was repeated several times until the blue bands were observed.

Finally, the photos of both gels were taken in transilluminator to then perform its quantification using the ImageJ Fiji software.

III.5.4. Evaluation of catalase activity by the Clark electrode

The catalase activity of the soluble protein extracts was measured using a Clark electrode to monitor the rate of production of O_2. The protein concentration of each sample was set to a final concentration of 0.03 µg/µl (optimized concentration) in 2 ml of 50 mM phosphate buffer (pH 7.0). After stabilization of the oxygen recorder containing the protein samples, 0.5 - 6 mM H_2O_2 substrate was added to measure catalase activity. For each sample/condition this process was repeated 3 times. Catalase activity was estimated as the number of divisions per sec per ng of protein **(Fig. 12)**.

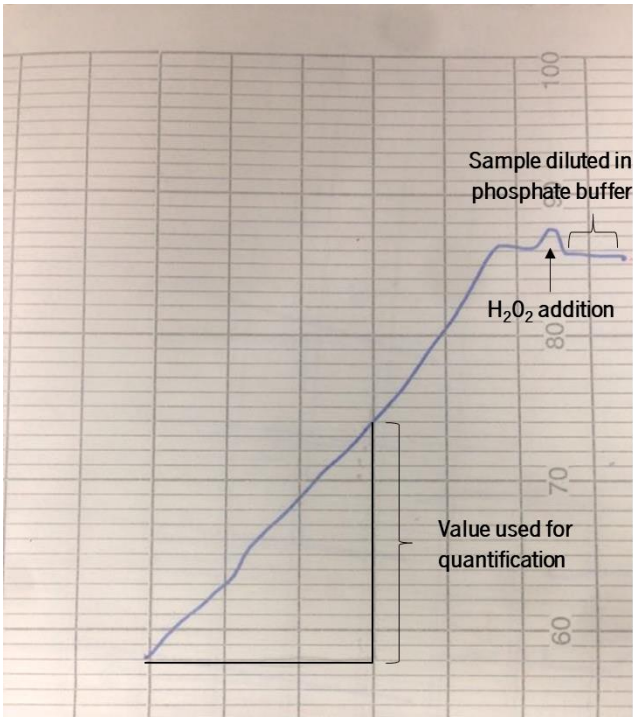


Figure 12 Representative example of recording change in response to oxygen production after H_2O_2 addition as a result of catalase activity. Catalase activity was estimated as the number of divisions ("value used for quantification" depicted in the figure) per sec (taking into account the paper velocity at 0.5 mm/s) per ng of protein (having as reference the initial protein concentration of 0.03 µg/µl).

III.6. Plasmid extraction

Escherichia coli was used to amplify the pRS413 Pep4-mCherry plasmid. For this, the cells already transformed with this plasmid were incubated overnight at 37˚C in liquid LB medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl] containing 100 μg/ml ampicillin to select positive transformants. The plasmid was extracted from cells from the overnight culture using the GenElute Plasmid Miniprep Kit (Sigma Aldrich). Initially, *E. coli* cells were harvested by centrifugation, resuspended in "Resuspension Solution" containing RNase and incubated for 5 min with "Lysis Buffer". After lysis, to precipitate cellular debris, "Neutralization Buffer" was used, and samples were centrifuged at maximum speed. A binding column was assembled in a microcentrifuge tube and the "Column Preparation Solution" was added to this column to maximize binding of the DNA to the membrane. The supernatant was then transferred to the column and centrifuged at full speed to retain only the DNA in the column. Then, two column washing steps were performed to eliminate possible residues and one centrifugation to dry it. Finally, DNA bound to the column was recovered by addition of "Elution Buffer" and centrifugation at maximum speed for 1 min. Subsequently, the extracted plasmid DNA was quantified in a NanoDrop device (**Table 3**). After quantification, the yeast cells were transformed, as described in the next section.

Table 3- NanoDrop quantification of extracted plasmid DNA and contamination ratios.

DNA quantification (ng/μL)	A_{260}/A_{280}	A_{260}/A_{230}
127,5	1,88	2,72

III.7. Yeast transformation using LiAc/SS carrier DNA/PEG method

For transformation with the pRS413 Pep4-mCherry plasmid, the strain *S. cerevisiae* BY 4741 *vtc4*Δ was grown overnight in YEPD medium. Cells were then diluted to an OD_{640nm}=0.2 in YEPD containing 4% (v/w) glucose, incubated at 30 ˚C with agitation at 200 rpm, and allowed to reach OD_{640nm}=0.8. Then, cells were collected by centrifugation at 3000 ×*g* for 5 min, washed to remove any remaining medium and resuspended in 1 ml of sterile deionized water. For transformation, 360 μl of transformation mix **(Table 4)** containing or not the plasmid DNA were added to 100 μl of the cellular suspensions and incubated at 42˚C for 40 min. Cells were then centrifuged at maximum speed to remove the transformation mix, resuspended in 50 μl of sterile deionized water, plated in synthetic complete medium

without histidine and incubated at 30˚C until the appearance of transformants. A negative control corresponding to the cells incubated with the same mix but without plasmid DNA was also performed.

Table 4- Composition of the transformation mix for one transformation.

Reagents	Plasmid Mix (µl)
Lithium acetate (LiAc) (1M)	36
PEG (50%)	240
H_20	30
ssDNA carrier (2 mg/ml)	50
Plasmid DNA	4

III.8. Assessment of Pep4p localization and vacuolar membrane permeabilization by fluorescence microscopy

The strains expressing Pep4-mCherry were treated as described above. To evaluate vacuolar membrane permeabilization (VMP), cells were collected and stained for 20 min in the dark with Celltracker™ Blue CMAC (7-amino-4-chloromethylcoumarin) stain at a final concentration of 2 µM (stock solution of 100 µM in DMSO). This probe is sequestered in the vacuole exhibiting a bright blue fluorescence. However, when the vacuolar membrane is compromised, this probe is no longer accumulated in the vacuole, exhibiting a diffuse fluorescence in the cell (Stewart & Deacon, 1995). This procedure was used to assess Pep4p localization and VMP during AA-induced cell death.

Fluorescence microscopy experiments were performed using a Leica Microsystems DM-5000B epifluorescence microscope coupled to a Leica DCF350 FXR2-193510309 digital camera. The photomicrographs obtained were acquired and processed using Leica Application Suite (LAS) AF 6000LX Microsystems software along with a 100× oil-immersion objective and the appropriate filter settings for differential phase contrast (DIC), red fluorescence (N21) and blue fluorescence (A). For quantification, at least 300 cells of independent experiments were counted.

III.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 software and p-values <0.05 were considered statistically significant in all experiments.

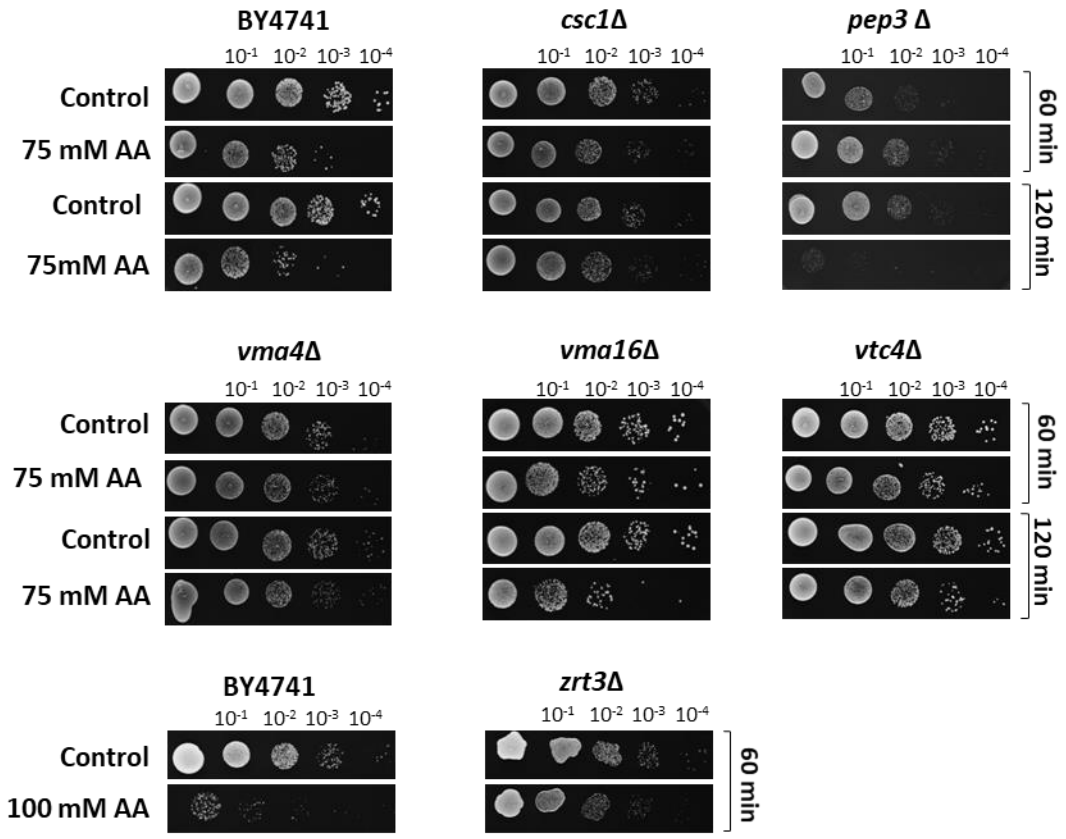
CHAPTER IV – RESULTS

IV.1 Role of the vacuolar membrane proteins Csc1p, Pep3p, Vma4p, Vma16p, Vtc4p and Zrt3p in acetic acid-induced cell death of *S. cerevisiae*

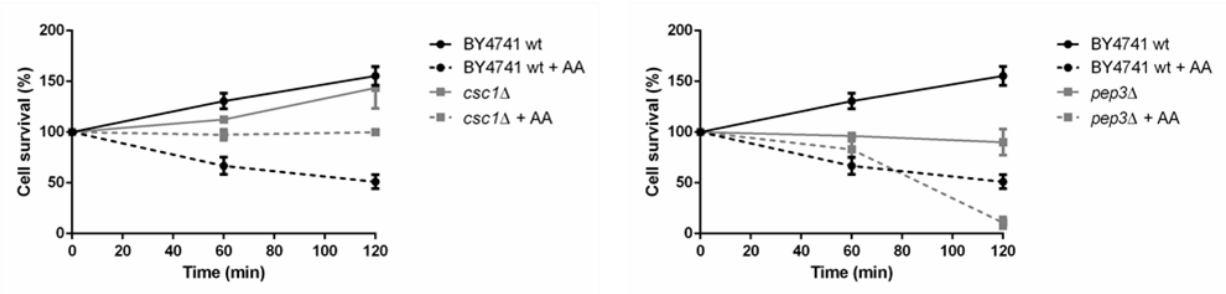
As referred in the Introduction, previous studies showed that acetic acid (AA) induces an apoptotic cell death in yeast associated with a selective vacuolar membrane permeabilization (VMP) and release of the vacuolar protease Pep4p in the cytosol. In order to further understand the putative role of vacuolar membrane proteins in this regulated cell death process, in particularly in these two events, we sought to characterise the phenotype of a set of mutant strains lacking the following vacuolar membrane proteins: Csc1p, a calcium permeable channel; Pep3p, a component of HOPS and COVERT tethering complexes; Vma4p and Vma16p, two subunits of V-ATPase; Vtc4p, a subunit of the vacuolar transporter chaperone complex; and Zrt3p, a zinc transporter.

We started by treating the deleted mutant strains as well as the wt strain in the absence or presence of AA along time. Results from the semi-quantitative spot assays **(Fig. 13A)** assays demonstrate that *CSC1*, *VMA4* and *VTC4* deletions increased cell survival to 75 mM AA treatment, pH 3.0, while *PEP3* deletion has the opposite effect. According to a previous work in our lab (Matos, 2018), the *zrt3*Δ strain exhibited a resistance phenotype when treated with 100 mM AA, pH 3.0, as we also attested in this study. Quantitative cell survival assessment revealed *vtc4*Δ and *vma4*Δ mutants are the most resistant mutants to 75 mM AA, followed by the *csc1*Δ mutant **(Fig. 13B)**. Also, the *zrt3*Δ mutant, exhibited a high cell survival in comparison with the wt strain upon treatment with 100 mM AA **(Fig. 13B)**. In contrast, the *pep3*Δ mutant showed sensitivity to AA treatment, while the *vma16*Δ mutant exhibits a similar phenotype in comparison with the wt strain **(Fig. 13B)**.

A


BY4741
csc1Δ
pep3 Δ
vma4Δ
vma16Δ
vtc4Δ
zrt3Δ
10-1 10-2 10-3 10-4
Control
75 mM AA
75mM AA
100 mM AA
60 min
120 min

B


csc1Δ
pep3Δ
Cell survival (%)
Time (min)
BY4741 wt
BY4741 wt + AA
csc1Δ
csc1Δ + AA
pep3Δ
pep3Δ + AA

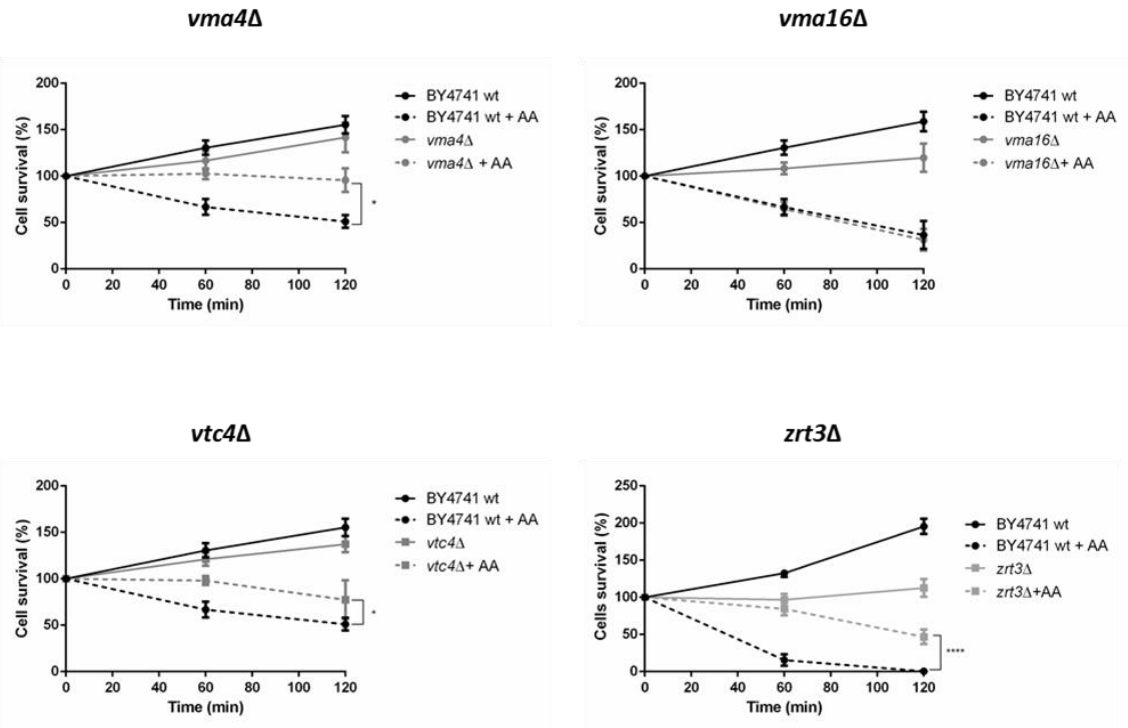


Figure 13- Assessment of cell viability of different mutant strains lacking the vacuolar membrane proteins Csc1p, Pep3p, Vma4p, Vma16p Vtc4p and Zrt3p upon acetic acid treatment. *S. cerevisiae* BY4741 wild type and indicated mutant strains were grown in YEPD until exponential phase and then harvested and resuspended in YEPD pH 3.0 without or with 75 mM AA (for *csc1*Δ, *pep3*Δ, *vma4*Δ, *vma16*Δ and *vtc4*Δ strains) or 100 mM AA (for *zrt3*Δ strain). The wt strain was treated with both AA doses. Cells were then incubated for 120 min. **(A)** Spot assay of undiluted culture and four serial dilutions of cells incubated or not with the indicated concentration of AA were plated in YEPD plates. **(B)** Cell survival of the indicated strains treated with AA was assessed by standard dilution plate counts, at each time point (0, 60 and 120 min) and expressed as a percentage of cell survival on YEPD plates in relation to time 0. Values are the % ± SEM of four independent experiments. Statistical analysis was performed by one-way ANOVA. *p<0.05, ****p<0.0001 in comparison with the wild type strain.

In the following topics, we will further explore the role of some of these proteins, first with emphasis on the zinc transporter and then on Vtc4p and Csc1p proteins.

IV.2 Exploring the role of the Zrt3p protein and zinc homeostasis during acetic acid-induced cell death

Several studies have been highlighting the role of zinc in cellular homeostasis and how it can contribute to determine cell survival or death (Dziedzic & Caplan, 2011; MacDiarmid *et al.*, 2002; Simm *et al.*, 2010; Wan *et al.*, 2015). In this respect, the Zrt3p zinc vacuolar transporter has been shown to be a crucial element, facilitating cellular zinc homeostasis. When a sudden decrease in cellular zinc levels

occurs, this zinc transporter mobilizes the accumulated zinc in the vacuole to meet the cell needs (MacDiarmid *et al.*, 2000).

Previous results from our group showed that the *zrt3*Δ mutant is resistant to cell death induced by AA associated with lower vacuolar acidification and lower accumulation of cellular and mitochondrial superoxide anion, in comparison with the wt strain (Matos, 2018). In addition, a delayed release of Pep4p from the vacuole to the cytosol was also observed in this mutant (Matos, 2018). In the following topics, we will further explore the role of zinc and Zrt3p in AA-induced cell death.

IV.2.1 Zinc limitation or supplementation affects *S. cerevisiae* cell survival and superoxide anion accumulation induced by acetic acid

As referred above, we confirmed that *zrt3*Δ displays increased cell viability than the wt strain after AA treatment **(Fig. 13)**, suggesting that zinc homeostasis may play a role in the cell death process. To address this hypothesis, we decided to analyse the behaviour of the wt strain treated with AA under zinc limitation, through addition of the chelating agent EDTA, or under zinc supplementation, through addition of $ZnSO_4$ **(Fig. 14)**.

Data on zinc limitation conditions showed that, when cells are pre-incubated with EDTA and subsequently incubated with both EDTA and AA, cell survival greatly decreases in comparison with cells treated only with AA **(Fig. 14A)**. In fact, c.f.u counts showed that, in AA-treated cells, cell survival decreases ca. 40% in comparison with the control without any treatment or with cells only treated with EDTA. In the case of cells pre-treated with EDTA and then co-treated with EDTA and AA, this decrease is ca. 80% **(Fig. 14B)**. These results indicate that zinc limitation makes cells more sensitive to AA. In these experiments, we used 75 mM AA since the co-treatment with 1 mM EDTA and AA was extremely toxic with a dose of 100 mM AA.

As for zinc supplementation experiments, results showed that addition of $ZnSO_4$ protects cells from AA-induced cell death as observed in the spots experiments **(Fig. 14C)**. Indeed, cells treated with 100 mM of AA suffer a decrease in cell survival assessed by c.f.u. counts of about 90% in comparison with the control. However, in cells co-treated with AA and $ZnSO_4$this decrease is only ca. 50% **(Fig. 14D)**. Altogether, the results suggest a protective role of zinc in AA-induced cell death.

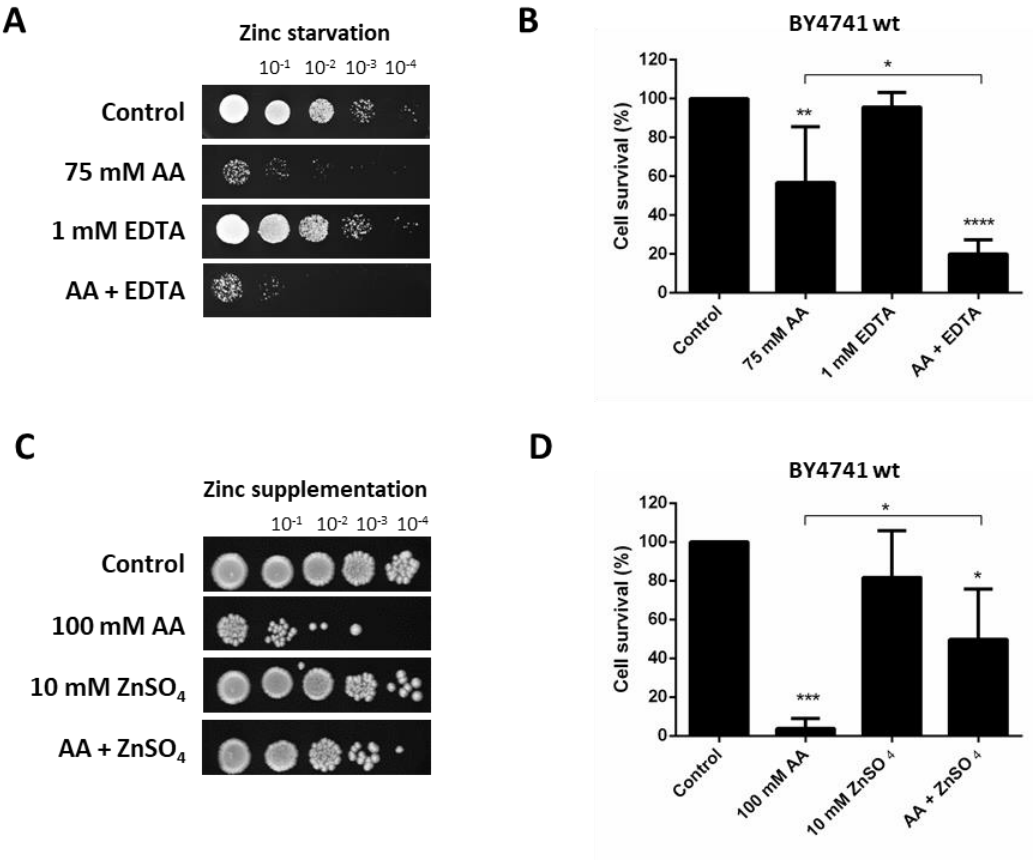


Figure 14 - Effect of zinc limitation or supplementation in AA-induced cell death in *S. cerevisiae* BY4741 wt strain. For the assessment of the effect of EDTA addition, cell survival was assessed by spots assay **(A)** and by standard dilution plate counts and expressed as a percentage of c.f.u. on YEPD plates in relation to time 0 **(B)**. Cells were pre-treated with and without 1 mM EDTA for 30 min and next treated with 1 mM of EDTA, 75 mM of AA or co-treated with both for 90 min. For the assessment of the effect of zinc addition by spots assay **(C)** and cell survival by the percentage of c.f.u. on YEPD plates in relation to time 0 **(D)**, cells were treated with 10 mM $ZnSO_4$, 100 mM AA or co-treated with both for 60 min. Values are the % of cell survival ± SEM of three independent experiments. The control was considered 100% cell survival. Statistical analysis was performed by one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

The same approach was performed using the *zrt3*Δ mutant, however, no differences were observed neither with zinc limitation nor with zinc supplementation, suggesting that zinc levels do not affect the phenotype of this resistant mutant to AA **(Fig. 15 A, B)**.

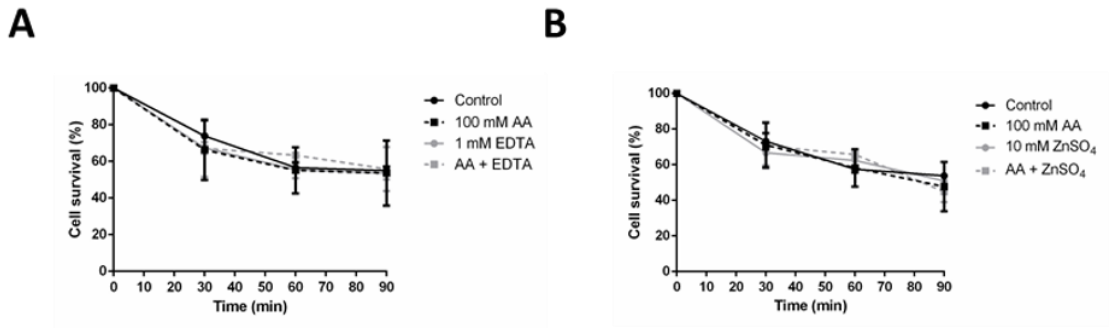


Figure 15 - Effect of zinc limitation or supplementation in AA-induced cell death in *S. cerevisiae* BY4741 mutant strain lacking the vacuolar membrane protein Zrt3p. **(A)** Cell survival under zinc limitation conditions. *zrt3*Δ cells were pre-treated with and without 1 mM EDTA for 30 min and next treated with 1 mM of EDTA, 75 mM of AA or co-treated with both for 90 min. **(B)** Cell survival under zinc supplementation conditions. *zrt3*Δ cells were treated with 10 mM $ZnSO_4$, 100 mM AA or co-treated with both for 60 min. Cell survival was assessed by standard dilution plate counts at each time point (0, 30, 60 and 90 min.), and expressed as a percentage of c.f.u on YEPD plates in relation to time 0. Values are the % of cell survival ± SEM of three independent experiments.

Previous results showed that cell death of *S. cerevisiae* treated with AA was associated with increased accumulation of ROS (Ludovico *et al.*, 2001; Matos, 2018). To find if ROS accumulation could be associated with the phenotypes observed with zinc limitation or supplementation, we next evaluated the accumulation of superoxide anion in these conditions, by flow cytometry using the DHE probe. The gates defined to quantitatively determine the percentage of cells that accumulate superoxide anion are depicted in **Fig. 16A**. Results showed that, 90 min after AA treatment, 30% of the wt cells were DHE-positive, but treatment with AA and EDTA, though not statistically significant, yielded an even higher percentage of DHE-positive cells (ca. 60%). In the control and EDTA treatment conditions, only ca. 5% in average of cells demonstrated superoxide anion accumulation **(Fig. 16B)**. In the zinc supplementation experiments, the percentage of DHE-positive cells after treatment with AA and $ZnSO_4$, though not statistically different, was slightly lower (ca. 10%) than after treatment with AA **(Fig. 16C)**.

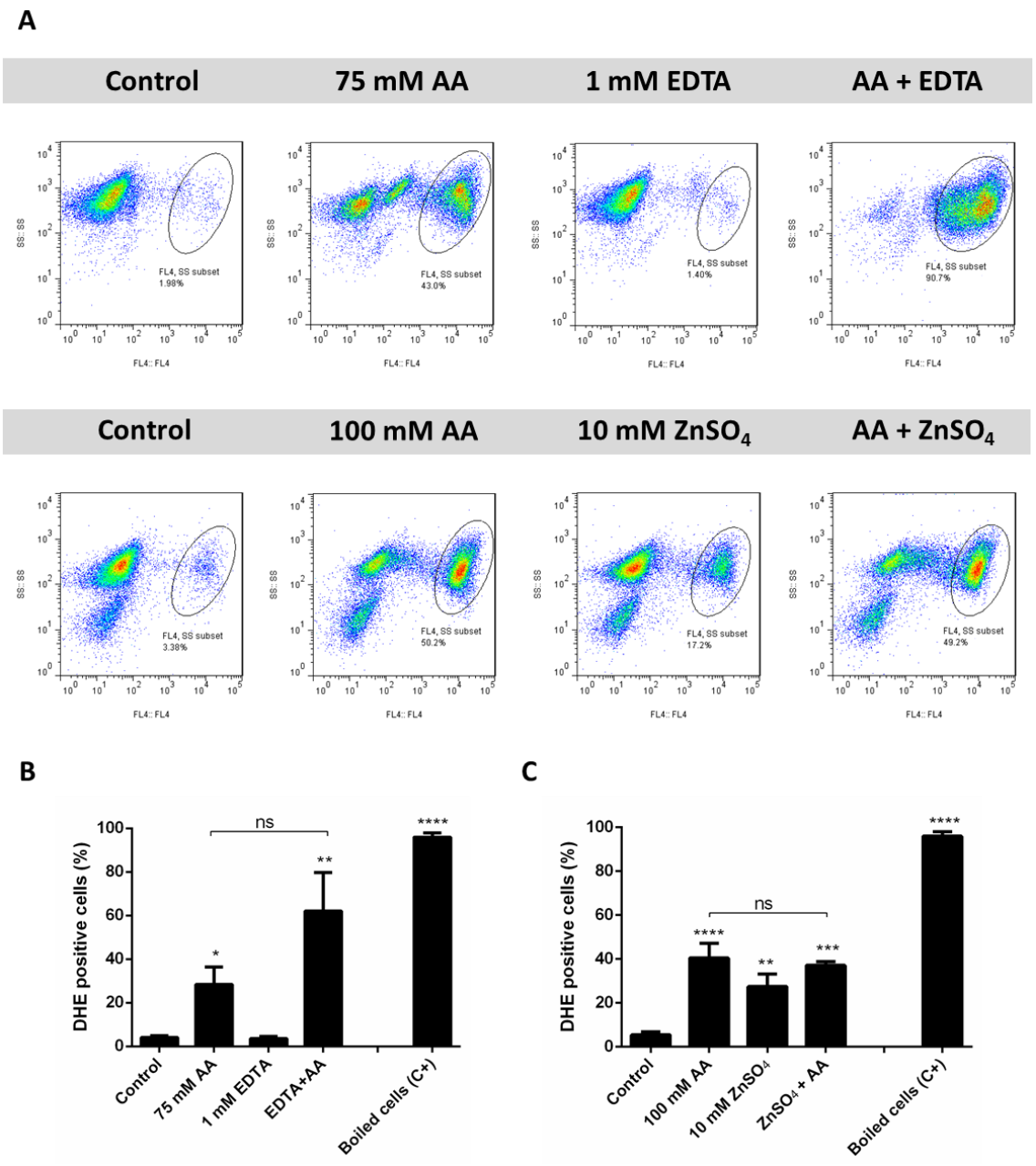


Figure 16 - Effect of zinc limitation or supplementation in AA-induced cellular superoxide anion accumulation in *S. cerevisiae* BY4741. (A) Representative biparametric histograms of FL4 *versus* SS of DHE stained cells and respective gates. FL4 measures red fluorescence intensity and SS the side scatter that reflects cell complexity. **(B)** Quantification of the percentage of cells with positive DHE staining after pre-treatment with and without 1 mM EDTA for 30 min, followed by treatment with 1 mM of EDTA, 75 mM of AA or both for 90 min. **(C)** Quantification of the percentage of DHE positive stained cells after treatment with 10 mM $ZnSO_4$, 100 mM AA or both for 60 min. Values are % of DHE positive cells ± SEM of three (B) and five (C) independent experiments. Statistical analysis was performed by one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 in comparison with the wt. Non-significant values are represented as ns.

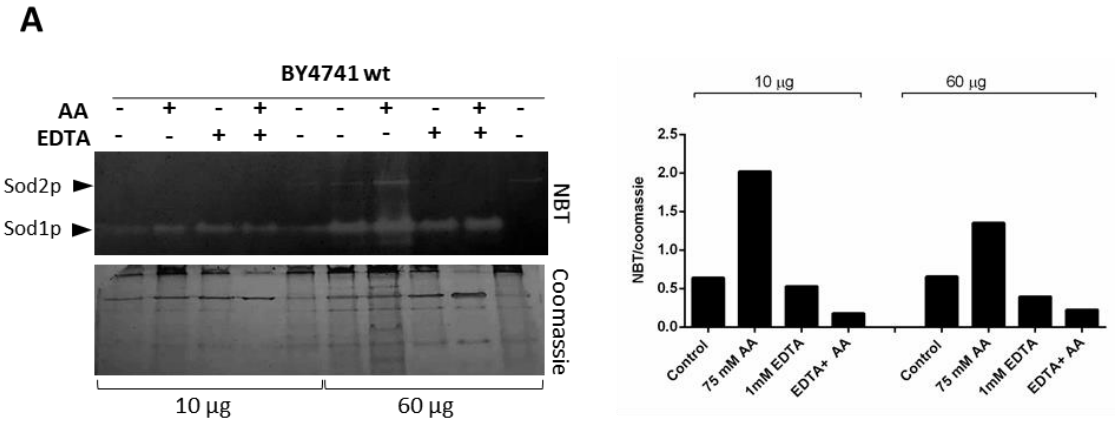
IV.2.2 Acetic acid treatment increases the activity of Sod1 but not of catalase in the wild type strain

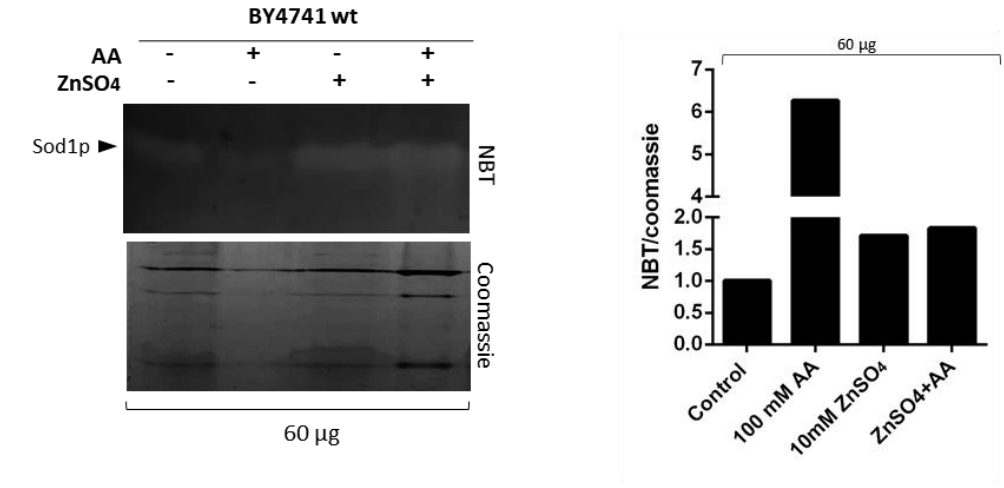
The antioxidant enzymes Sod1 and catalase T, which have zinc as a cofactor, play a direct role in regulating cellular ROS levels (Galiazzo & Labbe-Bois, 1993; Lushchak & Gospodaryov, 2005). Given that both zinc and ROS levels seem to mediate AA-induced cell death in yeast, we sought to study the activity of both enzymes under limitation and supplementation of zinc. It is reported that Sod1 is the first enzyme to act when there is production of superoxide anion (O^{2-}), as we found for AA-treated cells, being responsible for the production of hydrogen peroxide that is oxidized by catalase T (Galiazzo & Labbe-Bois, 1993).

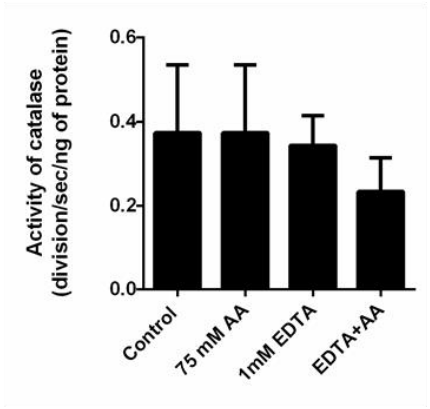
For this purpose, Sod1 activity was measured in cells treated with AA and/or EDTA, and treated with AA and/or $ZnSO_4$, using native gel electrophoresis and NBT method, as reported in the materials and methods section. By analysing the gels, it was possible to quantify the enzymatic activity of Sod1 in each condition **(Fig. 17A)**. The results showed that, in the absence of AA, zinc limitation does not change Sod1 activity compared to the control. However, when cells are incubated with AA there is a 4-fold increase in Sod1 activity in comparison with the control. In turn, co-treatment with AA and EDTA, decreased the activity of this enzyme below the levels of control cells **(Fig. 17A)**, probably because most cells are no longer viable.

When cells are supplemented only with $ZnSO_4$, there is a slight increase in Sod1 activity in comparison with the untreated cells. As for AA-treated cells, since in this experiments we use 100 mM instead of 75 mM AA, a higher increase (ca. 6-fold) in Sod1 activity was observed **(Fig. 17B)**. Nevertheless, when cells are treated with both AA and $ZnSO_4$, Sod1 activity levels decreased to values similar to those of the cells treated only with $ZnSO_4$ **(Fig. 17B)**.

The same samples were used to measure the catalase T activity using a Clark electrode to measure oxygen consumption. The results showed that, in the zinc limitation experiments, though not statistically significant, there was a small decrease in catalase activity when cells were co-treated with EDTA and AA compared to treatment with AA alone **(Fig. 17C)**. Also, in the zinc suplementation assays, although values are not statistically significant, a slight increase in catalase T activity in AA-treated cells is observed, and a decrease to levels similar to the control in cells co-treated with $ZnSO_4$ and AA **(Fig. 17D)**. It is nothworthy that this experiment was performed only one time and should be repeated in the future.


A
BY4741 wt
AA
EDTA
Sod2p
Sod1p
NBT
Coomassie
10 µg
60 µg
NBT/coomassie
Control
75 mM AA
1mM EDTA
EDTA+ AA


B
BY4741 wt
AA
ZnSO4
Sod1p
NBT
Coomassie
60 µg
NBT/coomassie
Control
100 mM AA
10mM ZnSO4
ZnSO4+AA


C
Activity of catalase
(division/sec/ng of protein)
Control
75 mM AA
1mM EDTA
EDTA+AA

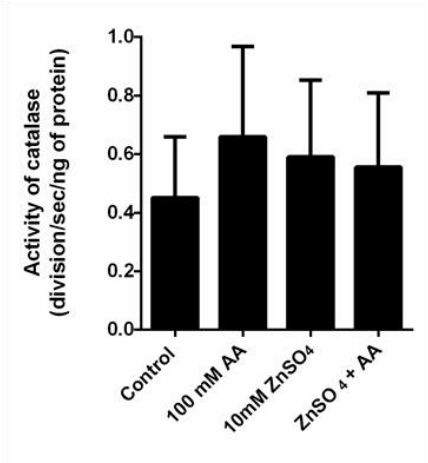

D
Activity of catalase
(division/sec/ng of protein)
Control
100 mM AA
10mM ZnSO4
ZnSO4 + AA

Figure 17- Effect of zinc limitation or supplementation in Sod1 and catalase T activity in *S. cerevisiae* BY4741 wt cells undergoing AA-induced cell death. In A and C, cells were pre-treated with and without 1 mM EDTA for 30 min following treatment with 1 mM EDTA, 75 mM AA or co-treatment with both for 90 min. In B and D, cells were treated without or with 10 mM $ZnSO_4$, 100 mM AA or co-treated with both for 60 min. **(A)** Whole cell protein extracts of EDTA experiment samples were run on native polyacrylamide gels and stained with NBT and Coomassie Blue (left panel). Gel bands intensity was quantified in Image J software and values are NBT/Coomassie intensities (right plot). **(B)** Same as in A but with samples from zinc supplementation experiments. **(C)** Determination of catalase activity after addition of 0.75 mM H_2O_2 in whole cell protein extracts from EDTA experiment samples measured in a Clark oxygen electrode. Catalase activity was evaluated by performing 3 repeats of each sample and values are the mean expressed as divisions/sec/ng protein. The samples were the same used to quantify Sod1 enzymatic activity. **(D)** Same as in C but with samples from zinc supplementation experiment (n=1 in both).

IV.2.3 Acetic acid treatment does not affect Sod1 activity but strongly decreases catalase activity in the *zrt3*Δ mutant

Since intracellular zinc levels seem to modulate the activity of the antioxidant enzymes under study, especially Sod1, next we decided to analyse the activity of these enzymes in the AA-resistant mutant strain lacking Zrt3p transporter in comparison with the wt strain. Regarding Sod1, as it can be observed in **Fig. 18A** and **B**, its activity increases when wt cells are treated with AA, in accordance with the above-mentioned results. However, the same trend was not observed in the *zrt3*Δ mutant, where the addition of AA did not affect Sod1 activity.

As for catalase, we compared the kinetics in both wt and *zrt3*Δ mutant in control cells and after AA treatment **(Fig. 18C)**. In all of the conditions tested, a Michaelis-Menten kinetics was observed, and though not statistically different, the *zrt3*Δ basal catalase activity was higher than in the wt strain. Moreover, while AA reduced the catalase activity of *zrt3*Δ it had no effect on the catalase activity of wt cells. The same pattern was observed in an independent assay, using the non-saturating concentration of 0.75 mM of H_2O_2 **(Fig. 18D)**.

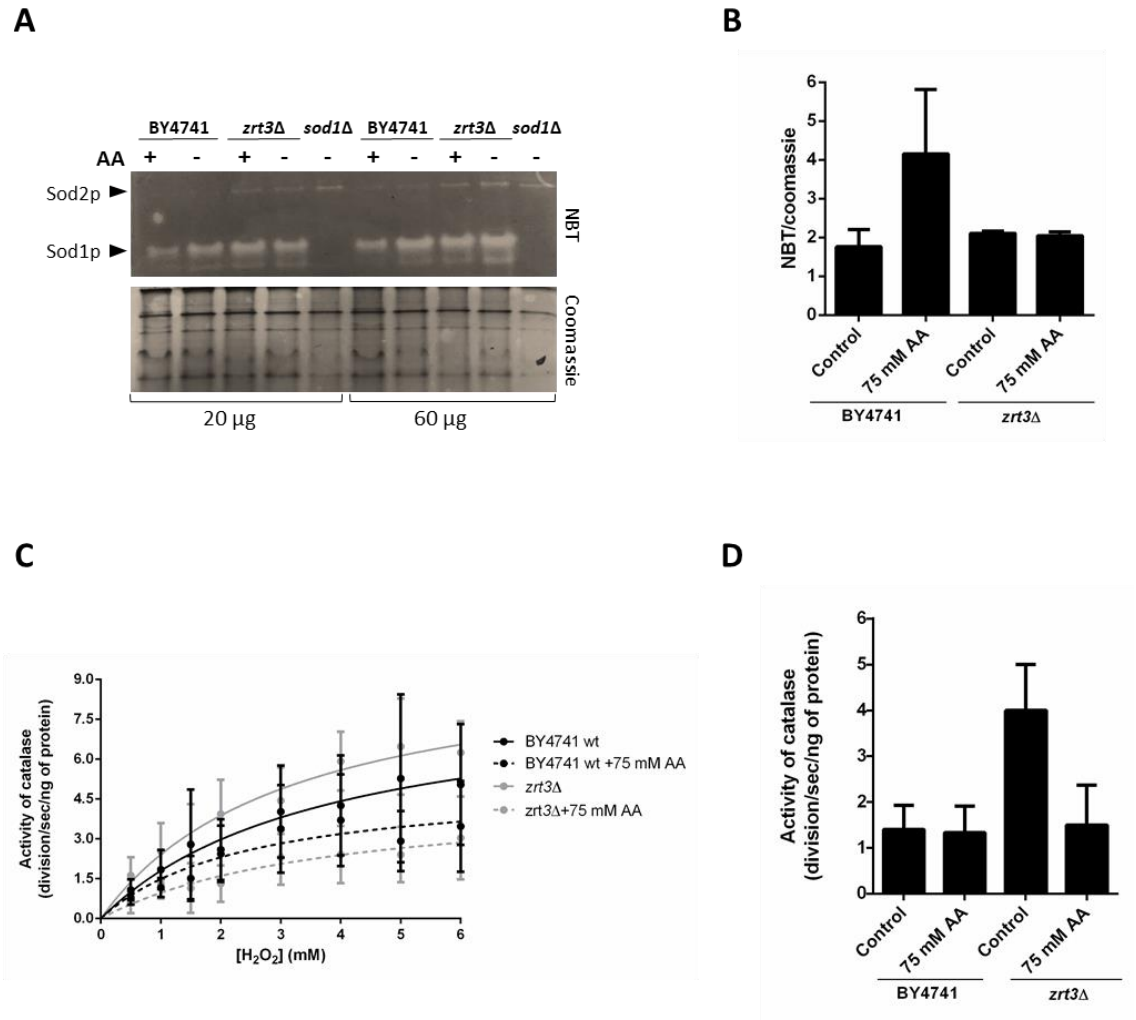


Figure 18 - Evaluation of Sod1 and catalase enzymatic activity in *S. cerevisiae* BY4741 wt and *zrt3*Δ mutant after treatment with AA. Cells were treated in the absence or presence of 75 mM AA during 60 min. **(A)** To evaluate Sod1 activity in wt and *zrt3*Δ mutant, protein extracts were run on native polyacrylamide gels and stained with NBT and Coomassie Blue. Representative gels of these assays are shown. **(B)** To measure enzyme activity, gel bands intensity was quantified in Image J software and values are NBT/Coomassie intensities of 20 µg and 60 µg of protein (n=3). **(C)** Determination of catalase activity in wt and *zrt3*Δ strains estimated by measuring oxygen consumption with a Clark electrode. Kinetics using 0.75 - 6 mM H_2O_2 of four independent experiments. Lines are derived by fitting the data points to a Michaelis-Menten kinetics. **(D)** Catalase activity was evaluated in 3 replicates of the same sample using 0.75 mM H_2O_2.

IV.3 Study of acetic acid resistance phenotype of *vtc4*Δ and *csc1*Δ strains

The majority of the mutants lacking vacuolar membrane proteins analyzed in this work exhibited an increased resistance to AA-induced cell death. Since the mechanisms that are associated with this resistance phenotype are unclear, we next pursued with different approaches to further understand the role of Csc1p and Vtc4p and associated functions in AA-induced cytotoxicity.

IV.3.1 The *csc1*Δ and *vtc4*Δ strains exhibit a delayed Pep4p release to cytosol and vacuolar membrane permeabilization during acetic acid treatment

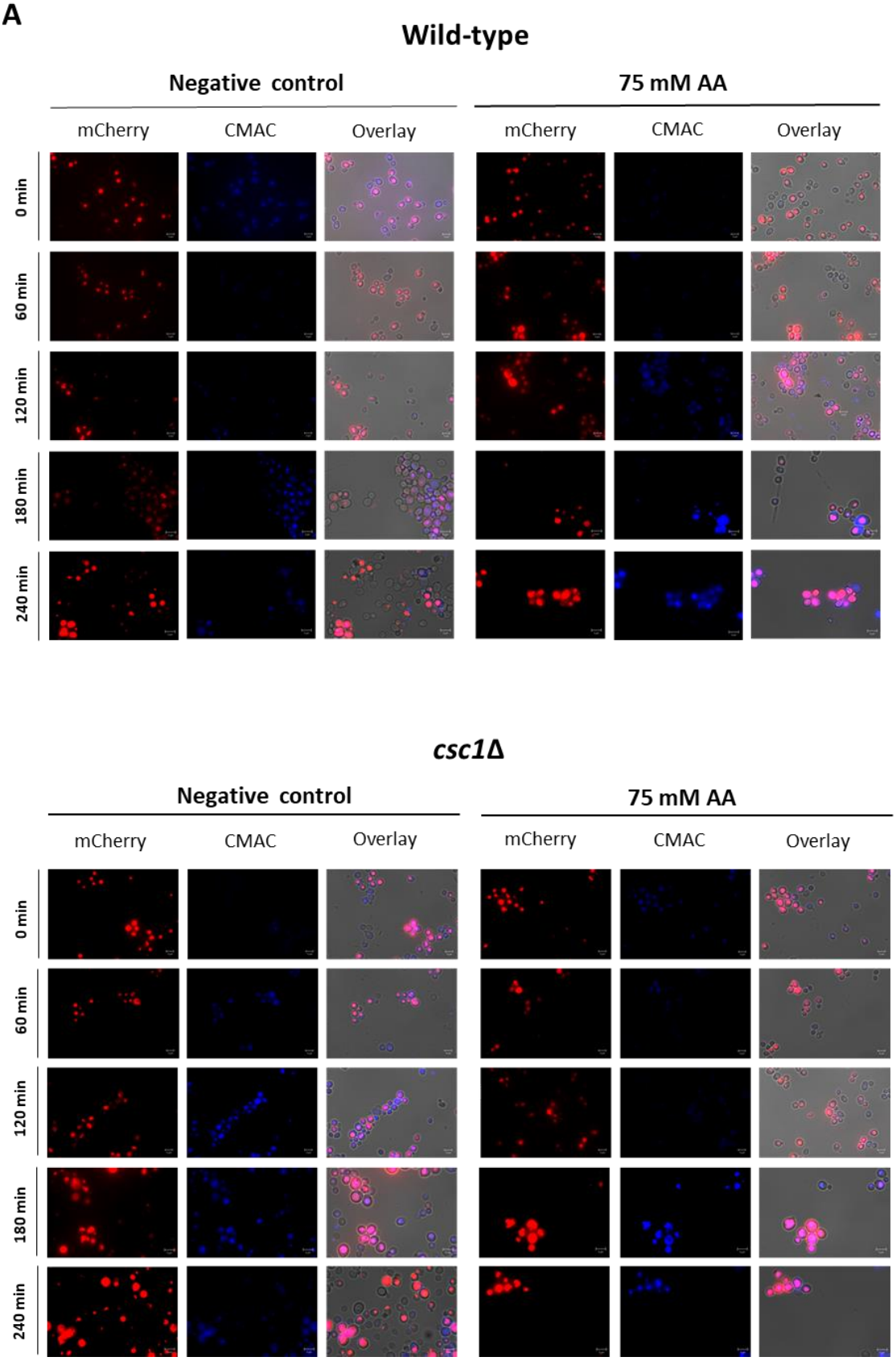
The vacuolar protease Pep4p has been shown to play both a protective and executing role in AA-induced cell death, depending on the genetic context. Studies have shown that, in the BY4741 strain, absence of Pep4p makes cells more resistant to AA (Sousa *et al.*, 2013), while in the W303-1A strain, the deletion of this protein confers higher susceptibility to AA treatment (Pereira *et al.*, 2010). This feature was hypothesized to be due to differences in mitochondrial mass content (Sousa *et al.*, 2013). Furthermore, it has been previously shown that Pep4p is released from the vacuole to the cytosol, which is associated with a partial and selective permeabilization of the vacuolar membrane, upon AA treatment (Pereira *et al.*, 2010).

In this context, to deeper understand the *csc1*Δ and *vtc4*Δ AA-resistance phenotype, we decided to investigate Pep4p localization upon AA treatment. For this purpose, the *csc1*Δ and *vtc4*Δ mutants were transformed with the pRS413 Pep4-mCherry plasmid, and stained with the vacuolar dye CMAC to evaluate VMP. This fluorescent dye accumulates in the vacuole when the vacuolar membrane is intact and diffuses in the cytosol when permeabilization occurs, thus allowing to monitor the integrity of the vacuolar membrane(Stewart & Deacon, 1995).Pep4-mCherry displays red fluorescence and CMAC blue fluorescence so, under the fluorescence microscopy, we can observe if they colocalize (purple fluorescence) or not, which also allows to establish a temporal kinetics of VMP and the release of Pep4p.

As expected, in the untreated wt, *csc1*Δ and *vtc4*Δ control cells, it was observed that, 60 min after incubation, Pep4-mCherry and CMAC co-localized essentially at the vacuole, with most cells exhibiting purple vacuolar fluorescence **(Fig. 19)**. As for 60 min AA-treated cells, results showed that the wt cells present essentially a vacuolar purple fluorescence **(Fig. 19A)**. Quantitatively, 65% of the cells had vacuolar Pep4p and CMAC and approximately 25% of the cells presented diffused Pep4p and CMAC staining. For 60 min timepoint, results were also similar to *vtc4*Δ and *csc1*Δ mutants, however the percentage of cells with Pep4p and CMAC vacuolar localization was slightly higher **(Fig. 19B)**. After 120

min of AA incubation, there were no major changes compared to 60 min. For this reason, longer incubation times were tested, and it was clearer that, in wt strain, there was a greater percentage of cells with cytosol diffused Pep4p and CMAC stainings, approximately 50% of the cells. However, in *csc1*Δ and *vtc4*Δ mutants, this percentage was much lower, approximately 12.5%. For all incubation times, it seems that there was no translocation of Pep4p without VMP/CMAC release as shown by the very low percentage of cells in the white bars category. In contrast, in all cases, there was a small percentage of cells that suffer VMP without Pep4p release (light grey bars), suggesting that VMP appears to precede Pep4p release to the cytosol (**Fig. 19B**). However, additional experiments should be performed to ascertain this assumption. Nevertheless, the results suggest that the AA-resistance phenotype of these mutants is associated with delayed VMP and translocation of Pep4p to the cytosol.

A


Wild-type
Negative control
75 mM AA
mCherry
CMAC
Overlay
mCherry
CMAC
Overlay
0 min
60 min
120 min
180 min
240 min
csc1Δ
Negative control
75 mM AA
mCherry
CMAC
Overlay
mCherry
CMAC
Overlay
0 min
60 min
120 min
180 min
240 min

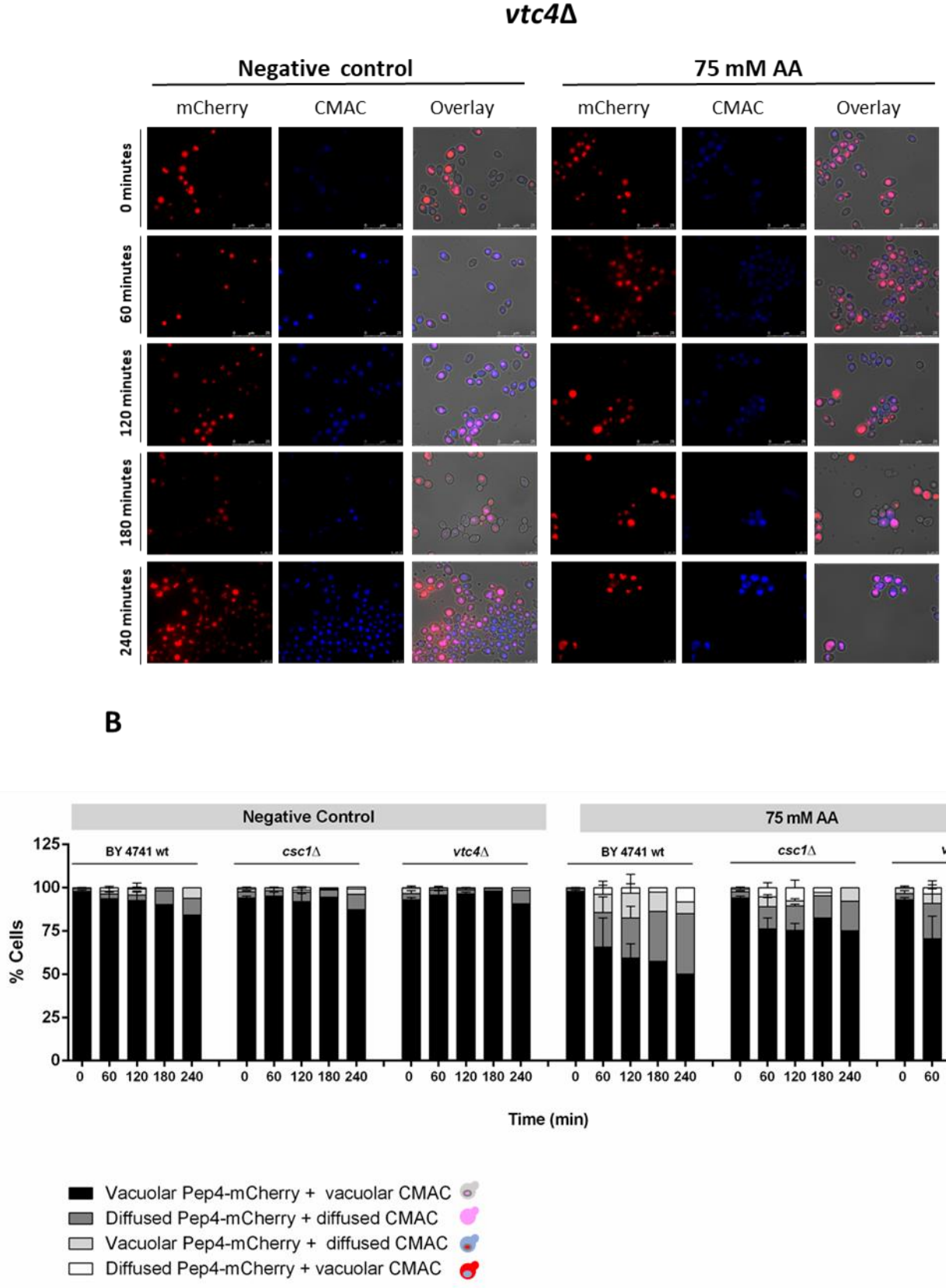

vtc4Δ
Negative control
75 mM AA
mCherry
CMAC
Overlay
mCherry
CMAC
Overlay
0 minutes
60 minutes
120 minutes
180 minutes
240 minutes
B
Negative Control
75 mM AA
BY 4741 wt
csc1Δ
vtc4Δ
BY 4741 wt
csc1Δ
vtc4Δ
% Cells
125
100
75
50
25
0
0 60 120 180 240
Time (min)
Vacuolar Pep4-mCherry + vacuolar CMAC
Diffused Pep4-mCherry + diffused CMAC
Vacuolar Pep4-mCherry + diffused CMAC
Diffused Pep4-mCherry + vacuolar CMAC

Figure 19 - Analysis of Pep4-mCherry localization and vacuolar membrane permeabilization in *S. cerevisiae* BY4741 wild type and *csc1*Δ and *vtc4*Δ mutant strains in response to 75 mM AA. **(A)** Representative fluorescence microscopy images of wt, *csc1*Δ and *vtc4*Δ strains transformed with Pep4-mCherry. Cells were treated with and without AA up to 240 min and then stained with 2 µM CMAC to evaluate vacuolar membrane integrity. Cells were observed under the fluorescence microscope with a 100× oil immersion objective. Bar: 5 µm. **(B)** Quantification of the percentage of cells with different localization of Pep4-mCherry (vacuolar /diffused) in combination with different CMAC vacuolar/diffused localization. At least 300 cells were counted for each condition. Values are the mean ± SEM of at least three independent experiments.

IV.3.2. Acetic acid increases intracellular calcium levels in the wild type strain but not in the *csc1*Δ mutant

Calcium ion has several functions as intracellular messenger, regulating various cellular processes. Changes in the homeostasis of this ion leads to its cytosolic accumulation, which has been shown to have a cytotoxic effect on the cell, thus triggering cell death (Carraro & Bernardi, 2017; Cui *et al.*, 2009). There are several calcium channels in yeast, however the focus of this study is on the role of the Csc1p channel.

As *csc1*Δ mutant exhibited a higher AA resistance as well as a delayed release of Pep4p to the cytosol compared to the wt strain, we decided to analyze whether perturbations of intracellular calcium levels could be involved in AA-induced cell death. For this purpose, we used the Fluo4-AM fluorescent probe, whose fluorescence intensity is dependent on Ca^{2+} binding, increasing with increasing intracellular calcium levels.

In order to validate the Fluo4-AM staining protocol, two inducers of cytosolic calcium accumulation were chosen to function as positive controls of calcium increase: H_2O_2 and calcimycin. H_2O_2 is an oxidant that can affect redox homeostasis of the cell, being also reported to induce intracellular calcium increase (Popa *et al.*, 2010). In turn, calcimycin, also known as the calcium ionophore A23187, is widely used to study Ca^{2+} involvement in the control of cellular functions since it binds to Ca^{2+} and increases the intracellular concentration of free calcium (Duffs & Patterson, 1974). Results showed an increase of the green fluorescence intensity up to 3-fold after addition to wt cells of 3 mM H_2O_2 and 4-6 µM calcymicin indicating an increase in calcium levels , which validated our staining protocol (**Fig. 20A, B**). It is also noteworthy that the Fluo4-AM fluorescence intensity was similar for all the calcymicin concentrations tested.

After optimizing the protocol, the effect of AA on calcium levels in the wt and in the *csc1*Δ mutant strain was analysed. Curiously, we observed a different response between the wt and *csc1*Δ mutant to

AA treatment. Regarding basal calcium levels, results suggest that the *csc1*Δ mutant has slightly higher calcium levels than the wt strain. As for AA treatment, we found that, in the wt strain, it increases intracellular calcium levels to more than 2-fold in comparison with the control. With respect to the *csc1*Δ mutant, no differences in intracellular calcium were observed in AA-treated cells compared to untreated cells **(Fig. 20C)**. These preliminary evidences suggest that the higher resistance of *csc1*Δ mutant to AA is associated with no increase in intracellular calcium levels.

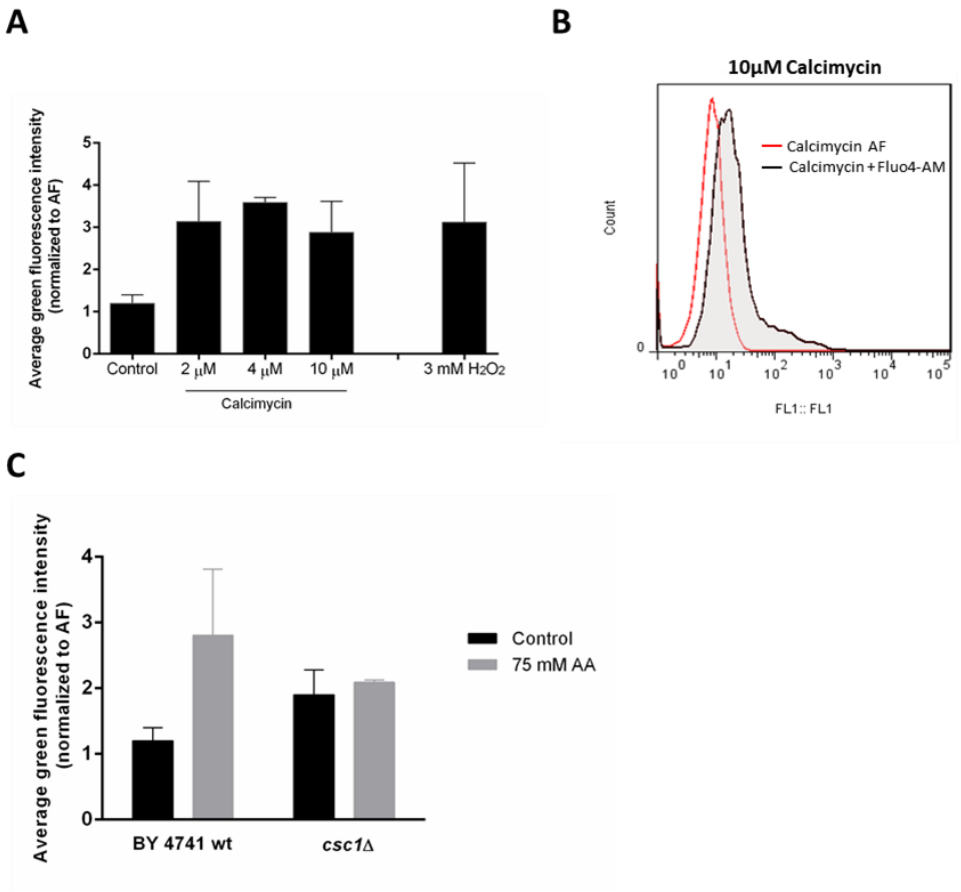


Figure 20 - Assessment of intracellular calcium levels in *S. cerevisiae* BY4741 wt and *csc1*Δ strains after treatment with calcimycin, H_2O_2 or AA. (A) Cells were treated with 2 µM, 4 µM and 10 µM calcimycin, and 3 mM H_2O_2 for 30 min. The control corresponds to untreated cells. Cells were then stained with 5 µM Fluo4-AM. Values correspond to the mean green fluorescence intensity normalized to the autofluorescence of each sample of 2 independent experiments. **(B)** Representative histogram of the increase in the green fluorescence intensity of wt cells incubated with 10 µM calcimycin for 90 min in relation to the respective autofluorescence (AF). **(C)** BY4741 wt and *csc1*Δ strains were treated in the absence or presence of 75 mM AA and then stained with 5 µM Fluo4-AM to evaluate intracellular calcium accumulation. Values are the mean green fluorescence intensity normalized to the autofluorescence of each sample of 2 independent experiments.

CHAPTER V – DISCUSSION

The various mechanisms that allow the normal functioning of a cell, as well as those that lead to its deregulation and cell death, have been extensively studied by numerous researchers. In this context yeast has been used as a simple eukaryotic model in particular to elucidate the mechanisms underlying regulated cell death in response to different stimuli. One of the well characterized lethal stimulus is acetic acid (AA) (Ludovico *et al.*, 2001; Madeo *et al.*, 1999). This acid is a normal by-product of alcoholic fermentation of *S. cerevisiae* that enters the cell in its undissociated form by simple diffusion under conditions where the extracellular pH is higher than the intracellular pH. Above a certain concentration, it becomes toxic mainly because of the accumulation of its dissociated form and concomitant intracellular acidification.

The cell death process induced by AA in yeast exhibits features typical of apoptosis in mammalian cells and is characterized by chromatin condensation, DNA fragmentation, release of cytochrome *c* (cyt *c*), accumulation of reactive oxygen species (ROS) and mitochondrial dysfunction (Ludovico *et al.*, 2001, 2002; Martins *et al.*, 2018; Pereira *et al.*, 2007). Furthermore, it is known that AA can induce vacuolar changes leading to partial vacuolar membrane permeabilization (VMP) and release of the protease Pep4p from the vacuole to the cytosol (Matos, 2018; Pereira *et al.*, 2010). Since the molecular basis of this selective VMP and release of Pep4p to the cytosol is not known yet, we decided to tackle this question by using a functional genomics approach, following a previous study in our lab (Matos, 2018). Specifically, we aimed to identify vacuolar membrane proteins involved in AA-induced apoptosis as well as to understand their putative involvement in the release of Pep4p to the cytosol. For this purpose, we analysed the impact of deleting the vacuolar proteins Pep3p, Vma4p, Vma16p, Zrt3p, Vtc4p and Csc1p to assess whether they modulate this cell death process. At the same time, the relationship between the deletion of these proteins and the kinetics of VMP and Pep4p release to the cytosol was studied.

The Pep3p protein is one of the components of two homologous tethering complexes called CORVET (class C core vacuole/endosome tethering) and HOPS (homotypic fusion and vacuole protein sorting). These complexes are of central importance for the biogenesis of endosomes and vacuole in yeast. They perform diverse biochemical functions in the endocytic pathway and CORVET complex acts upstream of HOPS (Peplowska *et al.*, 2007; Schauer *et al.*, 2009; Seals *et al.*, 2000).

Pep3p is located in the vacuolar membrane as well as in the cytosol and endoplasmic reticulum. Although having different locations, it is in the vacuolar membrane that it plays the most relevant role in membrane fusion processes (Preston *et al.*, 1991). Our cell survival experiments and a previous screening in our lab (Sousa *et al.*, 2013) showed that *pep3*Δ mutant is more sensitive to AA-induced cell death than the wt strain. These results are in line with previous reports (Preston *et al.*, 1991; Schauer *et al.*, 2009;

Ding *et al*, 2015). In fact, the *pep3*Δ strain was found to have a decreased chronological lifespan compared to the wt strain when treated with AA at pH 3.0. This was associated with increased ROS accumulation, and with a greater percentage of cells with compromised plasma membrane integrity assessed by propidium iodide staining, which means that *pep3*Δ cells suffer necrotic cell death upon AA treatment. Interestingly, these authors suggest that this phenotype is intrinsically linked to low pH (pH 3.0) since, at pH 4.05, this sensitivity phenotype was much less pronounced (Schauer *et al.*, 2009). On the other hand, different studies revealed that *PEP3* overexpression leads to altered fusion-fission balance and, consequently, increased number of fragmented vacuoles. Moreover, authors showed that *PEP3* overexpression provides protection from AA cytotoxicity by shortening the lag phase, increasing the vacuole surface area and also V-ATPase activity. All of these features result in an increased capacity to sequester protons from the cytosol, which is associated with the AA resistance phenotype, as it helps to counteract the intracellular proton accumulation induced by AA (Ding *et al*, 2015; Preston *et al.*, 1991). These data suggest that the Pep3p protein, or maybe the HOPS and COVERT complexes themselves and their role in maintaining the fusion-fission homeostasis and, consequently, functional vacuoles, seem to be essential to cope with the intracellular acidification induced by AA. Because these processes are altered in *pep3*Δ cells, they cannot easily get rid of the excess of protons in the cytosol, which results in a higher sensitivity to cell death.

Vacuolar ATPase (V-ATPase) is an ATP-dependent proton pump that uses ATP hydrolysis to actively transport protons from the cytosol to the vacuole (Martinez-Munõz & Kane, 2008). Yeast uses the proton gradient generated by the V-ATPase to maintain intracellular pH levels and regulate ion homeostasis. This enzyme is made up of multiple membrane-bound subunits and is divided into two functional domains: the peripheral ATP hydrolysis complex V_1 and the membrane-inserted proton translocating complex V_0 (Kane, 2006).

Contrary to what happens in higher organisms, in yeast, deletion of any subunit of V-ATPase is not lethal (Finnigan *et al.*, 2011). However, disruption of V-ATPase function leads to vacuolar alkalinization and cytosolic acidification. These alterations in pH homeostasis also perturb the function of the plasma membrane proton pump Pma1p, which is compromised due to loss of V-ATPase activity, indicating a relationship between these H^+-pumps (Martinez-Munõz & Kane, 2008). Generally, *vma* mutants are viable and share many features including inactivation of V-ATPase, organelle acidification defects, inability to grow at pH values lower than 3 or higher than 7, and extracellular calcium sensitive growth (Kane, 2006; Kane, 2007). Additionally, *vma* mutants are hypersensitive to multiple forms of oxidative stress, which may indicate an antioxidant role of V-ATPase (Kane, 2007). A study at pH 5.5 showed that the partial

acidification of the vacuoles that occurs in the *vma* mutants is due to passive proton transport independent of V-ATPase. These authors suggested that, although V-ATPase is the main responsible for vacuole acidification, alternative acidification mechanisms may act in conjunction with the proton pump mainly at low extracellular pH, where acids concentrations are permanently higher (Diakov & Kane, 2010).

Previous studies have shown that deletion of some V-ATPase subunits (Vma6p, Vma8p and Vma13p) makes cells more sensitive to AA at pH 3.0 (Schauer *et al.*, 2009). Since V-ATPase has two domains with different functions, we decided to study the effect of deleting a subunit of each functional domain, the subunits Vma4p and Vma16p, in AA-induced cell death. The subunit E (Vma4p) is reported to be one of the peripheral components of the V-ATPase V_1 domain and has a role in glucose-dependent V_0V_1 assembly. The subunit c" (Vma16p) belongs to the V_0 complex proton pore.

Ho *et al.* described that deletion of Vma4p subunit prevents the assembly of the V_0V_1 domains (Ho *et al.*, 1993). Three V-ATPase subunits (E, B and a) are reported to be the sites where aldolase binds, an enzyme involved in the assembly of V_0V_1 domains (Lu *et al.*, 2004). The aldolase modulates assembly/disassembly of V-ATPase by the interaction with these subunits (Marshansky & Futai, 2008). Lu *et al.* suggested that disruption of a single interaction between aldolase and V-ATPase, for instance with E subunit, can make the aldolase V-ATPase complex unstable. However, whether the aldolase-E subunit interaction is also required for V-ATPase assembly and activity, has not been determined yet (Lu *et al.*, 2007). In fact, the impact of Vma4p deletion on the cell is not yet fully understood.

V-ATPase V_0 c-ring domain contains three components: Vma3p, Vma11p and Vma16p, with different copy numbers. A study by Hirata *et al.* showed that cells without these subunits have identical phenotypes, exhibiting failure of V_0 subcomplex assembly and an increase in the degradation rate of Vph1p (a V_0 domain constituent) (Hirata *et al.*, 1997).

In our hands, *vma4*Δ mutant exhibited a higher resistance to AA, in comparison with the wt strain, while the *vma16*Δ mutant had a similar phenotype to the wt strain. However, in a screening performed in our lab the *vma4*Δ mutant exhibited a higher sensitivity to AA (Sousa *et al.*, 2013). The *vma4*Δ resistance to AA is likely associated with compensatory effects caused by deficiency in the Vma4p subunit that end up being efficient under these conditions. With respect to the *vma16*Δ mutant, the V-ATPase-independent alternative mechanisms of vacuole acidification like proton diffusion (Diakov & Kane, 2010) may compensate its absence, rendering a phenotype similar to the wt strain.

The vacuolar membrane protein that we deeper explored in the context of AA-induced cell death was the Zrt3p zinc transporter. Zinc has been shown to be essential in yeast, particularly at the level of antioxidant response of the cells, since it is a cofactor for many antioxidant enzymes (Eide, 2011). There

are several zinc carriers present in the cell, either at the plasma membrane or at the vacuole. Zrt3p is located at the vacuolar membrane and is responsible for transporting the vacuolar zinc to the cytosol (MacDiarmid *et al.*, 2000).

Previous work in the laboratory has shown that *zrt3*Δ cells are more resistant to AA than the wt strain, which is associated with less superoxide anion accumulation, less vacuolar pH perturbations, and also delayed VMP as well as release of Pep4p to the cytosol (Matos, 2018). In order to confirm the previously observed phenotype, we started by treating *zrt3*Δ cells with AA and found that, indeed, they are more resistant than the wt strain. These results suggest that either Zrt3p or its cell functions, namely regulation of zinc levels, are involved in AA-induced apoptosis. Thus, to uncover the possible role of zinc in AA cytotoxicity, we decided to starve cells from zinc using 1 mM EDTA (Simm *et al.*, 2007), or to supplement cells with 10 mM $ZnSO_4$ (Matos, 2018). EDTA forms very stable complexes with metals, thus acting as a zinc chelating agent, thereby restricting the free zinc available in the cell (Hart, 2000). The results gathered with this approach were very interesting. EDTA-induced zinc limitation made cells much more sensitive to AA, which was also associated with higher superoxide anion levels. In contrast, supplementation with $ZnSO_4$, protects cells from AA cytotoxicity, but no changes in superoxide anion levels were observed in this case. This indicates that zinc levels play a crucial role in AA-induced cell death. Surprisingly, zinc supplementation or limitation did not change the phenotype of the *zrt3*Δ mutant, as it remains highly resistant to AA in both conditions. This suggests that *zrt3*Δ cells may have an alternative mechanism to counteract the effect of intracellular zinc limitation. And, in fact, they have. MacDiarmid and colleagues, who described Zrt3p as a vacuolar zinc transporter for the first time, observed that Zrt3p mobilizes zinc from the vacuole under zinc extracellular limiting conditions. However, under these limiting conditions, *zrt3*Δ mutants do not have any growth defect because they compensate the defect by upregulating zinc uptake mechanisms. One of these mechanisms is the up-regulation of zinc transport mediated by Zrt1p and Zrt2p across the plasma membrane, which increases extracellular zinc uptake to maintain cellular homeostasis (MacDiarmid *et al.*, 2000). On the other hand, under zinc-replete conditions, there is higher zinc accumulation in *zrt3*Δ mutants than the wt, meaning that there is still zinc moving through the vacuole (MacDiarmid *et al*, 2000). These data show that *zrt3*Δ mutants have compensatory mechanisms to cope with the zinc pool alterations, which explains why the phenotype is similar regardless of zinc levels.

Since zinc pool and ROS levels are intimately related through the zinc-dependent antioxidant enzymes, such as cytosolic Cu/Zn-superoxide dismutase Sod1 and catalase T, we decided to analyze the activity of these enzymes in order to ascertain if their enzymatic activity is involved in the cell death

process induced by AA. Sod1 is responsible for the conversion of the superoxide anion (O_2^-), generated by the reduction of oxygen, in hydrogen peroxide (H_2O_2). In turn, catalase T converts H_2O_2 to H_2O and O_2 (Jamieson, 1998). A well-established set of reactions with these and other antioxidant enzymes allows cells to maintain their homeostasis (Morano *et al.*, 2011). In addition to Sod1, yeast also has Sod2, which is present in the mitochondrial matrix and is involved in oxygen radical detoxification. This enzyme is particularly required during stationary phase growth (Morano *et al.*, 2011). It also has catalase A, which is present in peroxisomes and is involved in degradation of hydrogen peroxide (Lushchak & Gospodaryov, 2005). However, since it was previously shown by our group that AA induces accumulation of superoxide anion mainly at the cellular than at the mitochondrial level, we focused in the cytosolic counterparts of these enzymes (Matos, 2018).

Our results show that, in response to AA, Sod1 activity increases in a concentration-dependent manner. This allows cells to cope with the accumulation of superoxide anion observed with the DHE staining. As for the *zrt3*Δ mutant, no changes in Sod1 activity were observed, which is also in line with the absence of superoxide accumulation. On the other hand, the increase of superoxide anion in cells treated with AA+EDTA in comparison with cells treated only with AA, is associated with a decrease in Sod1 activity and a decrease in cell survival. Though $ZnSO_4$ supplementation increases cell survival in response to AA, this protective effect is neither related with higher Sod1 activity nor lower superoxide anion accumulation. Indeed, increased zinc availability counteracts the increase of Sod1 activity in response to AA, but surprisingly this does not affect superoxide anion accumulation. However, it is noteworthy that these later results are preliminary and must be repeated to achieve reliable conclusions.

In turn, the activity of catalase was found to be similar in the wt strain with and without treatment with AA. Also, no changes in catalase activity were observed under the zinc limitation and zinc supplementation experiments, which indicates that the activity of this enzyme is not important for the observed decrease and increase of cell survival upon AA treatment, respectively. These results led us to believe that the sensitivity of wt cells to AA may be related to the high production of H_2O_2 by Sod1, and its inefficient degradation by catalase T. Accordingly, hydrogen peroxide is thought to play a role as the second messenger needed to start the AA-triggered RCD cascade (Guaragnella *et al.*, 2011). As for *zrt3*Δ mutant, a very intriguing result was achieved. While untreated *zrt3*Δ cells display a 3-fold higher catalase activity than the wt strain, treatment with AA decreases its activity to levels similar to the wt strain. The cause of the higher basal catalase activity of *zrt3*Δ cells and of its reduction after AA treatment is unknown, but it is reported that, in cells where catalase T is highly expressed, AA-induced cell death is avoided (Guaragnella *et al.*, 2011).

As above mentioned, in *zrt3*Δ mutants, VMP and Pep4p release to the cytosol are delayed upon AA treatment in comparison with the wt strain (Matos, 2018). Whether this is due to a higher vacuolar zinc accumulation in *zrt3*Δ cells (MacDiarmid *et al*, 2000) remains to be addressed.

Phosphate is present in small quantities in the environment and, like other microorganisms, yeast has developed complex mechanisms to cope with changes in its availability. Inorganic polyphosphate (polyP), known as a reservoir for intracellular phosphate, is accumulated in the vacuole and synthesized by the vacuolar transporter chaperone (VTC) complex, a vacuolar polyP polymerase (Mu *et al.*, 2002). Vtc4p is the catalytic subunit of the VTC complex known to synthetize polyP and to lead to its accumulation in the vacuole. In fact, Vtc4p assists in the process of fusion of phosphate-rich vesicles with the vacuole membrane (Hothorn *et al.*, 2009; Muller *et al.*, 2003; Ogawa *et al.*, 2000).

*vtc4*Δ mutants are unable to synthetize polyP, have delayed cell cycle progression and nucleotide production (Yu *et al.*, 2012); show increased sensitivity to Zn^{2+} (Dziedzic & Caplan, 2011); and have microautophagy defects (Uttenweiler *et al.*, 2007). Additionally, deletion of Vtc4p has been reported to decrease the number of V-ATPase V_1 subunits associated with the vacuole, and to lead to ATP accumulation in the cell (Muller *et al.*, 2003).

In this work, we found that *vtc4*Δ cells display a higher resistance to AA cytotoxicity. The same phenotype was also observed previously (Sousa *et al.*, 2013) This resistance phenotype is associated with a delayed release of Pep4p to the cytosol as well as VMP compared to the wt strain. Previous work in the lab showed that *vtc4*Δ resistance is also associated with less superoxide accumulation and less vacuolar acidification (Matos, 2018). Based on the data available and our own results, we can put forward some hypothesis to explain why VMP is delayed in this mutant upon AA treatment. AA-induced selective VMP may depend on the proton gradient generated by V-ATPase, and since the stability of V_1/V_0 complexes and, consequently, V-ATPase activity is reduced in *vtc4*Δ cells (Müller *et al*, 2003), this could delay the process. This fact could also explain why, in these cells, the vacuolar acidification induced by AA is lower. VMP can also be influenced by the deregulation of cellular phosphate concentration caused by the low polyP levels. However, much more work is needed in order to deeper understand this mutant resistance phenotype.

Calcium is known to be a regulator of several cellular processes. In yeast, one of the changes observed when apoptosis occurs is a significant increase in calcium levels (Giorgi *et al.*, 2008). After this increase, other changes occur such as increased ROS levels, mitochondrial depolarization and increased matrix volume. Several transporters in the cell are responsible for transporting calcium and maintain its

homeostasis, namely Csc1p, which is responsible for transporting calcium from the vacuole lumen to the cytosol.

In this work, we analysed the effect of AA in the *csc1*Δ strain and found that it is more resistant when compared to the wt strain. Moreover, the higher resistance was associated with delayed release of VMP as well as of Pep4p to the cytosol. To dissect the role of Csc1p and calcium in these observations, we measured calcium levels by flow cytometry and found that there is a great increase in intracellular calcium in the wt strain treated with AA. In contrast, in *csc1*Δ cells either treated with AA or not, the calcium levels were similar to the untreated wt cells. The increase in Ca^{2+} levels in the wt strain is in accordance with the apoptotic process triggered by AA in this strain. Since the *csc1*Δ strain is resistant to AA, and considering calcium increase as an apoptotic marker, it makes sense that no changes in calcium are observed this strain. However, there are many more plasmalemmal and organellar calcium transporters in yeast, being difficult to draw any further conclusions with our results. Notably, VMP is delayed in this mutant strain, which may indicate that calcium signalling may somehow modulate this process.

CHAPTER VI – FINAL REMARKS AND FUTURE PERSPECTIVES

Acetic acid (AA), a by-product of the alcoholic fermentation of the yeast *Saccharomyces cerevisiae*, has been reported to have impact on the biotechnological field. Indeed, its presence in the culture medium or accumulation during fermentation is known to inhibit yeast metabolic capacity and growth, and at higher concentrations to induce cell death. Thus, many efforts are being made to develop yeast strains with increased tolerance to acetic acid (Palma *et al.*, 2018). Moreover, acetate has been shown to induce cell death in colorectal cancer cells, which opens the possibility to exploit this short chain fatty acid in cancer therapy, thus with biomedical impact (Ferro *et al.*, 2016). In both scenarios, it is important to understand the mechanisms underlying AA/acetate-induced cell death to prompt its more effective application, which was precisely the goal of this work. Both AA and acetate lead to a selective vacuolar/lysosomal membrane permeabilization (VMP/LMP) associated with the release of the protease Pep4p or cathepsin D (the human ortholog of Pep4) from the vacuole to the cytosol. Since the mechanisms underlying VMP/LMP are not known, our aim was to use yeast as a model to unveil molecular components involved in the selective VMP and release of Pep4p in cells undergoing AA-induced cell death. Using a yeast functional genomic approach with a set of mutant strains lacking vacuolar membrane proteins, we were able to identify some putative proteins or their associated cellular functions that may be involved in these processes **(Fig. 21).**

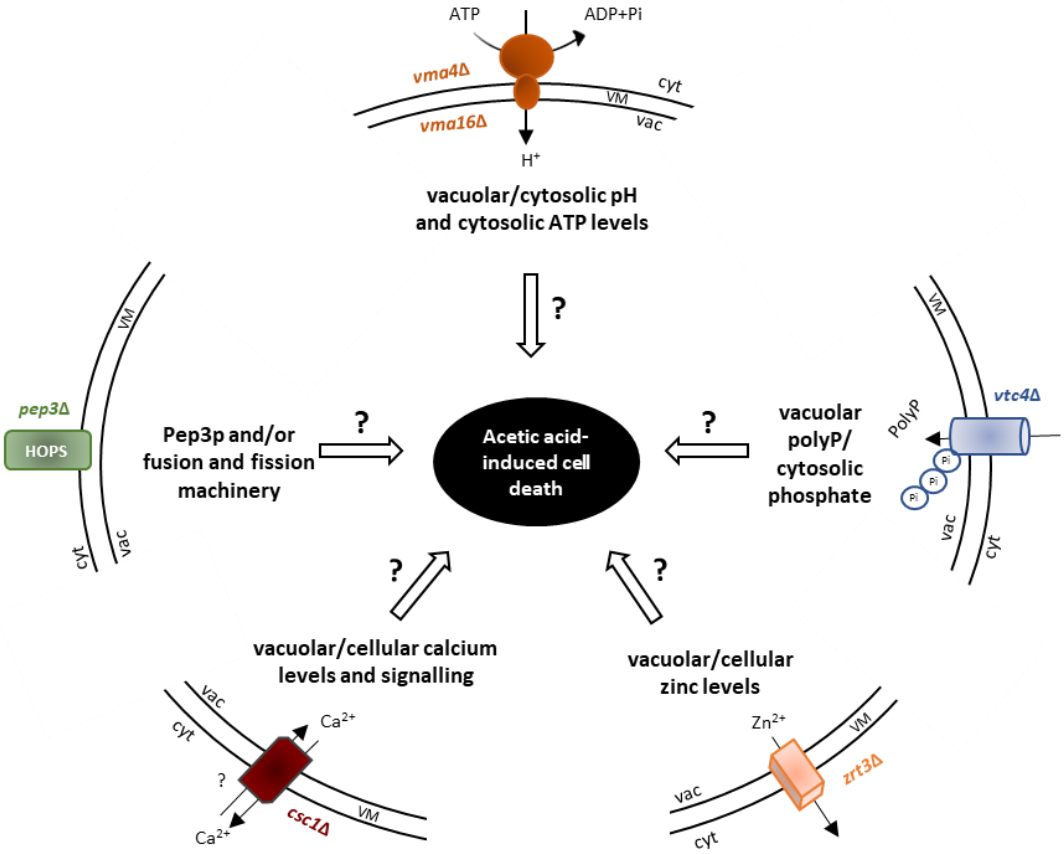


Figure 21 - Schematic representation of vacuolar membrane proteins as putative molecular components involved in AA-induced cell death. The use of mutant strains lacking the Zrt3p, Pep3p, Csc1p, Vct4p, and Vma4p proteins allowed to hypothesize their involvement in AA-induced cell death. Taking into account the functional role of these proteins, we can suggest that the levels of zinc, calcium, polyphosphate, ATP and H^+ may be involved in the selective vacuolar membrane permeabilization and translocation of Pep4p to the cytosol triggered by AA.

The experiments with the AA-sensitive *pep3*Δ strain allowed us to put forward the hypothesis that the Pep3p protein, or maybe the HOPS and CORVET complexes and their role in maintaining the fusion-fission homeostasis, are essential to cope with the intracellular acidification induced by AA. Since the results are very preliminary, it would be interesting to study the relationship of its AA-sensitivity phenotype with VMP and the release of Pep4p to cytosol. The same approach should also be performed with strains overexpressing *PEP3*. We could monitor the fission and fusion processes in these conditions and analyse the activity of V-ATPase, as it has been reported that *PEP3* overexpression protects cells from AA cytotoxicity by increasing the vacuole surface area and also V-ATPase activity (Ding *et al*, 2015). This may give us information about the putative involvement of the fusion and fission machinery in the selective

VMP induced by AA. Additionally, since the effect of *PEP3* overexpression is directly influenced by the pH (Ding *et al*, 2015), these experiments should be performed at different pHs.

The proteins Vma4p and Vma16p, constituents of V-ATPase, showed distinct phenotypes. The *vma4*Δ mutant was shown to be more resistant to AA-induced cell death, while the *vma16*Δ mutant has a phenotype similar to the wt strain. As for the *vma* mutants, much more work needs to be performed to uncover the role of V-ATPase or associated functions in AA cytotoxicity and specifically in VMP. Particularly, vacuolar acidification should be monitored in both mutants to understand whether proton movement through the vacuole occurs by other compensatory mechanisms. One next logical step would also be to monitor VMP and Pep4p release in different *vma* mutants with distinct cell survival phenotypes (similar to wt, more resistant or more sensitive) to try to get further insights. In order to compare the results with the *vma* mutants, it would be interesting to use concanamycin A, a potent inhibitor of V-ATPase, either by pre-incubation of cells with concanamycin A followed by treatment with AA, or co-incubation with concanamycin A and AA.

The results with the *zrt3*Δ mutant and zinc limitation/supplementation assays allowed us to identify a role of zinc levels in AA-induced cell death. In fact, our data indicates that zinc is essential to cope with the oxidative stress induced by AA. When it is limited, cells become hyper-sensitive to AA and the levels of superoxide anion increase. In contrast, when cells are incubated with zinc sulphate, they become more resistant to AA. Under the same conditions, the *zrt3*Δ mutant phenotype is unchanged, remaining resistant to AA. This suggests that the cell compensatory mechanisms in the absence of this vacuolar transporter owed to *zrt3*Δ cells the ability to cope with zinc limitation in response to AA, which in turn make cells insensitive to zinc protection. The evaluation of zinc levels and its cellular distribution with a fluorescence probe, like Fluozin, would help to confirm this hypothesis. Also, the possible involvement of zincosomes, vesicles that store zinc and are important for cell survival under zinc-limiting conditions, and the study of the regulation and activity of the other vacuolar and plasma membrane zinc transporters in *zrt3*Δ cells, could give additional clues. Regarding the activity of the antioxidant enzymes, we demonstrated that AA greatly increases the activity of Sod1, having no effect on catalase T activity in the wt strain. In opposition, in the *zrt3*Δ mutant, AA does not change Sod1 activity, but drastically decreases catalase T activity. In spite of our data, we still have many questions, and the reason why *zrt3*Δ cells have delayed VMP in response to AA is likely due to a lower superoxide anion accumulation, as reported by Matos, 2018. Pre-incubation of *zrt3*Δ mutant with a pro-oxidant, known to induce superoxide anion accumulation, followed by AA treatment would allow to address this question. Anyway, further studies are needed to find out why *zrt3*Δ cells accumulate lower levels of superoxide anion as their Sod1p

basal activity is similar to that of the wild type cells. We could also further explore the higher catalase T activity in the *zrt3*Δ mutant and its contribution to the *zrt3*Δ resistant phenotype. This could be addressed by assessing cell survival in *zrt3*Δ lacking catalase T. Additionally, the activity of other antioxidant enzymes that have zinc as a cofactor should be studied.

In this work, we showed that the *vtc4*Δ mutant is resistant to AA-induced cell death when compared to the wt strain, which is associated with a delayed VMP as well as Pep4p release from the vacuole to the cytosol. Based on our results, we can speculate that VMP delay is related with the deregulation of phosphate concentration caused by low polyP levels in this mutant strain. However, there is still much more work to do to understand why *vtc4*Δ mutant strain displays these phenotypes. There are different features associated with Vtc4p deficiency, however how they contribute to AA-induced cell death is still elusive. In future work, it would be interesting to quantify changes in polyP cellular content in wt and *vtc4*Δ mutant treated with AA. In addition, it would be important to determine the activity of the polyP kinase Vtc4p upon AA treatment in the wt strain. The study of the effect of AA in other *vtc* mutants (e.g. *vtc1*Δ, *vtc2*Δ and *vtc3*Δ) would also reveal if this phenotype is or not specific for Vtc4p deletion.

Finally, the use of the *csc1*Δ mutant strain opened the window to explore the role of calcium signaling in the selective VMP triggered by AA. Indeed, this mutant showed a higher resistance to AA-induced cell death than the wt strain, which was also associated with a delayed release of Pep4p to cytosol and VMP. Furthermore, we concluded that AA treatment increased intracellular calcium levels in the wt strain. Future work could rely on the study of the effect of AA in yeast cells lacking other plasma membrane or organellar calcium transporters such as Cch1/Mid1 complex, Vcx1 and Pmc1, regarding their levels of expression and activity. The study of calcium uptake in AA-treated cells and the effect of extracellular calcium concentration on AA-induced cytotoxicity and VMP could also give us additional insights.

With this work, we raised several hypotheses that open new avenues for the research of the mechanisms underlying AA-induced cell death, especially related to the selective VMP/release of Pep4p triggered by this compound. We believe that the better understanding of these mechanisms and their possible modulation will have a relevant impact on industrial processes and applications on cancer therapy.

CHAPTER VII – REFERENCES

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CHAPTER VIII – SUPPLEMENTARY MATERIAL

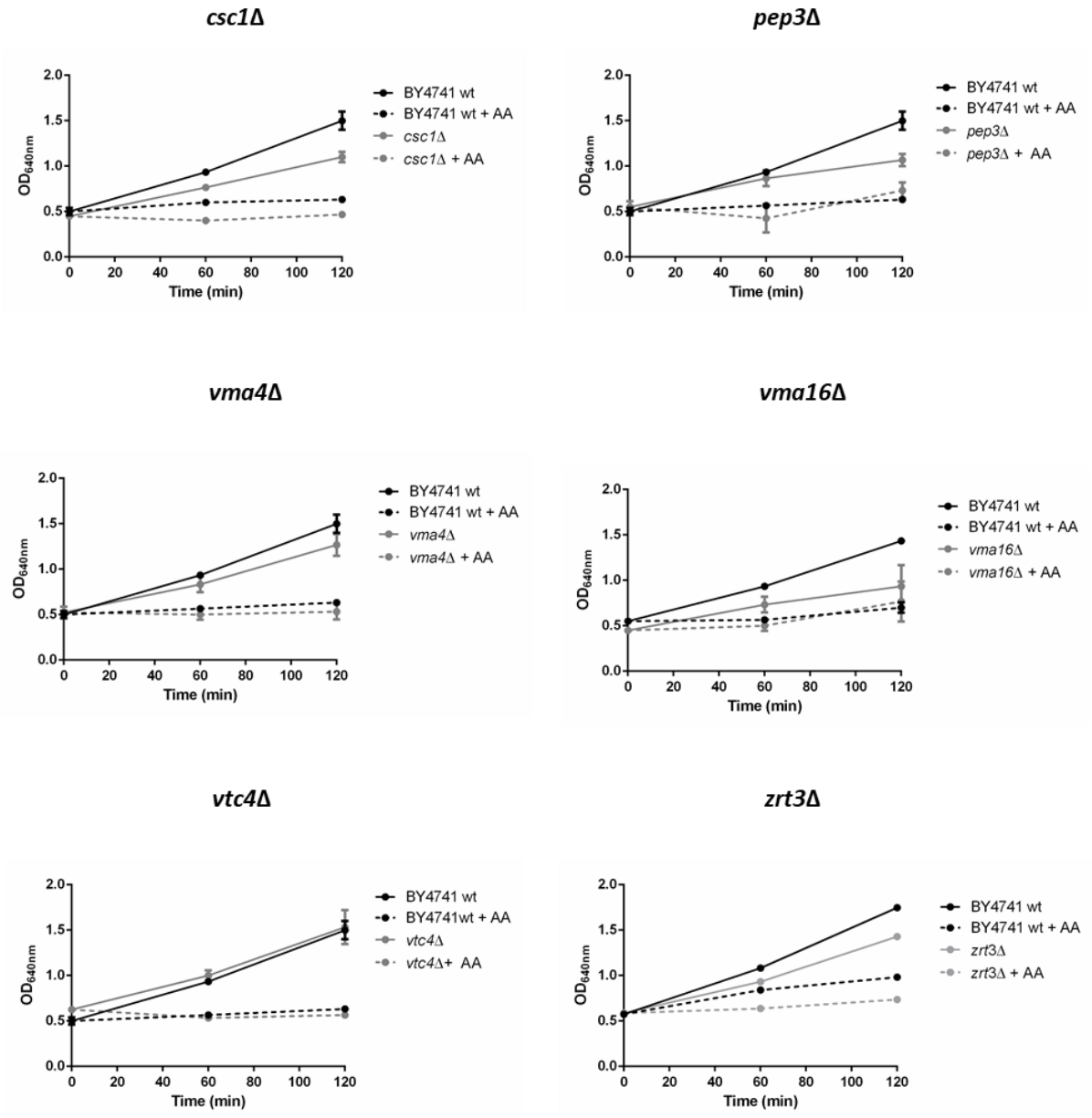

Figure 22- Assessment of growth of different mutant strains lacking vacuolar membrane proteins upon acetic acid treatment.

S. cerevisiae BY4741 wild type and indicated mutants were grown in YEPD until exponential phase and then harvested and resuspended in YEPD pH=3 without or with 75 mM AA (for *csc1*Δ, *pep3*Δ, *vma4*Δ, *vma16*Δ and *vtc4*Δ strains) or 100 mM (for *zrt3*Δ strain). The wt strain was treated with both AA doses. Cells then incubated for 120 min. Growth was followed by optical density (OD) at 640 nm. Values are the growth ± SEM of four independent experiments