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The role of Cks proteins in apoptosis

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Doutora Susana Alexandra Rodrigues Chaves

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Ana Rita Correia Pacheco

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The role of Cks proteins in apoptosis

Abstract

Cyclin-dependent kinase subunits (Cks) proteins are evolutionary conserved small proteins that interact with Cyclin-dependent kinase (Cdk) and are frequently overexpressed in several types of cancer, which is correlated with poor prognosis and aggressive behavior. Lower eukaryotes as budding yeast express one Cks protein (Cks1), whereas mammalian cells express two (Cks1 and Cks2).

Cks proteins were most frequently associated with a direct role in cell cycle regulation, but are also involved in efficient transcription of multiple genes. However, despite their similarity, Cks proteins can have specific functions. For instance, mammalian Cks1, but not Cks2, is involved in ubiquitination of p27, a cyclin-dependent kinase inhibitor. There is some indication that modulation of Cks protein levels can impact apoptosis in some mammalian cell lines, but there is a lack of focused research on this subject and the mechanism involved remains uncharacterized. A role of Cks proteins in apoptosis has therefore yet to be explored as a target in cancer therapy.

The yeast model has already demonstrated its potential for application in the apoptosis field, as evidenced by the increasing number of studies using yeast as a model for neurotoxicity and cancer. Most functions attributed to Cks proteins are conserved in yeast, and indeed mammalian Cks proteins can functionally substitute for yeast Cks1p. Our aim was therefore to determine whether modulation of Cks1p levels alters apoptotic signaling in the yeast *Saccharomyces cerevisiae*, which would be indicative of a conserved function in apoptosis. We found that deletion of yeast *CKS1* led to significantly increased sensitivity to short-term exposure to the chemotherapeutic agent cisplatin, but not to the other DNA damaging agents - methyl methanesulfonate and 5-fluorouracil, nor to the general apoptosis inducers - acetic acid and hydrogen peroxide. This is a strong indication of a specific regulated role for Cks1p in DNA damage-induced cell death, independent from its role in cell cycle. Further studies to unravel its function, as well as the pathways involved, could therefore provide novel targets to exploit in the treatment of cancers where Cks proteins are up-regulated.

O papel das proteínas Cks na apoptose

Resumo

Subunidades Kinase dependente de ciclina (Cks) são pequenas proteínas conservadas evolucionariamente, que interagem com kinases dependentes de ciclina (Cdks) e encontram-se sobreexpressas em diversos tipos de cancro, relacionado com mau prognóstico e comportamento agressivo. Eucariotas inferiores como a levedura expressam uma proteína Cks (Cks1), enquanto as células de mamífero expressam duas proteínas (Cks1 e Cks2).

Proteínas Cks estão mais associadas a um papel direto na regulação do ciclo celular, mas também estão envolvidas na transcrição eficiente de vários genes. Contudo, apesar das suas semelhanças, as proteínas Cks podem ter funções específicas. Por exemplo, a Cks1 de mamífero está envolvida na ubiquitinação do p27, um inibidor da kinase dependente de ciclina. Há algumas indicações que a modulação dos níveis de proteínas Cks podem ter impacto na apoptose em algumas linhas celulares de mamífero, mas há pouca investigação neste assunto e o mecanismo continua por caracterizar. Um papel das proteínas Cks na apoptose tem ainda que ser explorado para ser usado como alvo na terapia de cancro.

O modelo da levedura tem mostrado potencial para aplicação na área da apoptose, como é evidenciado pelo aumento do número de estudos a usarem a levedura como modelo para neurotoxicidade e cancro. Muitas funções atribuídas a proteínas Cks são conservadas em levedura, e de facto proteínas Cks de mamífero podem substituir funcionalmente a proteína Cks1p de levedura. O nosso objetivo foi determinar se a modulação dos níveis de Cks1p altera a sinalização apoptótica na levedura *Saccharomyces cerevisiae*, o que seria indicativo da conservação da função na apoptose. Determinamos que a deleção de *CKS1* em levedura leva ao aumento significativo da sensibilidade a uma exposição curta à cisplatina, mas não a outros agentes danificadores de ADN, metanosulfato metil e 5-fluorouracil, nem a indutores gerais de apoptose, ácido acético e peróxido de hidrogénio. Isto é uma forte indicação para um papel de regulação específico de Cks1p em morte celular induzida por danos de ADN, independente do seu papel no ciclo celular. Estudos futuros para desvendar esta função, assim como as vias envolvidas, podem fornecer novos alvos para explorar no tratamento de cancros onde as proteínas Cks estão sobreexpressas.

Index

Abbreviations

5-FU – 5-Fluoururacil

AA – Acetic Acid

AIF – Apoptosis Inducing Factor

ANT – Adenine Nucleotide Translocator

APAF1 – Apoptotic Proteasome Activating Factor 1

ATP – Adenosine Triphosphate

BER – Base Excision Repair

Bid – BH3-interacting domain death agonist

c.f.u. – Colony-forming units

cDDP – cis-diamminedichloroplatinum (II)

Cdk – Cyclin-dependent kinase

Cks – Cyclin-dependent kinase subunit

CypD – Cycliphilin D

DHE – Dihydroethidium

DIC – Differential Interference Contrast

DISC – Death Inducing Signaling Complex

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic acid

FASL – FAZ ligand

FdUMP – 5-Fluorodeoxyuridine monophosphate

GFP – Green Fluorescent Protein

H_2O_2 – Hydrogen peroxide

HTRA2 – High Temperature Requirement Protein A2

HU – Hydroxyurea

IAP – Inhibitor of Apoptosis Protein

MMR – Mismatch Repair

MMS – Methyl methanesulphonate

MOMP – Mitochondrial Outer Membrane Permeabilization

MPF – Cdk1/Cyclin B complex

NER – Nucleotide Excision Repair

PCD – Programmed Cell Death

pI – Isoelectric point

PI – Propidium Iodide

PTP – Permeability Transition Pore

RNA – Ribonucleic acid

ROS – Reactive Oxygen Species

siRNA – small interfering RNA

SMAC/DIABLO – Second Mitochondria-derived Activator of Caspases/Direct IAP-binding protein with low pI

tBid – Truncated Bid

TNF – Tumor Necrosis Factor

TS – Thymidylate Synthase

UV – Ultra-violet

VDAC – Voltage-dependent Anion Channel

$\Delta\psi m$ – Mitochondrial transmembrane potential

INTRODUCTION

Cks proteins: Relevance in cancer

Cyclin-dependent kinase subunit (Cks) proteins are evolutionary conserved small (9-18 kDa) proteins [1-3]; lower eukaryotes express only one Cks1 protein, whereas mammals, and possibly other vertebrates, express two orthologs, Cks1 and Cks2 [2-6]. Cks proteins have been the subject of increased attention in recent years, since they are frequently overexpressed in several cancers, which is correlated with poor prognosis. Cks1 overexpression has been strongly associated with various clinical and pathological features that are commonly used to determine aggressive tumor behavior [7-9]. High expression of Cks1 may be involved in the pathogenesis of non-small cell lung carcinoma [10], oral squamous cell carcinoma [11], colorectal carcinoma [8, 12], gastric carcinoma [13], lung carcinoma [11] and breast cancer [14]. Overexpression of Cks1 correlates with the increased radiotherapy resistance of esophageal squamous cell carcinoma [15] and tumor stage and positive lymph node metastasis in breast cancer [14]. It has also been found that Cks2 is expressed at significantly higher levels in tumors with metastasis [16] and can be responsible for aggressive behavior of tumours [17]. Cks2 levels were also increased in cervical cancer [18], metastatic androgen-independent prostate cancer [19] and cholangiocarcinoma [17]. In addition, *CKS2* has been proposed as a biomarker for the diagnosis and staging of bladder cancer [20] and melanocytic tumour progression [21]. Expression of Cks1 and Cks2 was elevated in prostate tumors and forced expression of both Cks1 and Cks2 in benign prostate tumor epithelial cells accelerated cell growth [22]. Therefore, Cks proteins may be considered as potential novel prognostic marker and target for the future development of specific therapeutic interventions. Targeting these proteins may be a promising therapeutic option for the treatment of cancers where they are up-regulated.

Cks proteins: Structure and functions

Cks proteins were originally identified through their ability to genetically suppress defective alleles of the cyclin-dependent kinase (Cdk) of fission and budding yeast [1-3], which pointed to a prominent role in the cell cycle.

The cell-cycle machinery is distinguished by a series of coordinated events essential to ensure faithful DNA replication and segregation of replicated chromosomes into two separate cells. It consists of specific phases: the G1 phase, during which the cell prepares for DNA synthesis; the S phase, during which active replication of the chromosomes occurs; G2 gap

period, prior to chromosome segregation and cytokinesis in M phase (mitosis) (Figure 1) [23-24]. Cells in G1 can, before committing to DNA replication, enter a quiescent state, called G0 [25].
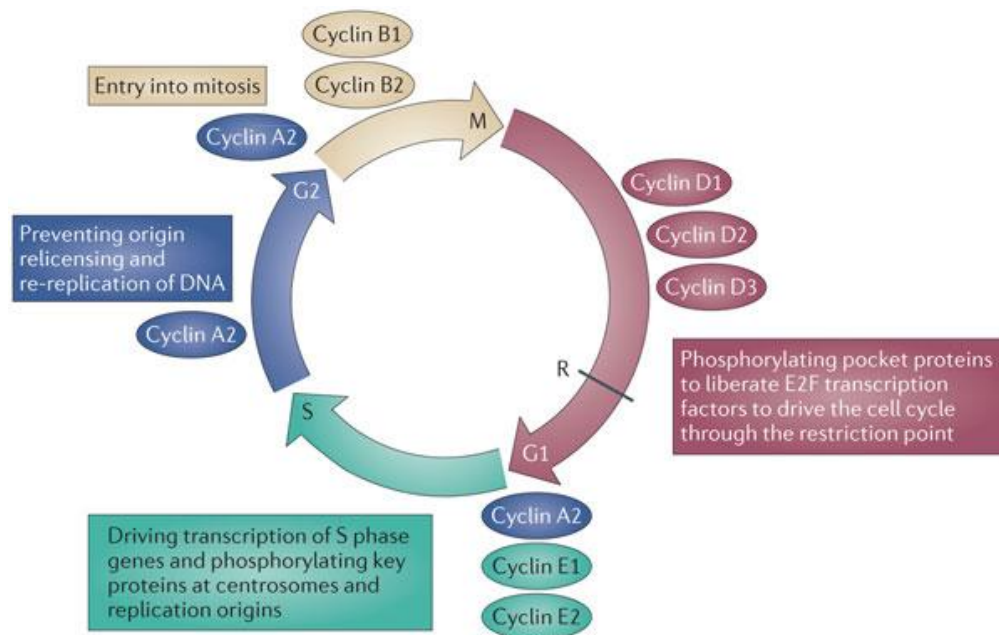

Figure 1. Cell Cycle regulation in mammals. Cyclins D are synthesized in response to mitogenic signals and they direct bind CDK4 (and/or CDK6) to phosphorylate pocket proteins. CDK2–cyclin E functions at the G1/S transition to trigger chromosomal DNA replication and to initiate centrosome duplication. The longer-lived CDK2–cyclin A complex collaborates with CDK2–cyclin E to regulate DNA replication both positively and negatively thus that DNA is only replicated once. The final wave of CDK1–cyclin B activity reorganizes the cell for mitosis (Moore (2013) [26]).

Progression through each cell-cycle phase and transition from one phase to the next are monitored by specific checkpoints, which maintain the correct order of events [25-26]. If these sensors detect aberrant or incomplete cell-cycle events, checkpoint pathways relay a signal that can lead to cell cycle arrest until the problem is resolved [27-28]. These checkpoints are governed by a tight relationship of specific Cdks [29], serine/threonine kinases that must bind to cyclin proteins to become active [30] and regulate the cell cycle division [1]. Cdks are required for the G1 to S phase cell cycle transition, initiation of DNA replication, the G2 to M phase cell cycle transition, and initiation of multiple mitotic events [31]. Cks proteins bind a subset of Cdks with high affinity at a position remote from the ATP and cyclin binding sites, functioning as activating partners. However, unlike cyclins, they are not required for the general activation of the Cdk activity [4], but seem to modulate substrate choice or the extent of phosphorylation [1].

Cks proteins are structurally and functionally conserved in eukaryotes, and share over 80% sequence identity [4, 22, 32-35]. The Cks structure consists of a four-stranded β-sheet capped at one end by two or three short α-helices and a highly conserved sequence, the so-called "hinge loop" that links the exchanged β-strand with the rest of the protein [5]. The Cdk-binding and phosphate-binding surfaces are also conserved in Cks proteins. In contrast, the major structural differences are located on the two α helices and also at the carboxyl terminus, which generate a largely contiguous surface. It is likely that these regions perform Cks-specific functions [1, 3].

It has been shown that the yeast Cks1p and Cdk1 are involved in chromatin remodeling and play a role in gene expression [36], and that one function of Cdk1/Cks1p and the 19S subunit of the proteasome is to evict nucleosomes from chromatin in the context of gene induction [37]. A role for Cks1p in cyclin B degradation has been suggested in *S. cerevisiae*. Both genetic and biochemical data indicate that Cks1p is required for efficient proteasomal targeting of ubiquitinated cyclin B [33].

Despite being 87% similar, Cks proteins in mammalian can have specific functions [1, 3]. For example, Cks1 plays an important role in facilitating the ubiquitin-mediated proteolysis of p27, a cyclin-dependent kinase inhibitor, through interaction with Skp2 (Figure 2) [38]. Cks1 is part of the substrate-binding surface, and is necessary for efficient ubiquitination of T187-phosphorylated p27^{kip1} [11, 39]. Tissues from mice lacking Cks1 accumulate p27^{Kip1} and exhibit proliferative defects. Accordingly, Cks1$^{-/-}$ mice exhibit a small body size, resembling the size phenotype of Skp2$^{-/-}$ mice. Overexpression of Cks1, in general, is correlated with decreased p27 levels [40], which are associated with high aggressiveness, poor prognosis and aggressive behavior in a large variety of malignant tumors [13, 39]. However, Cks1 supports hepatocarcinogenesis independently of Skp2, indicating it can play a separate role in carcinogenesis [41]. In contrast, Cks2 seems to counter Cks1 and stabilize p27; absence of Cks2 results in increased cyclin A/Cdk2 activity, shortening of the cell cycle, and DNA damage [34]. Cks2 is also essential for the first metaphase/anaphase transition of meiosis [22, 35].
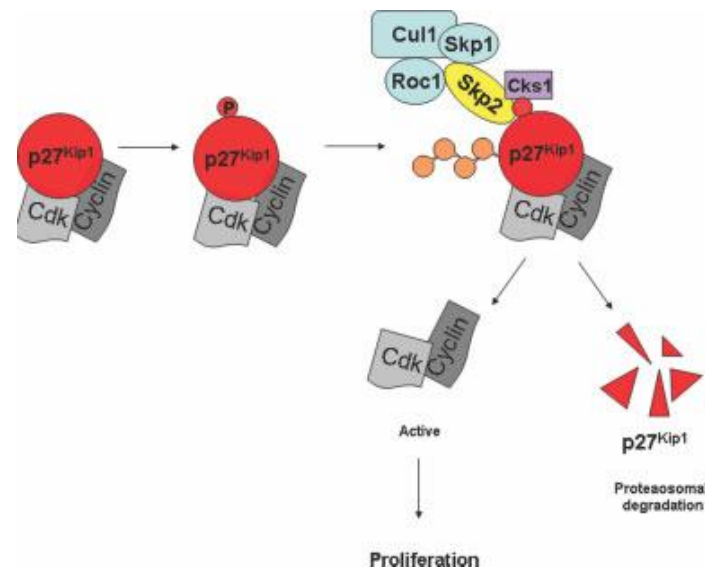
Figure 2. Skp2-dependent degradation of p27. After phosporylation of p27^{Kip1} at T187 by cyclin E/A-Cdk2, p27^{Kip1} is recognized by Skp2 and Cks1, which stimulates its targeting for ubiquitylation by the SCF^{Skp2} complex. The ubiquitylated p27^{Kip1} is then rapidly destroyed by the proteasome, allowing the activity of cyclin E/A-Cdk2 and progression to the S phase (Hershko (2008) [38]).

Cks proteins: Potential link to apoptosis

There is some indication that modulation of Cks proteins levels can impact apoptosis in select mammalian cell lines. Knockdown of Cks1 expression inhibited the growth of oral squamous cell cancer cells both in cultured cells and *in vivo* [11] and increased the activity of caspase 3, promoting apoptosis of breast cancer cells [42]. Inhibition of Cks1 by siRNA also induced accumulation of cells at the G2/M phase and apoptosis in Cks1-overexpressing lung cancer cells, but not in normal lung fibroblasts [6]. Down-regulation of Cks2 expression inhibited cell proliferation, colony formation and tumorigenesis by suppressing cell cycle progression and enhancing the susceptibility of cholangiocarcinoma cells to Bax-mediated mitochondrial caspase-dependent apoptosis [17]. In prostate tumor cells, knockdown of Cks2 expression induced apoptosis *in vitro* and compromised tumorigenic activity of the cells *in vivo* [22]. Additionally, knockdown of both Cks1 and Cks2 in malignant prostate tumor cells inhibited cell growth, anchorage-independent growth, and migration activity [22]. However, due to the lack of focused research on this subject, it is still not clear what role Cks proteins play in apoptosis, and especially if overexpression of Cks1 or Cks2 does indeed protect cells from undergoing apoptosis,

particularly in response to antitumor drugs. A role of Cks proteins in apoptosis has thus yet to be explored as a target in cancer therapy.

The importance of apoptosis in cancer therapy

Apoptosis is an important process in normal organ development and cellular regulation, playing a role in a variety of physiological and pathological conditions [43]. Apoptosis is also an important variable in cancer development, prevention and therapy [44]. It is well-established that a large number of anticancer agents induce apoptosis, and that disruption of apoptosis programs can reduce treatment sensitivity [45]. Indeed, abundant therapeutic opportunities have been uncovered by investigations of the fundamental mechanisms of apoptosis regulation and identification of the various cell survival genes that become de-regulated in tumors [46]. Since apoptotic programs can be manipulated to produce massive changes in cell death, apoptosis regulators are potential drug targets. Two observations suggest that such strategies are feasible. Firstly, most anti-apoptotic mutations act relatively upstream in the program, implying that tumor cells retain the machinery and latent potential for apoptosis. Secondly, tumor-specific alterations in apoptotic programs provide opportunities to target cell death in a selective manner. Several new strategies have been developed: for instance, apoptosis can be impaired by dominant oncogenes, and agents that inhibit their anti-apoptotic function can lead to a remarkable increase in cell death. In addition, hyperactivation of cell survival signaling may accompany tumor development, and these pathways are particularly exciting targets for small molecule inhibition. When apoptosis is lost by a recessive mutation, restoring the dysfunctional gene or activity can promote massive cell death. Indeed, strategies using this approach are currently in clinical trials. By enhancing the effects of pro-apoptotic mutations, it is also possible to directly harness the pro-apoptotic forces produced by certain oncogenic mutations to selectively destroy tumor cells [45].

In past years, there has been an extraordinary increase in the understanding of apoptosis, and its contribution to cancer development and cancer therapy. It seems likely that rational strategies to manipulate cell suicide programs will produce new therapies that are less toxic and mutagenic than current treatments [45].

Apoptosis regulation

The term apoptosis stems from the Greek language, to describe a process reminiscent of the "dropping off" or "falling off" of petals from flowers, or leaves from trees [47]. Apoptosis is a type of programmed cell death (PCD), categorized as a type of cell death that is not accidental (necrosis), but a genetically controlled sequence of steps that lead to morphological and biochemical changes [48], involving the cytoplasm, nucleus and plasma membrane [49]. There are particular morphological changes in the apoptotic process: rounding of the cell, retraction of pseudopodes, reduction in cellular volume, condensation of chromatin, fragmentation of the nucleus, plasma membrane blebbing and maintenance of an intact plasma membrane until the late stages of death [44, 50-54]. In contrast, during accidental necrosis, cells first swell, and then the plasma membrane collapses and cells are rapidly lysed, resulting in damage to neighboring cells and a strong inflammatory response in the corresponding tissue [55].

The apoptotic process is mediated by two classical pathways: the extrinsic and intrinsic pathways, which are activated by the binding of ligands to death receptors, or by stress triggered by intrinsic factors, such as oncogenes, exposure to irradiation and other environmental stresses, respectively.

I.   Extrinsic apoptosis

The term extrinsic apoptosis has been used to designate apoptotic cell death induced by extracellular stress signals that are received and propagated by specific transmembrane receptors. Extrinsic apoptosis can be initiated by the binding of lethal ligands, such as FAS/CD95 ligand (FASL/CD95L), tumor necrosis factor α (TNFα) and TNF ligand superfamily, to various death receptors. Alternatively, an extrinsic pro-apoptotic signal can be dispatched by the netrin receptors, which only exercise lethal functions when the concentration of their specific ligands is lower than a critical threshold level [56-57].

There are several transmembrane proteins that, at least under special circumstances, can transduce lethal signals in response to ligand binding. Most of these proteins have a double function, depending on the cellular context and triggering stimulus, and they can convey either pro-survival or pro-death signals [56]. A signaling pathway leading to extrinsic apoptosis is illustrated by FAS ligation (Figure 3). In the absence of FAS ligand (FASL), FAS subunits spontaneously assemble at the plasma membrane to produce trimers. Ligand binding stabilizes these trimers while at the same time inducing a conformational change that allows the assembly

of a dynamic multiprotein complex at the cytosolic tail of the receptor. The resulting supramolecular complex, which has been called death-inducing signaling complex (DISC), constitutes a platform that regulates the activation of caspase -8 (or -10) [49, 56, 58]. In some cell types, such as lymphocytes, so-called type I cells, active caspase -8 directly catalyzes the proteolytic maturation of caspase -3, triggering the executioner phase of caspase-dependent apoptosis in a mitochondrion-independent mode. In other cells, type II cells, including hepatocytes and pancreatic b cells, caspase -8 mediates the proteolytic cleavage of BH3-interacting domain death agonist (Bid), leading to the generation of a mitochondrion-permeabilizing fragment, known as truncated Bid (tBid). Therefore, while type I cells undergo extrinsic apoptosis irrespective of any contribution by mitochondria, type II cells give way to the activation of death receptors while showing signs of mitochondrial outer membrane permeabilization (MOMP), including the dissipation of mitochondrial transmembrane potential ($\Delta\psi$m) and the release of toxic proteins that are normally retained within the mitochondrial intermembrane space [56].
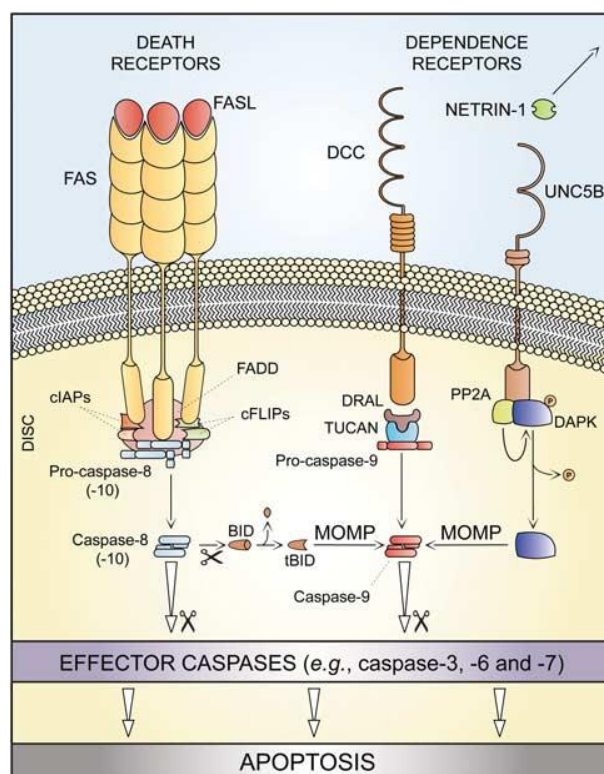

Figure 3. Extrinsic apoptosis. FASL: FAS ligand; FADD: FAS-associated protein with a death domain; cIAPs: cellular Inhibitor of Apoptosis Proteins; DISC: Death Inducing Signaling Complex; MOMP: Mitochondrial Outer Membrane Permeabilization; DAPKI: Death-associated Protein Kinase 1; PP2A: Protein Phosphatase 2A (Galluzzi *et al* (2012) [56]).

Extrinsic apoptosis features one of three major lethal signaling cascades: **(i)** death receptor signaling and activation of the caspase-8 (or -10) → caspase-3 cascade; **(ii)** death receptor signaling and activation of the caspase-8 → tBid → Mitochondrial outer membrane permeabilization → caspase-9 → caspase-3 pathway; or **(iii)** ligand deprivation-induced dependence receptor signaling followed by (direct or mitochondrial outer membrane permeabilization-dependent) activation of the caspase-9 → caspase-3 cascade. Extrinsic apoptosis is thus a caspase-dependent cell death process, and hence can be suppressed, at least theoretically, by pancaspase chemical inhibitors. [56].

II. Intrinsic apoptosis

Apoptosis can also be triggered by an excess of intracellular stress, including DNA damage, oxidative stress, cytosolic Ca^{2+} accumulation, excitotoxicity, related to glutamate receptor overstimulation in the nervous system, accumulation of unfolded proteins in the endoplasmic reticulum and many others [56, 59]. Although the processes that trigger intrinsic apoptosis are highly heterogeneous as far as the initiating stimuli are concerned, they are all associated with a mitochondrion-centered control mechanism.

Intrinsic apoptosis is mediated by MOMP and thus is associated with generalized and irreversible $\Delta\psi_m$ dissipation, release of mitochondrial intermembrane space proteins into the cytosol (and their possible relocalization to other subcellular compartments), and respiratory chain inhibition [56]. The connection between the extrinsic and intrinsic pathways and amplification of death signal is mediated by Bid, a pro-apoptotic Bcl-2 family member (Figure 4). Bid is cleaved by caspase-8 and when the truncated form is translocated into the mitochondria it acts to induce MOMP and release of pro-apoptotic proteins [60]. MOMP can occur due to the pore-forming activity of pro-apoptotic members of the Bcl-2 protein family such as Bak and Bax [61]. Bcl-2 and Bcl-x$_L$ can directly induce changes in conformation of the proteins Bax and Bak, preventing their activation and oligomerization, and blocking the release of pro-apoptotic mitochondrial factors that lead to apoptosis [62]. Another model suggests a permeability transition pore (PTP) that is formed, which allows the passage of solutes and water into the mitochondria matrix, causing mitochondrial depolarization, uncoupling of oxidative phosphorylation and osmotic swelling. The precise localization and composition of the PTP has not been fully determined, but it appears to be localized at the site of contact between the inner and outer mitochondrial membranes and to contain as main components as the voltage-

dependent anion channel (VDAC), the adenine nucleotide translocator (ANT), and cyclophilin D (CypD) [63]. After MOMP, cytosolic cytochrome *c* participates with apoptotic proteasome activating factor 1 (APAF1), cytoplasmic adaptor protein, and dATP in the formation of the apoptosome, which triggers the caspase-9 → caspase-3 proteolytic cascade (Figure 4) [64].
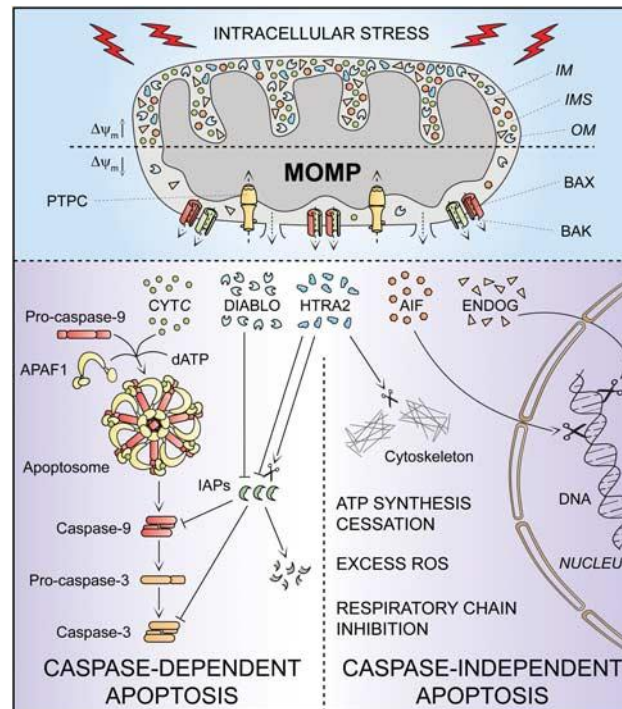

Figure 4. Intrinsic apoptosis. MOMP: Mitochondrial Outer Membrane Permeabilization; $\Delta\psi_m$: mitochondrial transmembrane potential; ROS: Reactive Oxygen Species; IMS: Mitochondrial Intermembrane Space; CYTC: cytochrome *c*; DIABLO: Direct IAP-binding protein with low pI; HTRA2: High Temperature Requirement Protein A2; IAP: Inhibitor of Apoptosis Protein; AIF: Apoptosis Inducing Factor; ENDOG: endonuclease G; IM: Mitochondrial Inner Membrane; OM: Mitochondrial Outer Membrane; PTPC: Permeability Transition Pore Complex (Galluzi *et al* (2012) [56]).

Independently of the precise biochemical and physical mechanisms through which it develops, irreversible mitochondrial outer membrane permeabilization affecting most mitochondria within a single cell has multiple lethal consequences: the dissipation of the $\Delta\psi_m$, with cessation of mitochondrial ATP synthesis and $\Delta\psi_m$-dependent transport activities; the release of pro-apoptotic proteins that circulate freely in the mitochondrial intermembrane space into the cytosol, like cytochrome *c*, apoptosis inducing factor (AIF), endonuclease G, direct IAP-binding protein with low pI (DIABLO, also known as second mitochondria-derived activator of caspases, SMAC) and high temperature requirement protein A2 (HTRA2); and the inhibition of the

respiratory chain (favored by the loss of cytochrome c), eliciting or aggravating reactive oxygen species (ROS) overproduction and hence activating a feed-forward circuit for the amplification of the apoptotic signal [56, 65-66].

Saccharomyces cerevisiae as model system to study apoptosis regulation

Like mammalian cells, yeast cells can trigger apoptosis showing characteristic markers, such as DNA fragmentation and chromatin condensation, externalization of phosphatidylserine to the outer leaflet of the plasma membrane and cytochrome c release from mitochondria [67]. Accumulating evidence points toward the phylogenetic conservation of the core machinery and the core regulators of cell death between yeast and mammals. This entails the possibility of using yeast as a research tool that can provide new hints to elucidation of cell death pathways [68].

An apoptotic phenotype in yeast was first described in a cdc28 temperature-sensitive mutant (Figure 5) [67]. When incubated above the restrictive temperature, cdc48 mutant cells showed an apoptotic phenotype characterized by phosphatidylserine exposure, DNA damage, chromatin condensation and fragmentation, release of cytochrome c and ROS production [69-70]. Since then, _CDC48_, an essential gene that encodes an AAA-ATPase localized in the endoplasmic reticulum and necessary for vesicle trafficking/translocation of ubiquitinated proteins from the endoplasmic reticulum to the proteasome for degradation [67], has been confirmed as a regulator of mammalian apoptosis, with anti-apoptotic functions, particularly apparent in neuronal pathology [71]. Various other yeast orthologues of vital apoptotic regulators have been identified, such as the caspase orthologue _YCA1_ (Figure 5) [72]. Yca1p belongs to the family of metacaspases that are found in fungi, plants and protists [68], but not in organisms containing "classical" caspases, and might represent an alternative form of caspase that has developed from the same ancient ancestor as human caspases [67]. Several studies also identified yeast orthologs of several other members of the mammalian apoptotic machinery, including AIF [73], inhibitor of apoptosis protein (IAP) [74], the apoptotic serine protease HTRA2/Omi [75] and endonuclease G [76].
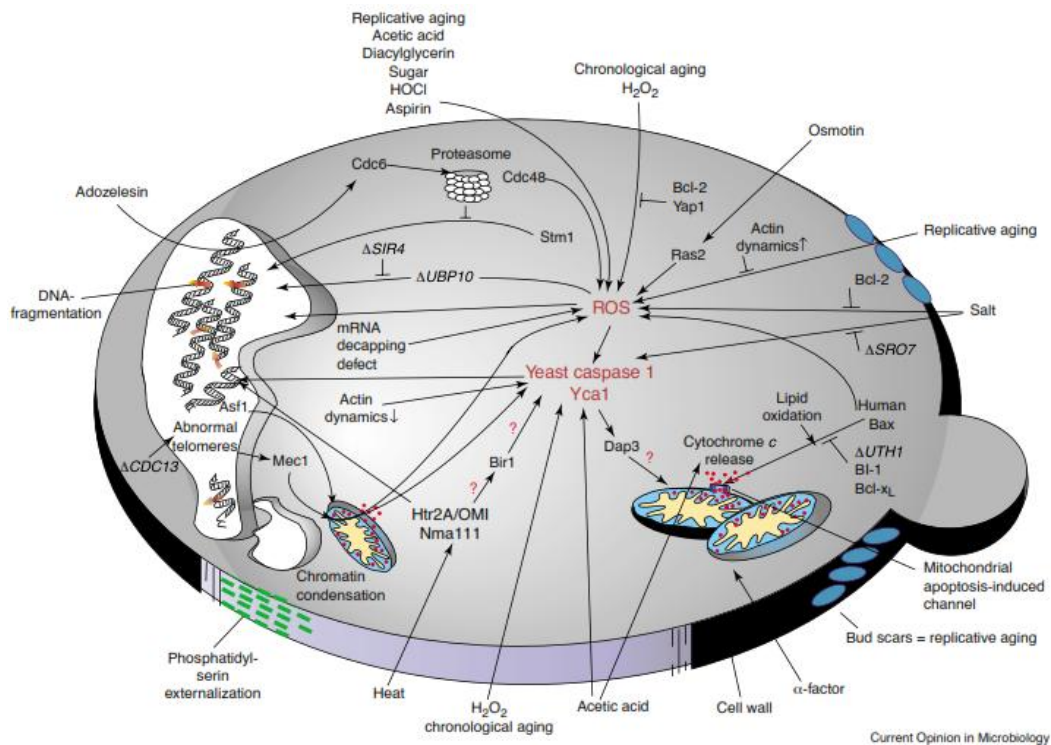
Figure 5. The apoptotic yeast cell. Red question marks indicate pathways that are known in mammals but not in yeast thus far (Madeo *et al* (2004) [67]).

Regulators such as Apaf-1 and most members of the Bcl-2 family proteins seem to be absent in yeast. Until now, only a putative yeast BH3-only protein was identified. Ybh3p translocates to the mitochondria and is capable of mediating the mitochondrial pathway of apoptosis [77]. However, heterologous expression of Bax in yeast leads to apoptotic cell death that can be prevented by heterologous expression of anti-apoptotic Bcl-2 and Bcl-x_L, suggesting the function of Bcl-2 family proteins is potentially conserved in yeast [78].

OBJECTIVES

AND

RESEARCH

PLAN

The major objective of this work is to determine whether Cks proteins have a specific role in apoptosis that could contribute to tumorigenesis and/or resistance of Cks-overexpressing tumors to treatment or prevention of cancer. The yeast apoptotic model has already demonstrated its potential for application in the human system, as evidenced by the increasing number of studies using yeast as a model for neurotoxicity and cancer. Since most functions attributed to Cks proteins are also conserved in yeast, and mammalian Cks proteins can functionally substitute for yeast Cks1p, we aimed to use *S. cerevisiae* as a model system to determine:

I: Whether deletion of *CKS1* alters apoptotic signaling in yeast;

II: Whether overexpression of Cks proteins alters apoptotic signaling in yeast.

MATERIALS

AND

METHODS

Reagents

Yeast nitrogen base, bactopeptone and tryptone were purchased from Difco Laboratories and yeast extract from Cultimed. The carbon source used was glucose (Fisher). All aminoacids were purchased from Formedium. 5-Fluorouracil (5-FU) (Sigma) was dissolved in dimethyl sulfoxide (DMSO) and keep at 4°C. Cisplatin (Sigma) was stored in aliquots of up to 2mg in the dark and dissolved in the medium immediately prior to use. Hydroxyurea (Formedium) was dissolved in the medium immediately before use.

Strains and plasmids

All *Saccharomyces cerevisiae* strains used in this study and respective phenotypes are listed in table 1. Plasmids YEp13 and YEp13-*CKS1* [79], and pYX232-*mtGFP* [80], expressing a GFP tagged with a mitochondrial targeting sequence, have been previously described.

All plasmids were amplified by transforming 100 ng of DNA into *Escherichia coli* DH5α chemically competent cells by transformation using standard procedures (reviewed in [81]) and selected on Luria Bertani (LB) medium [LB: 1 % (w/v) tryptone, 0,5 % (w/v) yeast extract, 1 % (w/v) NaCl and 2 % (w/v) agar] supplemented with 100 µg/mL ampicillin. One colony was inoculated in LB-Amp media, cultures were grown overnight, and DNA was extracted using the GenElute Plasmid Miniprep kit according to manufacturer's instructions (Sigma).

BF264-15D (15D) and *cks1*Δ cells were transformed with plasmid YEp13, YEp13-*CKS1* or pYX232-*mtGFP* using the lithium acetate method [82]. Transformants were selected on Synthetic Complete (SC) medium [SC: 0.17 % (w/v) Yeast nitrogen base without aminoacids and ammonium sulfate, 0.5 % (w/v) ammonium sulfate, 0.14 % (w/v), dropout mixture lacking histidine, leucine, tryptophan and uracil, 0.008 % (w/v) histidine, 0.04 % (w/v) leucine, 0.008 % (w/v) Tryptophan and 0.008 % (w/v) uracil] lacking the appropriate aminoacids plus 2 % (w/v) of carbon source and 1,5 % agar.

Table 1. *S. cerevisiae* strains used in this work

Strain	Phenotype	Reference
BF264-15D	Mat a, *leu2, trp1, ade1, his3*	[83]
cks1Δ	BF264-15D *cks1Δ* :: KanMX4	[84]
15D YEp13	BF264-15D harboring YEp13	This study
15D YEp13-*CKS1*	BF264-15D harboring YEp13-*CKS1*	This study
***cks1Δ* YEp13**	*cks1Δ* harboring YEp13	This study
cks1Δ* YEp13-*CKS1	*cks1Δ* harboring YEp13-*CKS1*	This study
15D pYX232-*mtGFP*	BF264-15D harboring pYX232-*mtGFP*	This study
cks1Δ* pYX232-*mtGFP	*cks1Δ* harboring pYX232-*mtGFP*	This study

Fluorescence Microscopy

Cells were harvested by centrifugation and resuspended in 1x PBS (80 mM Na_2HPO_4, 20 mM NAH_2PO_4 and 100 mM NaCl). Cells were visualized in a Leica Microsystems DM5000B epifluorescence microscope using appropriate filter settings with a 100x oil immersion objective. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software.

Growth conditions and treatments

S. cerevisiae strains 15D and *cks1Δ* were grown overnight in rich medium [YPD: 2 % (w/v) bactopeptone, 1 % (w/v) yeast extract, 2 % (w/v) glucose] at 30°C, 200 rpm. Cells were collected by centrifugation at OD_{600} 0,5-0,8 and resuspended in fresh YPD medium (pH 3.0 set with HCl in the case of acetic acid treatments) with or without 120 mM acetic acid (AA), 0,025 % methyl methanesulphonate (MMS), 0,2 mg/mL cis-diamminedichloroplatinum (II) (cDDP) or 2 % hydrogen peroxide (H_2O_2) (in this case cells were diluted to an OD_{600} ~ 0,2) for up to 180 min, at 30°C, 200 rpm. Growth conditions and treatments of transformed strains were performed in the same manner, but using SC medium lacking the appropriate aminoacid instead of YPD.

Viability assays

I. Colony-forming units (c.f.u.)

At specific time points, cells were collected by centrifugation, ressuspended in water, diluted and plated on YPDA plates [YPDA: 2 % (w/v) bactopeptone, 1 % (w/v) yeast extract, 2 % (w/v) glucose and 1,5 % (w/v) agar]. After 2 days of incubation at 30°C, colony-forming units (c.f.u.) were counted. OD_{600} was measured for each sample and c.f.u. at each time point was normalized to the OD_{600}. Percentages of viability were calculated in relation to time 0 (corresponding to 100 % of viability). Statistical analysis and cell viability quantification were performed with GraphPad Prism 6 software.

II. Spot assays

For semi-quantitative viability assays, 4 µL of cell suspensions and of ten-fold serial dilutions (10^{-1} to 10^{-4}) were spotted onto YPDA plates. After 2 days of incubation at 30°C, plates were photographed using a ChemiDoc XRS (BioRad) and Quantity One® software (BioRad).

III. Chronic exposure to DNA damaging agents

Strains were grown overnight in liquid medium, cells were collected by centrifugation at OD_{600} 0,5-0,8 and resuspended in water to an OD of 0,5. 4 µL of cells suspensions and of ten-fold serial dilutions (10^{-1} to 10^{-4}) were spotted onto YPDA or SCGLU (in case of exposure to 5-FU or cDDP) plates, containing different concentrations of HU (0, 200, 400 and 600 mM), MMS (0, 0075 and 0,015 %), 5-FU (0, 200 and 500 µg/mL) or cDDP (0, 50 and 100 µg/mL). After 2 days of incubation at 30°C, plates were photographed using a ChemiDoc XRS (BioRad) and Quantity One® software (BioRad).

Cell cycle analysis

For cell cycle analysis, cells were collected, fixed in 70 % (v/v) ethanol and stored at 4°C. Then, cells were washed and resuspended in 50 mM sodium citrate (pH 7.5). RNAse A (Sigma-Aldrich) and proteinase K (NZTech) were added to a final concentration of 0,25 mg/mL and 1 mg/mL, respectively, and samples incubated overnight at 37°C. Afterwards, cells were centrifuged and resuspended in 50 mM sodium citrate buffer (pH 7.5) containing 1 µM of sytox green (Molecular probes) and kept overnight in the dark at 4°C. Samples were sonicated briefly

(3 impulses 30 %, 1-2 sec each) prior to analysis in an Epics® XL™ flow cytometer (Beckman Coulter), with excitation and emission wavelengths of 497nm and 520nm, respectively (FL-1 channel). Analysis of the results was performed using FlowJo 7.6 software (Tree Star, Inc.).

Detection of apoptotic markers

I.   Plasma membrane integrity

50 µL of cells were collected at specific time points, centrifuged and resuspended in 500 µL 1x PBS. Propidium iodide (PI) (Sigma) was added to a final concentration of 2 µg/mL, and samples incubated for 10 minutes in the dark. Fluorescence was detected in an Epics® XL™ flow cytometer (Beckman Coulter). Cells with red fluorescence (FL-3 channel (488/620nm)) were considered to contain plasma membrane disruption.

II.   Production of ROS

50 µL of cells were collected at specific time points, diluted into 450 µL 1x PBS and incubated with 5 µg/mL dihydroethidium (DHE) (Molecular Probes), for detection of superoxide anion, for 30 min in the dark. Fluorescence was measured in an Epics® XL™ flow cytometer (Beckman Coulter). Cells with red fluorescence (FL-3 channel (488/620nm)) were considered to contain superoxide anion.

RESULTS

Deletion of yeast *CKS1* does not alter mitochondrial morphology

It has previously been reported that depletion of Cks1/2 in murine embryonic fibroblasts led to a significant increased in the number of fragmented mitochondria, which could affect a mitochondrial-mediated apoptotic pathway [85]. To evaluate whether the absence of yeast *CKS1* altered mitochondria morphology, wild-type strain 15D and strain *cks1Δ* were transformed with a plasmid expressing a mitochondrial green fluorescent protein and visualized by fluorescence microscopy. As shown in Figure 6, even though *cks1Δ* cells had an "aberrant" cellular morphology, the tubular mitochondrial network was visible in both strains and no difference in mitochondrial morphology was found.
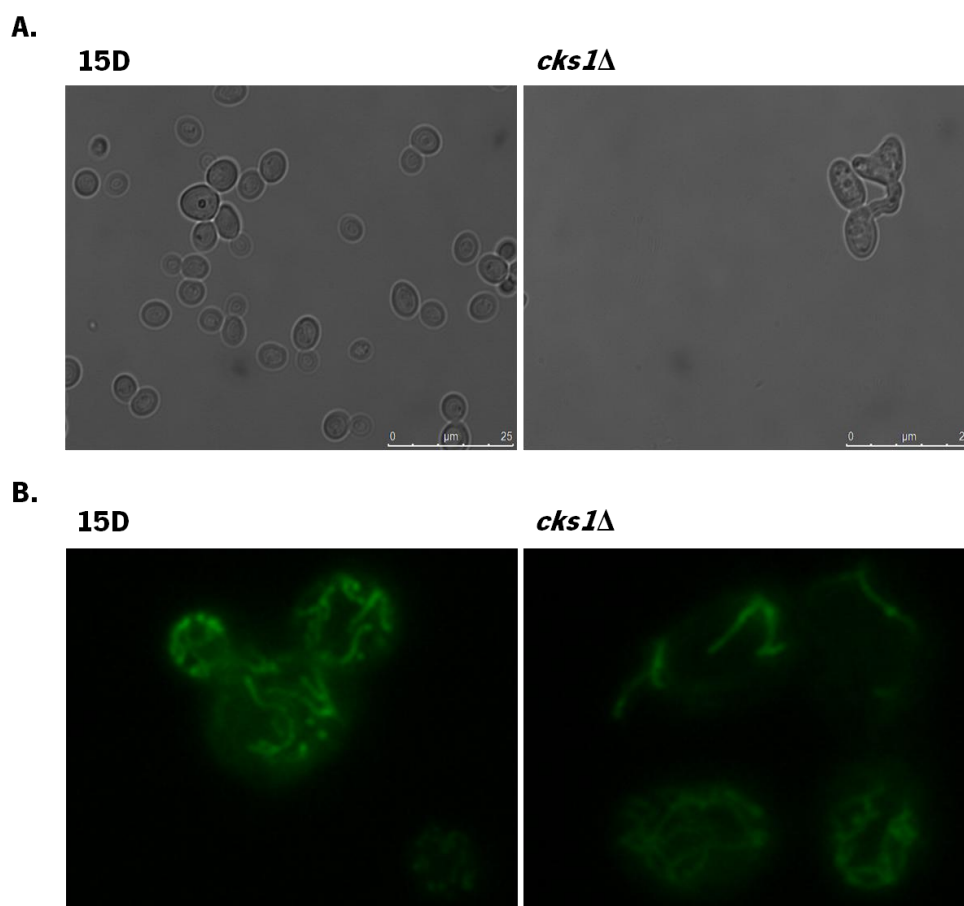

Figure 6. Morphology of *S. cerevisiae* 15D and *cks1Δ* cells. Strains 15D and *cks1Δ* were transformed with pYX232-*mtGFP* and visualized by fluorescence microscopy **(A)** DIC, **(B)** GFP fluorescence.

Deletion of yeast *CKS1* does not alter sensitivity to the yeast apoptosis inducers acetic acid or hydrogen peroxide

In order to determine whether *CKS1* could play a general role in the apoptotic process, we first tested if absence of Cks1p affected viability of *S. cerevisiae* cells exposed to the most commonly used yeast apoptosis inducers, acetic acid and hydrogen peroxide.

Acetic acid is a weak acid that can be formed as an end sub-product of alcoholic fermentation by *S. cerevisiae* [86]. After acetic acid enters the cell, it dissociates (when the intracellular pH is higher than the extracellular pH), compromising cell viability [87], leading to the intracellular acidification [88] and induction of apoptosis [89]. Exposure of *S. cerevisiae* to low doses to acetic acid at pH 3.0 results in cell death with features of mammalian apoptosis. Cells exhibit chromatin condensation, exposure of phosphatidylserine and DNA strand breaks [89]. Like in mammalian cells, yeast apoptosis induced by acetic acid was linked to mitochondria. It was shown that acetic acid can lead to the release of cytochrome *c*, ROS accumulation, transient hyperpolarization of mitochondria followed by depolarization, decrease of mitochondrial respiration associated with decrease in cytochrome oxidase activity [90] and mitochondrial ultrastructural changes, namely decrease of cristae number, formation of myelinic bodies and swelling [91].

Hydrogen peroxide is a reactive oxygen species described as an apoptotic stimulus at low doses [92], and is known to induce apoptosis both in *S. cerevisiae* and in mammalian cells [93]. High concentrations of hydrogen peroxide result in cell death associated with disintegration of intracellular structures but without the phenotypic markers of apoptosis [92]. Apoptotic cell death induced by hydrogen peroxide in mammalian cells, promotes decrease in the intracellular superoxide anion (O_2) concentration, reduction and acidification of the intracellular melieu, activation of caspases [94], namely caspase-9 and -3, release of cytochrome *c* [95], among others. In yeast, apoptosis induced by hydrogen peroxide is accompanied by accumulation of ROS [92], phosphatidylserine exposure [96], cytochrome *c* release [96], DNA fragmentation [92], and plasma membrane vesicles reminiscent of blebbing observed in mammalian cells [96].

Strain 15D and *cks1Δ* were exposed to apoptosis-inducing concentrations of acetic acid and hydrogen peroxide for up 180 min, at 30°C, and viability assessed by semi-quantitative spot assay and c.f.u. counts.
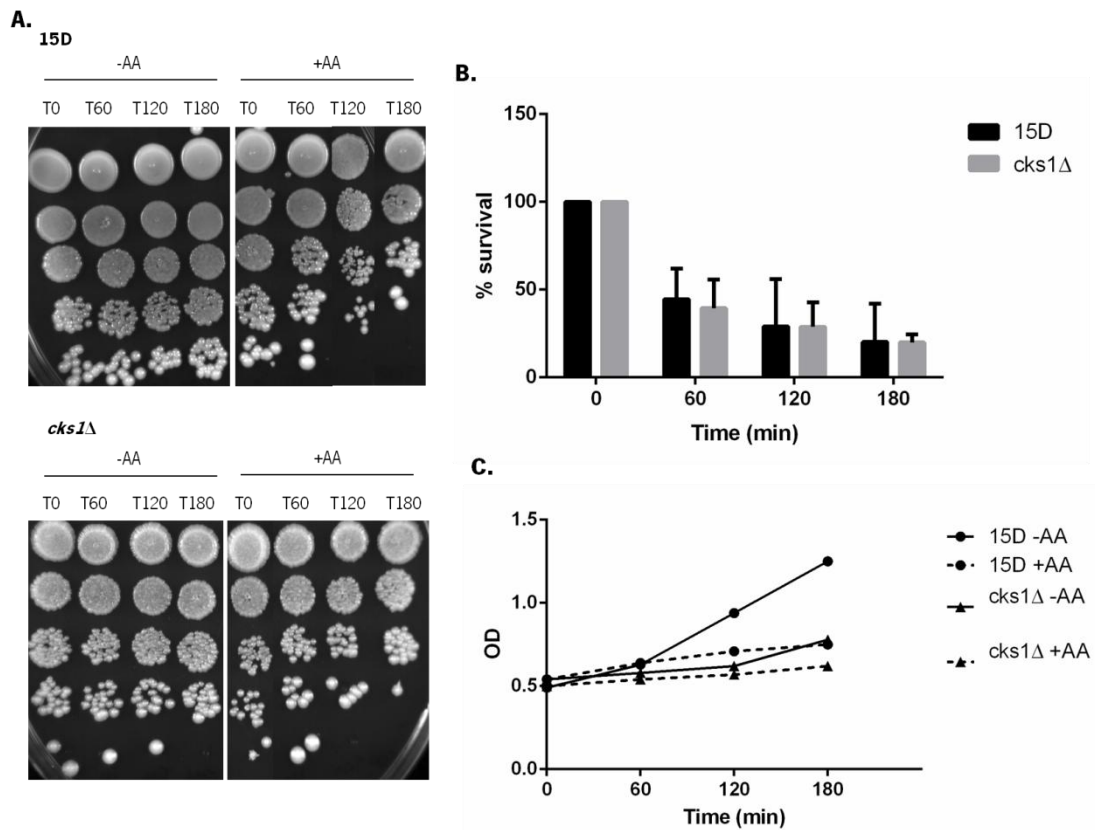
Figure 7. Sensitivity of wild-type strain 15D and deleted strain *cks1*Δ to AA. Exponential cultures of 15D and *cks1*Δ strains grown in YPD medium at 30°C, were transferred to fresh YPD medium (pH 3) with (+AA) or without (-AA) 120 mM AA. Cells were grown for 180 minutes at 30°C. Samples were taken after 0, 60, 120 and 180 min. **(A)** Serial dilutions (1:10) were spotted onto YPD plates and incubated for 2 days at 30°C. **(B)** For c.f.u. measurements, dilutions were plated on YPD plates, incubated for 2 days at 30°C and colonies counted. Values represent means and standard deviations of 3 independent experiments. **(C)** OD$_{600}$ of the cultures.
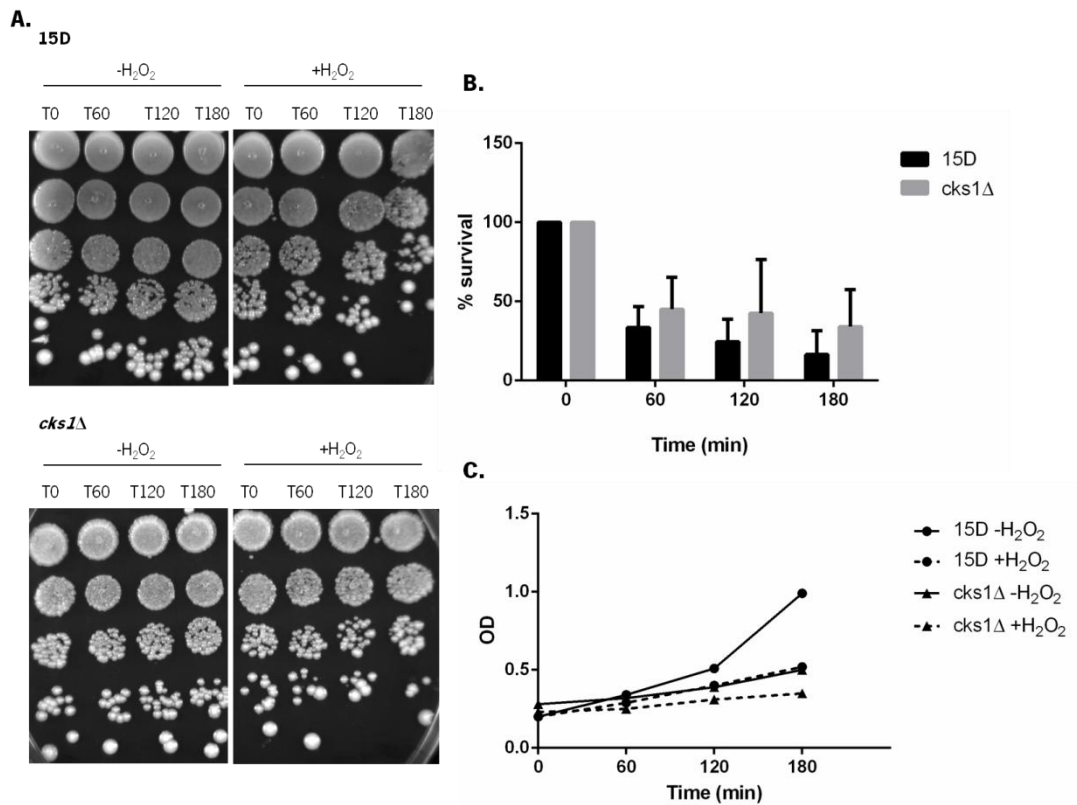
Figure 8. Sensitivity of wild-type strain 15D and deleted strain *cks1*Δ to H₂O₂. Exponential cultures of 15D and *cks1*Δ strains grown in YPD medium at 30°C, were transferred to fresh YPD medium with (+H₂O₂) or without (-H₂O₂) 2 % H₂O₂. Cells were grown for 180 minutes at 30°C. Samples were taken after 0, 60, 120 and 180 min. **(A)** Serial dilutions (1:10) were spotted onto YPD plates and incubated for 2 days at 30°C. **(B)** For c.f.u. measurements, dilutions were plated on YPD plates, incubated for 2 days at 30°C and colonies counted. Values represent means and standard deviations of 3 independent experiments. **(C)** OD$_{600}$ of the cultures.

There were no differences in sensitivity of the wild-type strain 15D and the strain *cks1*Δ to acetic acid (Figure 7B) or hydrogen peroxide (Figure 8B), indicating that Cks1p is not involved in a general stress response.

Deletion of yeast *CKS1* results in differential sensitivity to DNA damaging agents

Our global aim is to determine whether Cks proteins have a role in apoptosis contributing to tumorigenesis and/or resistance of Cks-overexpressing tumors to treatment regimens. We therefore sought to determine whether Cks1p plays a role in the DNA damage response, as DNA damaging agents are among the most commonly used anti-cancer agents.

It had previously been reported that deletion of yeast *CKS1* renders cells more sensitive to growth in the presence of hydroxyurea, but not to UV irradiation [84]. We next tested whether *cks1Δ* mutants were sensitive to other DNA damaging agents.

The DNA alkylating agent methyl methanesulfonate causes base mispairing and replication blocks [97-98] and induces both inter-chromosomal and intra-chromosomal recombination [97]. Exposure to MMS causes a checkpoint-independent reduction in the rate of replication fork progression, likely due to a physical impediment of fork progression caused by alkylated DNA or some intermediate in lesion processing [98]. Strains 15D and *cks1Δ* were exposed to MMS for up to 180 min, at 30°C and viability assessed by semi-quantitative spot assay and c.f.u. counts.
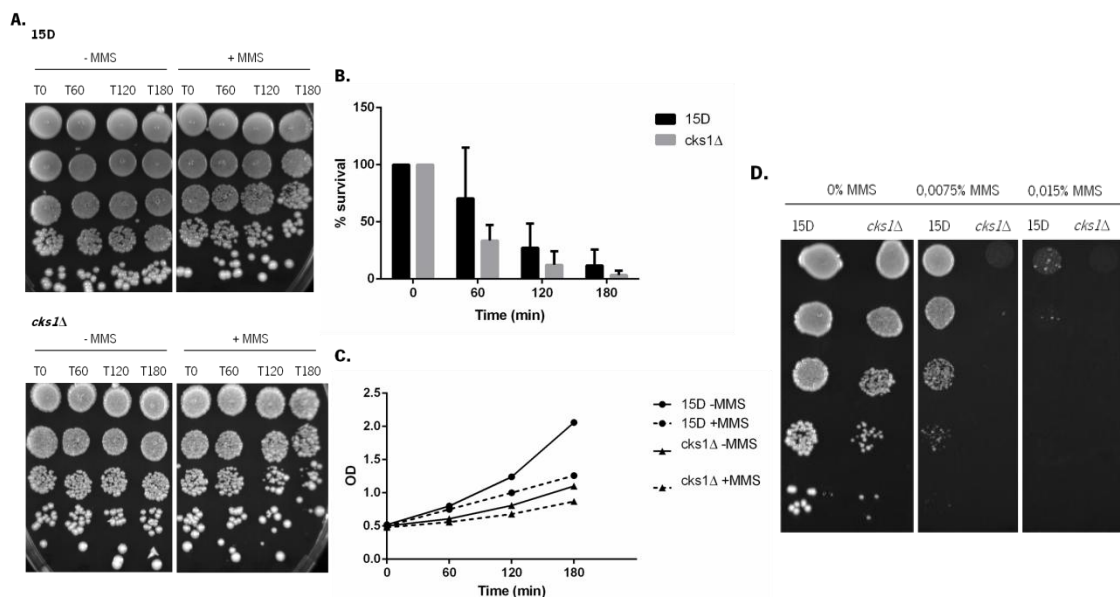

Figure 9. Sensitivity of wild-type strain 15D and deleted strain *cks1Δ* to MMS. Exponential cultures of 15D and *cks1Δ* strains grown in YPD medium at 30°C, were transferred to fresh YPD medium with (+MMS) or without (-MMS) 0,025 % MMS. Cells were grown for 180 minutes at 30°C. Samples were taken after 0, 60, 120 and 180 min. **(A)** Serial dilutions (1:10) were spotted onto YPD plates and incubated for 2 days at 30°C. **(B)** For c.f.u. measurements, dilutions were plated on YPD plates, incubated for 2 days at 30°C and colonies counted. Values represent means and standard deviations of 3 independent experiments. **(C)** OD_{600} of the cultures. **(D)** Serial dilutions (1:10) were spotted onto YPD plates containing 0, 0,0075 and 0,015 % MMS and incubated for 2 days at 30°C.

cks1Δ cells were only mildly more sensitive to transient MMS exposure than the wild-type strain, though there were no significant differences (Figure 9B). However, growth of the *cks1Δ*

strain on plates containing MMS was severely impaired (Figure 9D), indicating Cks1p is important for the ability of cells to survive chronic exposure to MMS, but not to overcome its short-term effects.

5-Fluorouracil is a pyrimidine analog antimetabolite commonly used in cancer treatment [99-102]. The cytotoxic mechanism of 5-FU occurs through inhibition of thymidylate synthase (TS), an enzyme involved in nucleotide synthesis [99], as well as through incorporation of fluoronucleotides into DNA and RNA, and disruption of RNA processing [101-102]. Since yeast lacks thymidine kinase, and therefore is unable to convert 5-FU into FdUMP, and inhibit TS, only the latter processes occur [103]. In order to determine whether yeast Cks1p affects sensitivity to 5-FU, strains 15D and *cks1Δ* were exposed to 5-FU for up to 24 hours, at 30°C, and viability assessed by semi-quantitative spot assay.
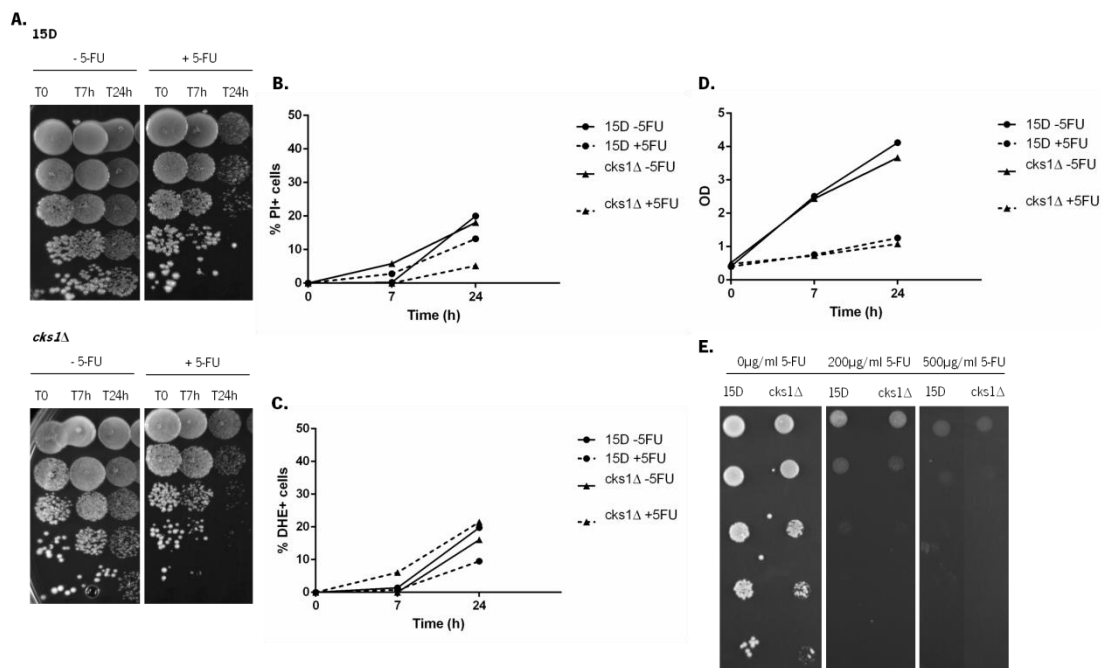

Figure 10. Sensitivity of wild-type strain 15D and deleted strain *cks1Δ* to 5-FU. Exponential cultures of 15D and *cks1Δ* strains grown in SC medium at 30°C, were transferred to fresh SC medium with (+5FU) or without (-5FU) 10 mM 5-FU. Cells were grown for 24 hours at 30°C. Samples were taken after 0, 7 and 24 hours. **(A)** Serial dilutions (1:10) were spotted onto YPD plates and incubated for 2 days at 30°C. **(B)** Samples were incubated with PI (5 μg/mL) and fluorescence measured by flow cytometry. Increase of cells with PI staining is shown. **(C)** Samples are stained with DHE (5 μg/mL) and the fluorescence measured by flow cytometry. Increase of cells stained with DHE is shown. **(D)** OD$_{600}$ of the cultures. **(E)** Serial dilutions (1:10) were spotted onto SC plates containing 0, 200 and 500 μg/mL 5-FU and incubated for 2 days at 30°C.

As seen in figure 10, there was no difference in the sensitivity of wild-type strain 15D and strain *cks1Δ* to 5-FU. Exposure to 5-FU did not lead to an increase in the number of cells stained with DHE or PI in comparison with non-treated cells (Figure 10B and 10C), indicating it does not cause an accumulation of ROS, typical of mitochondria-mediated apoptosis, but also does not lead to loss of plasma membrane integrity, typical of necrosis. Further studies will be required to characterize the mechanism mediating 5-FU-induced cell death in yeast, and confirm whether it is an active process. Nonetheless, there was no difference in the sensitivity of both strains to long-term exposure to 5-FU (Figure 10E), indicating *CKS1* does not play a role in the cellular response to 5-FU.

Cisplatin is a platinum-based chemotherapy drug with activity against a wide spectrum of tumors [104-108]. It acts by forming a platinum complex inside the cell which binds to DNA and forms adducts, leading to inter-strand and intra-strand DNA cross-links, as well as DNA-protein cross-links [107, 109-111]. However, nuclear DNA is not the only target of cisplatin. It also binds to mitochondrial DNA, interacts with phospholipids and phosphatidylserine in membranes, disrupts the cytoskeleton and affects the polymerization of actin [112]. When bound to DNA, cisplatin inhibits DNA replication and chain elongation [113-114]. Cunha *et al*, showed that cisplatin induces an atypical programmed cell death pathway in *S. cerevisiae*, which is active, but mitochondria-independent, and that proteasome inhibition protects yeast cell from cisplatin-induce cell death [115]. In order to determine whether deletion of *CKS1* affects sensitivity to cisplatin, wild-type strain 15D and *cks1Δ* strain were exposed to cisplatin for up to 180 min, at 30°C and viability assessed by semi-quantitative spot assay and c.f.u. counts.
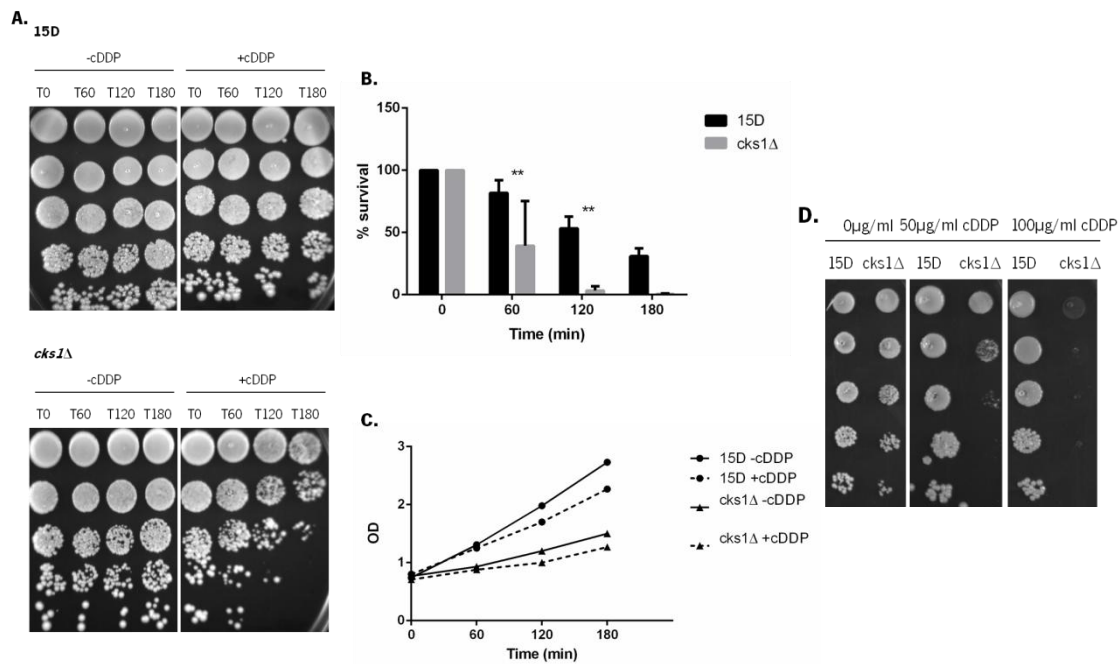
Figure 11. Sensitivity of wild-type strain 15D and deleted strain *cks1*Δ to cisplatin. Exponential cultures of 15D and *cks1*Δ strains grown in YPD medium at 30°C, were transferred to fresh YPD medium with (+cDDP) or without (-cDDP) 0,2 mg/mL cisplatin. Cells were grown for 180 minutes at 30°C. Samples were taken after 0, 60, 120 and 180 min. **(A)** Serial dilutions (1:10) were spotted onto YPD plates and incubated for 2 days at 30°C. **(B)** For c.f.u. measurements, dilutions were plated on YPD plates, incubated for 2 days at 30°C and colonies counted. Values represent means and standard deviations of 3 independent experiments (**p<0,005). **(C)** OD_{600} of the cultures. **(D)** Serial dilutions (1:10) were spotted onto SC plates containing 0, 50 and 100 µg/mL cDDP and incubated for 2 days at 30°C.

We found that the *cks1*Δ mutant strain is more sensitive to transient exposure to cisplatin than the wild-type strain 15D, which was more significant at time 60 and 120 min, and also to growth on plates containing cisplatin (Figure 11B, 11D), demonstrating that Cks1p is involved in the cellular resistance to cisplatin. We therefore proceeded to explore whether there was any alteration in apoptotic markers.

Cell membrane damage is considered a marker of necrotic cell death. Therefore, we tested if cisplatin-induced cell death in this strain background is accompanied by loss of membrane integrity. For this purpose, yeast cells were stained with PI and the fluorescence was evaluated by flow cytometry. As described for W303 cells [115], wild-type 15D and *cks1*Δ cells remained impermeable to PI after 180 minutes of exposure to cisplatin (Figure 12A), even

though there was a significant loss of cell viability under the same conditions (Figure 11B). This indicates cisplatin-induced yeast cell death occurs without loss of membrane integrity, supporting the hypothesis that cisplatin-induced death is an active process in both strains, and that the increased cell death observed in the *cks1*Δ mutant is not necrotic in nature.

ROS have been widely recognized as crucial cell death regulators and have been connected to many of the known apoptotic pathways in yeast [69, 116]. Huang *et al* (2003) showed that DNA damaging agents such as γ-irradiation and cisplatin induce apoptosis in Jurkat cells by increased ROS formation, leading to the generation of hydrogen peroxide and superoxide anion [117]. However, it has previously been reported that exposure of yeast W303 cells to cisplatin did not result in an increased percentage of cells stained with DHE, which detects the accumulation of intracellular superoxide anion [115]. In order to test the involvement of ROS in cisplatin-induced death in the 15D background, ROS levels were determined by flow cytometry using DHE.
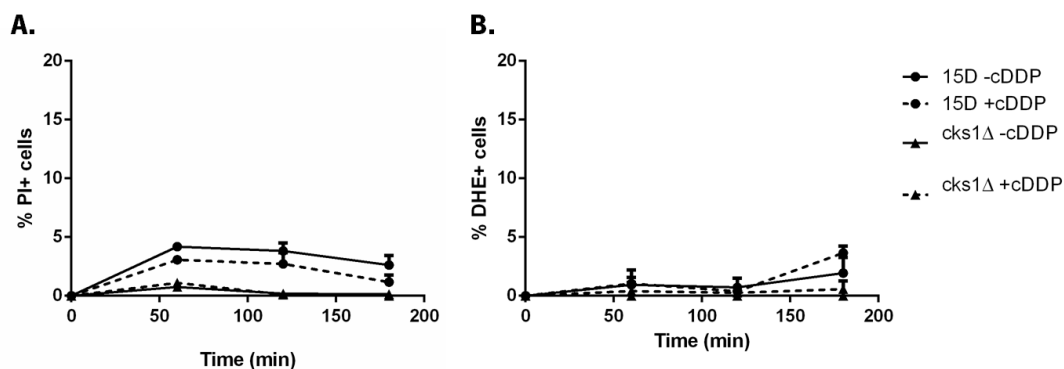

Figure 12. Plasma membrane integrity and ROS accumulation of wild-type strain 15D and mutant strain *cks1*Δ exposed to cisplatin. Exponential cultures of 15D and *cks1*Δ strains grown in YPD medium at 30°C, were transferred to fresh YPD medium with (+cDDP) or without (-cDDP) cisplatin. Cells were grown for 180 minutes at 30°C. Samples were taken after 0, 60, 120 and 180 min. **(A)** Incubated with PI (5 μg/mL) and fluorescence measured by flow cytometry. Increase of cells with PI staining is shown. **(B)** Stained with DHE (5 μg/mL) and the fluorescence measured by flow cytometry. Increase of cells stained with DHE is shown. Values represent means and standard deviations of 3 independent experiments.

We could not detect a significant increase in the percentage of cells stained with DHE after exposure to cisplatin (Figure 12B), indicating that, under our experimental conditions and in the 15D strain background, cisplatin-induced death is also not mediated by intracellular

superoxide anion formation, and that the increased sensitivity of the *cks1Δ* strain to cisplatin is not likely a result of increased ROS levels.

Sorensen *et al* (1990) and Grossman *et al* (1999) reported that DNA cross-links caused by cisplatin do not inhibit S-phase but cause a G2/M arrest in *S. cerevisiae* cells [113, 118]. Cunha *et al* (2013) showed that cisplatin-treated cells had an increased percentage of cells with sub-G0/G1 DNA content than untreated cells, especially after recovery in cisplatin-free media [115]. We therefore next tested whether the increased sensitivity of *cks1Δ* cells to cisplatin was due to higher levels of DNA fragmentation/degradation. For cell cycle analysis, cells were untreated or treated with cisplatin for 240 min, fixed and stained with sytox green, and DNA content was analyzed by flow cytometry. Representative histograms are shown in Figure 13A, the cell cycle distribution is shown in Figure 13B, and the quantification of the percentage of cells with sub G0/G1 DNA content after 240 min of cisplatin exposure and an additional 4 h recovery period are shown in Figure 13C.
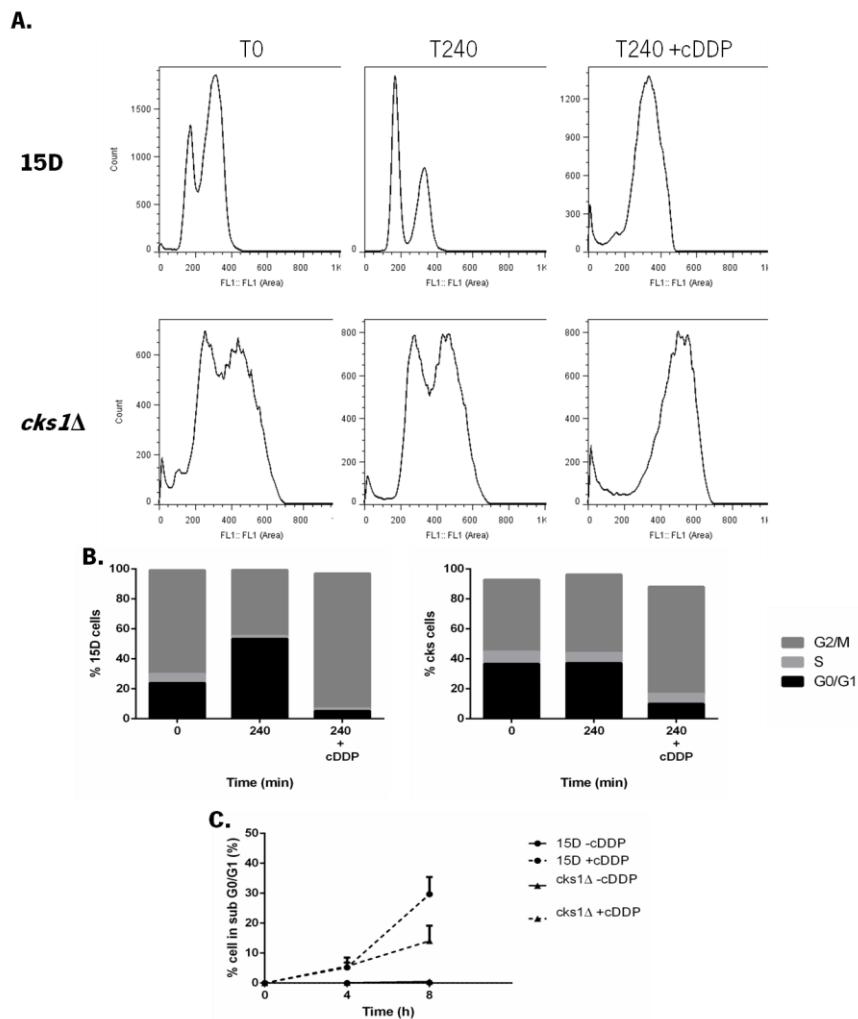
Figure 13. The effect of cisplatin on cell cycle distribution. Exponential cultures of 15D and *cks1*Δ strains grown in YPD medium at 30°C, were transferred to fresh YPD medium with (+cDDP) or without (-cDDP) 0,2 mg/mL cisplatin. Cells were grown for 240 minutes at 30°C. Samples were taken after 0 and 240 min, stained with sytox green (1 μM) and the fluorescence measured by flow cytometry. **(A)** Representative histograms. **(B)** Bars indicated the frequence (%) of cells in each cell cycle phase. **(C)** Cells were washed and resuspended in fresh medium without cisplatin and grown an additional 4h at 30°C. The percentage of cells with Sub G0/G1 DNA content was measured by flow cytometry. Values represent means and standard deviations of 3 independent experiments.

In agreement with published data, we also observed that exposure to cisplatin leads to an accumulation of cells in G2/M in both the wild-type strain 15D and strain *cks1*Δ (Figure 13A and 13B). The percentage of cells in the sub G0/G1 region was also higher in cisplatin-treated cells than in cells grown in cisplatin-free media, both after 240 min of treatment and after a 4 h recovery in YPD (Figure 13C). The percentage of 15D and *cks1*Δ cells in sub G0/G1 was similar

after the treatment period, but slightly lower in *cks1*Δ cells after recovery, despite higher levels of cell death (Figure 11B). This difference was statistically significant, showing that the higher sensitivity of *cks1*Δ cells to cisplatin is not due to increased DNA fragmentation/degradation.

Expression of yeast *CKS1* from a multicopy plasmid does not alter sensitivity to cisplatin

Since deletion of *CKS1* resulted in higher sensitivity to cisplatin, it is conceivable that overexpression could result in higher resistance, potentially causing the observed higher resistance of Cks-overexpressing tumours to therapy. In order to characterize the phenotype of overexpression of Cks1p in yeast, strains 15D and *cks1*Δ were transformed with a multi-copy plasmid expressing Cks1 (YEp13-*CKS1*) and respective empty vector control (YEp13). As expected, the "aberrant" morphology of the *cks1*Δ strain (*cks1*Δ YEp13) was reversed by expressing Cks1p from the YEp13 vector (*cks1*Δ YEp13-*CKS1*) (Figure 14).
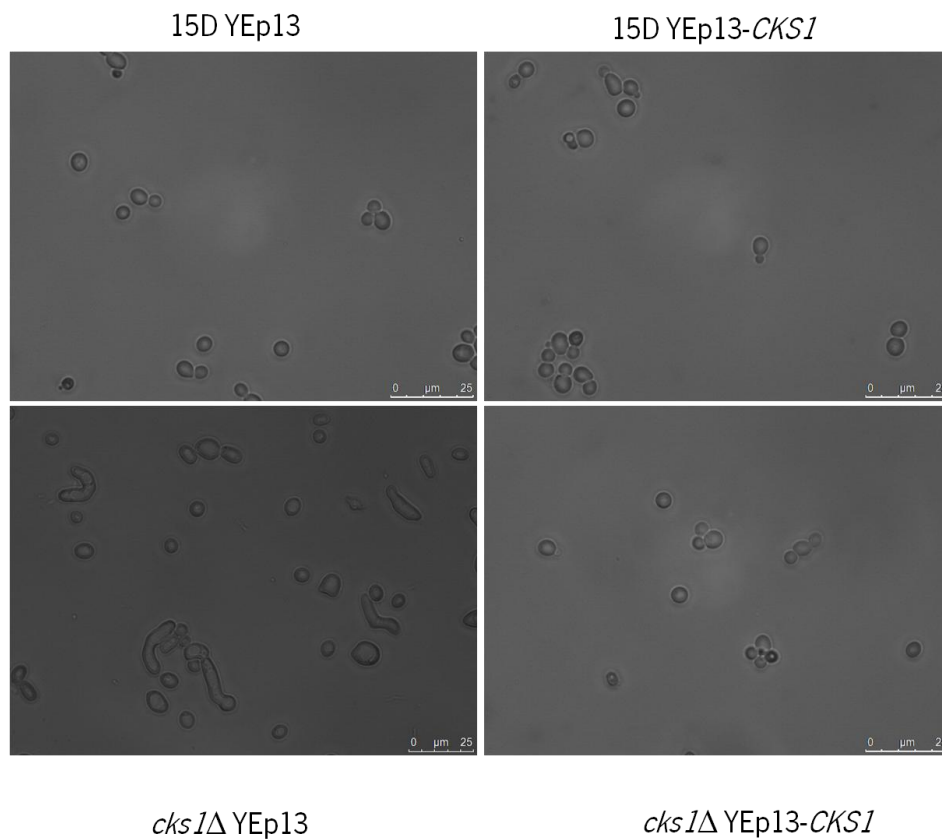

Figure 14. Morphology of *S. cerevisiae* strains 15D and *cks1*Δ expressing YEp13 and YEp13-*CKS1*.

To test whether overexpression of Cks1p resulted in increased resistance to cisplatin, all strains were exposed to cisplatin for up 180 min, at 30°C and viability assessed by semi-quantitative spot assay and c.f.u. counts.
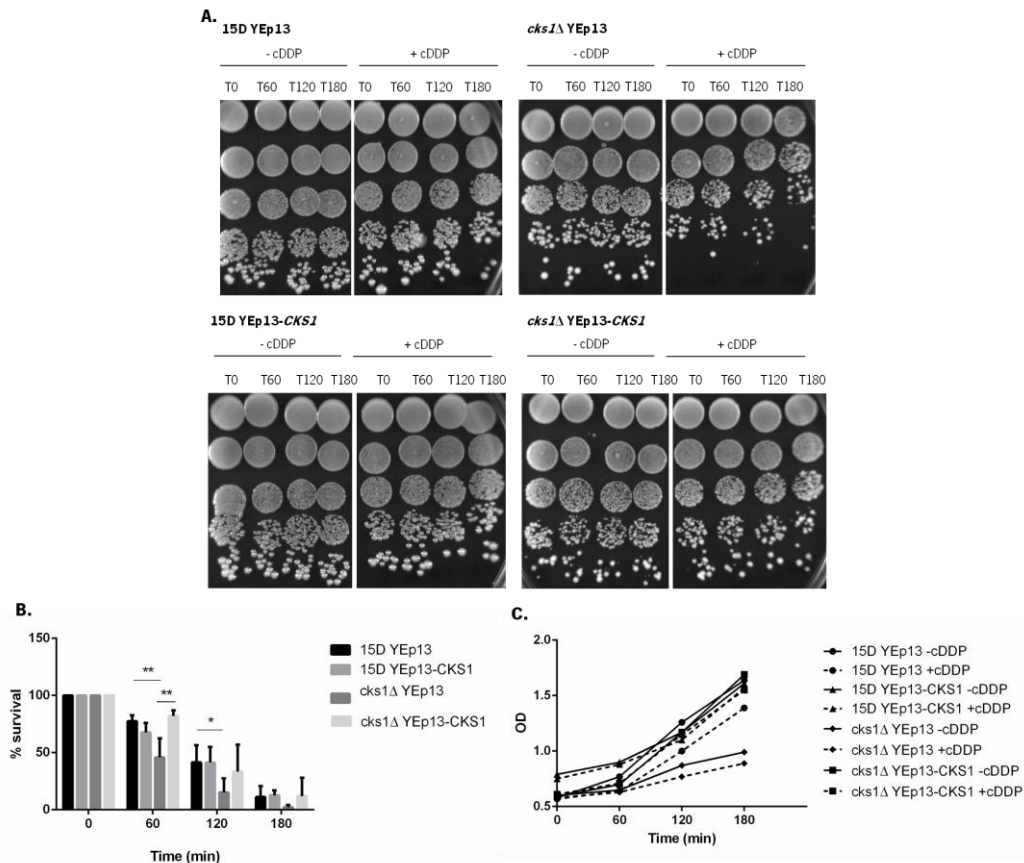

Figure 15. Sensitivity of *S. cerevisiae* strain 15D and *cks1*Δ expressing YEp13 and YEp13-*CKS1*. Exponential cultures of 15D YEp13, 15D YEp13-*CKS1*, *cks1*Δ YEp13 and *cks1*Δ YEp13-*CKS1* strains grown in SCGLU-Leu medium at 30°C, were transferred to fresh SCGLU-Leu medium without (-cDDP) or with (+cDDP) 0,2 mg/mL cDDP. Cells were grown for 180 min at 30°C. Samples were taken after 0, 60, 120 and 180 min. **(A)** Serial dilutions (1:10) were spotted onto YPD plates and incubated for 2 days at 30°C. **(B)** For c.f.u. measurements, dilutions were plated on YPD plates, incubated for 2 days at 30°C and colonies counted. Values represent means and standard deviations of 3 independent experiments (* p<0,05; **p<0,005). **(C)** OD$_{600}$ of the cultures.

There was no difference in the viability of 15D cells transformed with the empty vector and expressing YEp13-*CKS1*. However, expression of Cks1p in *cks1*Δ reverted the sensitivity of this strain to that of the wild-type strain. This indicates that either overexpression of Cks1p does

not have a phenotype or that expression of Cks1p from the YEp13 vector is not sufficient to induce an overexpression phenotype.

Expression of yeast *CKS1* from a multicopy plasmid does not alter sensitivity to hydroxyurea

Hydroxyurea is a DNA replication inhibitor that represses both the elongation and initiation phases of replication [119]. HU inhibits ribonucleoside diphosphate reductase, thereby blocking DNA synthesis and repair [120-122]. HU slows down or inhibits S-phase progression and can compromise genetic integrity by increasing the rate of recombination [119]. HU also induces the generation of reactive oxygen species. The production of ROS induces oxidative stress, adversely affecting cellular metabolism and leading to cell cycle arrest and cell death [123].

It had previously been descrived that overexpression of human Cks1 or Cks2 in human mammary epithelial and breast cancer-derived cell leads to override of the intra–S-phase checkpoint that blocks DNA replication in response to replication stress [35]. To determine whether a similar phenotype could be observed in yeast, 15D YEp13, 15D YEp13-*CKS1*, *cks1*Δ YEp13 and *cks1*Δ YEp13-*CKS1* strains were exposed to hydroxyurea for up to 240 min, at 30°C. Cells were stained with sytox green, and DNA content was analyzed by flow cytometry. Representative histograms are shown in figure 16.
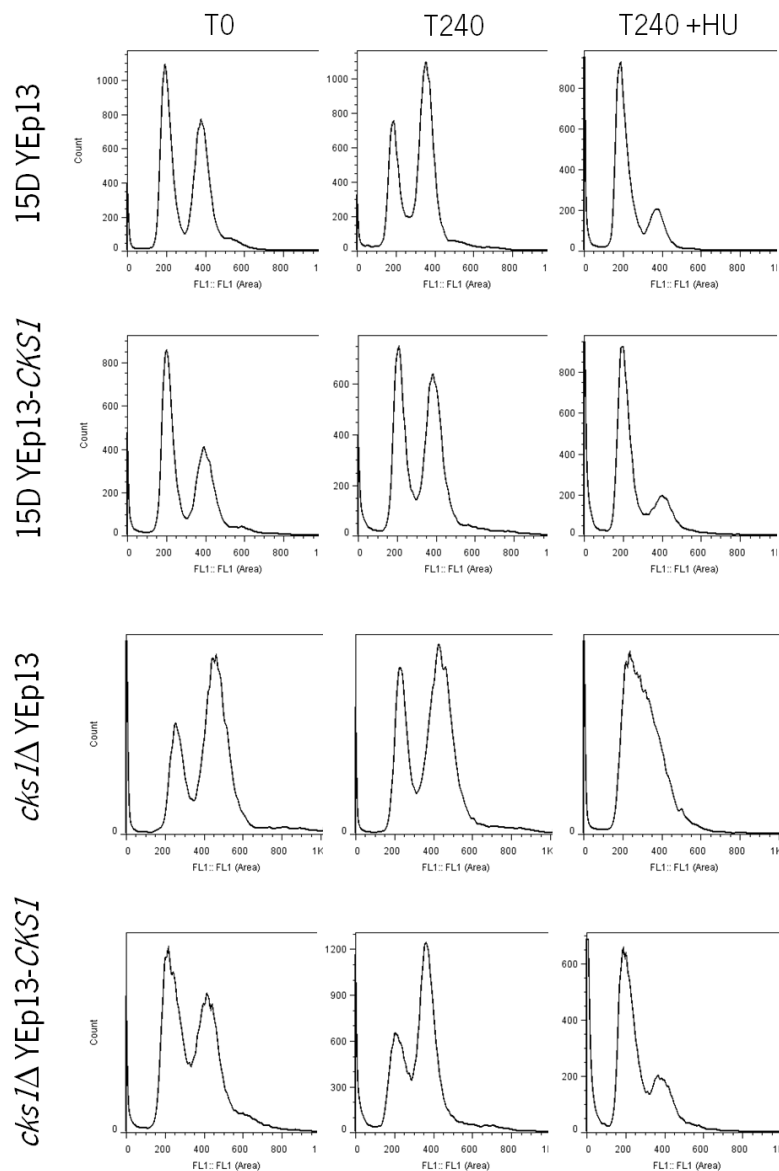
Figure 16. The effect of hydroxyurea on cell cycle distribution. Exponential cultures of 15D YEp13, 15D YEp13-*CKS1*, *cks1*Δ YEp13 and *cks1*Δ YEp13-*CKS1* strains grown in SCGLU-Leu medium at 30°C were transferred to fresh medium without or with (+HU) hydroxyurea. Samples were collected after 0 and 240 min at 30°C, stained with sytox green (1 μM) and the fluorescence measured by flow cytometry.

As expected, we observed that exposure to HU leads to an accumulation of cells in G1/S. There were no significant differences in the cell cycle profiles of the 15D YEp13 and 15D YEp13-*CKS1* strains. Strain *cks1*Δ YEp13 still accumulated in G1/S, though the peak was broader, likely reflecting the "abnormal" morphology and cell cycle defects of this strain. Expressing YEp13-*CKS1* in the *cks1*Δ strain reverted this phenotype, and the cell cycle profiles were similar

to those of the 15D strain. These results also indicate that either overexpression of Cks1p does not bypass the HU-induced cell cycle arrest in yeast or that expression of Cks1p from the YEp13 vector is not sufficient to induce an overexpression phenotype.

We next determined whether there were any differences in the viability of strains exposed to HU. Strains 15D YEp13, 15D YEp13-*CKS1*, *cks1Δ* YEp13 and *cks1Δ* YEp13-*CKS1* were exposed to hydroxyurea for up to 240 min, at 30°C and viability assessed by semi-quantitative spot assay.

Figure 17. Sensibility of *S. cerevisiae* strains to HU. Exponential cultures of 15D YEp13, 15D YEp13-*CKS1*, *cks1Δ* YEp13 and *cks1Δ* YEp13-*CKS1* strains grown in SCGLU-Leu medium at 30°C were transferred to fresh medium without or with 200 mM HU. Samples were taken after 0, 120, 180 and 240 min. Serial dilutions (1:10) were spotted onto YPD plates and incubated for 2 days at 30°C.

There was no cell death observed even when exposing cells to a HU concentration as high as 600 mM for 240 min (data not shown), indicating under these experimental conditions HU was cytostatic but not cytotoxic.

Strains 15D YEp13, 15D YEp13-*CKS1*, *cks1Δ* YEp13 and *cks1Δ* YEp13-*CKS1* were then spotted onto plates containing HU to evaluate their survival after long-term HU exposure (Figure 18).

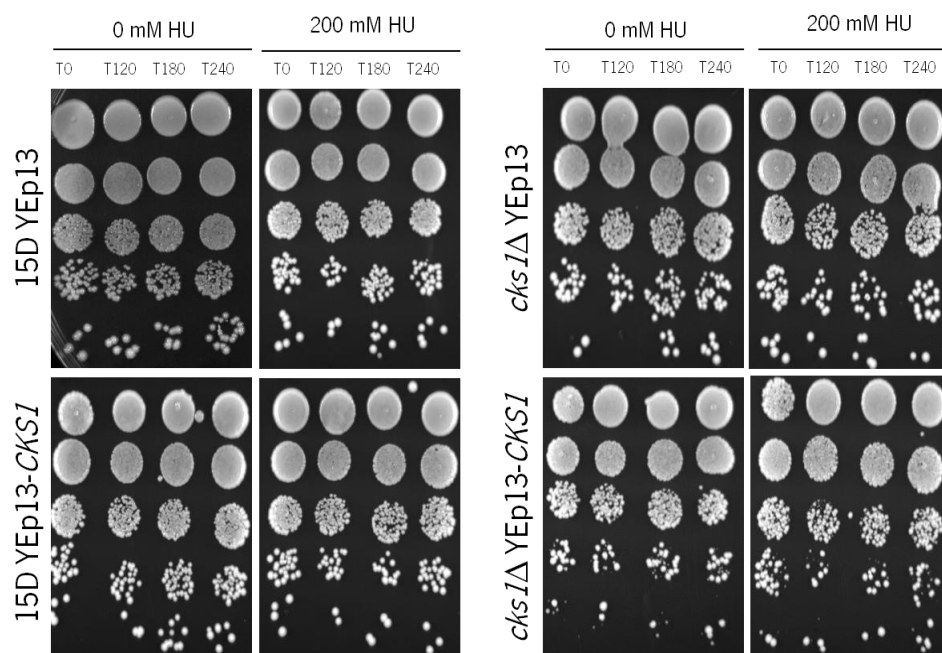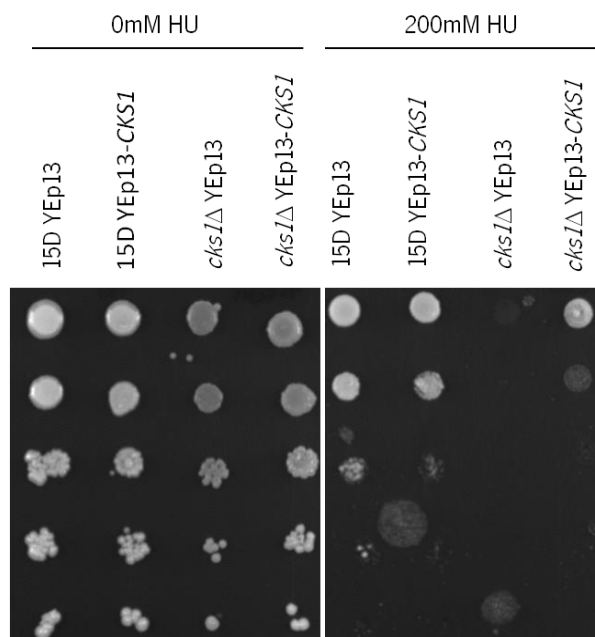**Figure 18. Chronic exposure of _S. cerevisiae_ strains to HU.** Exponential cultures of 15D YEp13, 15D YEp13-_CKS1_, _cks1_Δ YEp13 and _cks1_Δ YEp13-_CKS1_ strains grown in SCGLU-Leu medium at 30°C. Serial dilutions (1:10) were spotted onto SCGLU-Leu plates containing 200mM HU and incubated for 2 days at 30°C.

It has previously been described that _cks1_Δ cells are more sensitive to growth in the presence of HU [84]. We found that the viability of strain 15D expressing YEp13-_CKS1_ and strain 15D with the empty vector was identical, and, as expected, strain _cks1_Δ YEp13 was hyper-sensitive to HU. Expression of Cks1p (_cks1_Δ YEp13-_CKS1_) only partially reverted this phenotype, indicating that the protein levels of Cks1p when expressed from the YEp13 plasmid are low. This suggests that expression of Cks1p from the YEp13 plasmid is not sufficient to induce an overexpression phenotype, and that another expression system would be required to express high levels of Cks1p.

DISCUSSION AND FUTURE PERSPECTIVES

Cks proteins are frequently overexpressed in several cancers, correlating with poor prognosis, and may therefore be considered a potential prognostic marker and target for future development of specific therapeutic interventions in various types of cancers. However, despite the progress on elucidating functions of Cks proteins, strategies to eliminate tumors that overexpress Cks proteins have not yet been developed. Defective apoptosis induction is a major causative factor in the development and progression of cancer, and plays an important role in the resistance of tumors to conventional chemotherapy. Notably, mammalian Cks proteins seem to protect some cell lines from apoptosis. Several studies suggest that Cks protein levels can impact apoptosis, but the mechanism involved has not been elucidated or exploited as a target in cancer therapy. We therefore aim to determine whether Cks proteins have a specific role in apoptosis contributing to tumorigenesis and/or resistance of Cks-overexpressing tumors to treatment regimens.

The budding yeast *Saccharomyces cerevisiae* has proven to be a powerful and simple model system to study complex biological processes, often conserved through evolution. These studies have recently been extended to apoptosis, and it has been shown that an apoptotic-like death with common features of apoptosis in mammalian cells can also occur in yeast both under physiological conditions and after exposure to many exogenous stimuli [69, 124]. Most functions attributed to Cks proteins are conserved in yeast, and indeed mammalian Cks proteins can functionally substitute for yeast Cks1p. Therefore, in this work we aimed to study whether yeast Cks1p plays a role in apoptosis. We determined that the yeast *cks1Δ* mutant is significantly more sensitive than the wild type strain 15D to transient exposure to cisplatin and, to a lesser extent, MMS, but not to acetic acid, hydrogen peroxide, or 5-FU, which is a strong indication of a specific regulated role for Cks1p in DNA damage-induced cell death, but not in general apoptosis.

DNA damage can derive from three main sources: environmental agents such as ultraviolet (UV) light, ionizing radiation, and numerous genotoxic chemicals; ROS generated by respiration and lipid peroxidation; and spontaneous hydrolysis of nucleotide residues [125-126]. Yeast cells have integrated DNA repair systems with mechanisms to delay the cell cycle, called checkpoints [127]. This cell cycle arrest is thought to provide time for cells to repair damaged DNA before completing mitosis and segregating sister chromatids. Five genes (*RAD9*, *RAD17*, *RAD24*, *MEC3* and *DDC1*) have been shown to be required for a proper DNA damage response, and are proposed to act at an early step of damage recognition at any stage of the cell cycle [128-129].

Genetic and biochemical studies have established a general framework for DNA damage checkpoint signaling in yeast (Figure 19). The central player is the phosphatidylinositol 3' kinase-like kinase, *MEC1* [127]. Mec1p is part of a sensor mechanism that detects DNA damage in the form of single-stranded DNA and relays the checkpoint signal to a pair of transducing kinases. These kinases amplify the signal and regulate the cell cycle machinery to effect checkpoint arrest prior to mitosis [130].
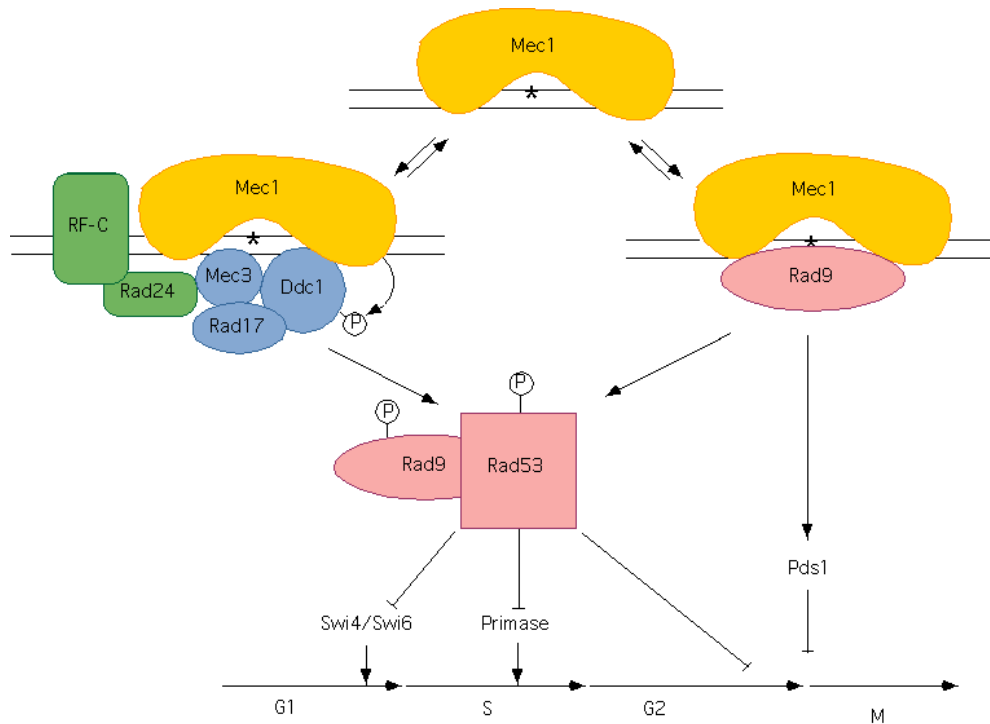
Figure 19. Model of the DNA damage checkpoint response in budding yeast. *MEC1* is an essential checkpoint factor, might be able to recognize specific DNA or protein–DNA structures. This function could be influenced by interaction with checkpoint proteins like Rad9p and the Rad24p group. *DDC1* phosphorylation depends on *MEC1*. Mec1p may participate, together with Rad24p, Rad17p, Mec3p and Ddc1p, at an early step of the DNA damage recognition process. It has been found that Rad9p is phosphorylated after DNA damage and that this modification depends on Mec1, Tel1p and the Rad24p group of proteins. Rad9p phosphorylation appears to be physiologically relevant since it correlates with checkpoint activation, and phosphorylated Rad9p interacts with Rad53p. *PDS1* is a rate-limiting negative regulator of anaphase that is degraded at the metaphase to anaphase transition, is phosphorylated in response to DNA damage in a Rad9p and Mec1p dependent manner, leading to metaphase arrest. *indicates damage in DNA (Longhese *et al* (1998) [128]).

RAD53 is a protein kinase involved in cell cycle arrest, transcriptional induction of repair genes, inhibition of late replication origin firing, and stabilization of stalled replication forks [131]. Rad53p is phosphorylated in response to genotoxic treatments, and this phosphorylation depends on the function of several DNA damage checkpoint genes [132] such Mec1p, Rad9p and the proteins of the Rad24p complex (*RAD24*, *MEC3*, *DDC1* and *RAD17*) [133]. It has been reported that Rad53p is phosphorylated in response to exposure to HU and MMS [131], cisplatin [134] and UV irradiation [135]. Whether Rad53p is phosphorylated in response to 5-FU has not yet been described. If not, and Cks1p functions downstream of Rad53p, it could explain lack of sensitivity of the *cks1Δ* strain to 5-FU, in contrast to its sensitivity to HU, MMS and cisplatin. However, Rad53p is phosphorylated in response to UV irradiation, and the *cks1Δ* strain is not more sensitive to this type of DNA damage [84], and thus this hypothesis is not likely. In the future, it would be interesting to determine whether Rad53p is phosphorylated under our experimental conditions, after exposure to MMS, cisplatin, 5-FU and UV, and determine whether there is a correlation. It would also be interesting to determine the phenotype of a *cks1Δrad53Δ* or a *cks1Δmec1Δ* double mutant to determine whether they function in the same pathway.

Exposure to 5-FU could result in Rad53p phosphorylation, like most other DNA damaging agents, suggesting that yeast Cks1p plays a role upstream of DNA-damage checkpoints. Mechanisms to repair the diverse types of DNA lesions are multiple and largely distinct. Repair of cisplatin-induced damage occurs mainly through nucleotide excision repair (NER) and mismatch repair (MMR) pathways [109]. NER removes a broad spectrum of single-strand lesions that cause local helix-destabilization [136]. Two different modes of damage detection are operational in NER: **(a)** transcription-coupled NER, which efficiently removes transcription-stalling lesions and allows quick resumption of transcription, and **(b)** global genome NER, which localizes lesions anywhere in the genome [125, 136]. MMR detects of mismatches and insertion/deletion loops and triggers a single-strand incision that is then acted upon by nuclease, polymerase and ligase enzymes [136]. Bases with small chemical alterations that do not strongly disturb the DNA double-helix structure are substrates for the base excision repair (BER) pathway, which is the main pathway used to repair 5-FU-induced lesions [137]. This group of lesions is targeted by lesion-specific DNA glycosylases that both recognize and remove the damaged base from the sugar-phosphate backbone [136]. While BER removes the damaged base, NER removes a large patch around the damage, even though there may be only a single bad base to correct. The difference between the

pathways used to repair damage caused by cisplatin and 5-FU could underlie the difference in phenotype obtained.

In this work, we used a small number of DNA damaging agents. In the future, we should test the sensitivity of *cks1Δ* cells to a variety of other DNA damaging agents, with different modes of action; although they may all lead to phosphorylation of *RAD53*, preferred repair mechanisms will differ, and a correlation may emerge.

It has recently been shown in cancer cell lines that cell cycle perturbations may be involved in acquired 5-FU resistance. There might be a slowdown in cell cycle progression preventing incorporation of 5-FU metabolites into DNA, providing sufficient time to correct the misincorpated nucleotides [138]. As deletion of Cks proteins leads to problems in the cell cycle, the slower growth of the *cks1Δ* strain may allow for increased repair of 5-FU damage, counteracting a role that would foster sensitivity, though this scenario is unlikely.

Unlike the phenotype obtained by deleting *CKS1*, overexpression of Cks1p didn't result in a readily observable phenotype, particularly regarding cisplatin sensitivity, which was unexpected. However, it is not always the case that overexpression of a given protein results in the opposing phenotype to its absence. Therefore, it is possible that overexpression of Cks1p does not cause resistance to cisplatin or to hydroxyurea, whereas deletion of *CKS1* does result in increased sensitivity. It is also possible that our expression system using the YEp13 plasmid does not lead to high enough levels of Cks1p to result in a phenotype. To address this issue, another expression system could be used to express high levels of Cks1p. For this purpose, we will next use plasmids expressing yeast *CKS1* and human *CKS1* and *CKS2*, under the control of the *GAL1* promoter, as well as empty plasmid controls (CpG2-*CKS1*, YCpG2-*CKShs1*, YCpG2-*CKShs2*, and YCpG2) and determine the viability of all strains in response to different DNA damaging agents. If no phenotype is observed, assessing the role of Cks proteins in apoptosis would require the use of a mammalian model system. We should test if overexpression of Cks1 or Cks2 renders mammalian cells more resistant to apoptosis induced by several drugs normally used in cancer treatment, especially those used in the treatment of breast and prostate cancers, where Cks proteins are frequently overexpressed. Of special interest would be how controlled overexpression of Cks proteins in the same cell background affects the response to multiple antitumor agents, which would require a control cell line and cell lines stably overexpressing Cks1 or Cks2, already available [35].

In summary, our results indicate that *CKS1* deletion results in sensitivity to some, but not all, DNA damaging agents, which could underlie a function in apoptosis. Further studies will be required to determine whether overexpression Cks1p specifically leads to resistance to particular anti-tumor drugs, both in yeast and in mammalian cells, as well as the pathways involved. Elucidating this mechanism should provide novel targets to exploit in the treatment of cancers where Cks proteins are up-regulated.

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