Ana Marta Gomes Duarte

Identification of genes and the signal transduction pathways involved in the regulation of acetic acid-induced programmed cell death

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Trabalho realizado sob a orientação de
Orientadora: Professora Doutora Maria João Sousa
Orientadora: Doutora Susana Chaves

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DECLARAÇÃO

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Identification of genes and the signal transduction pathways involved in the regulation of acetic acid-induced programmed cell death

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1. É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE, APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

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Ana Marta Gomes Duarte

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Identification of genes and the signal transduction pathways involved in the regulation of acetic acid-induced programmed cell death

Abstract

After many years of research, *S. cerevisiae* was accepted as a powerful model that allows increasing our comprehension about the underlying mechanisms of apoptosis in more complex and less accessible organisms. So, to better understand these apoptotic mechanisms we performed a functional analysis, at whole-genome scale, with the Euroscarf mutants collection. This analysis reveled 2159 resistant mutants and 391 mutants more sensitive to acetic acid induced cell death than the parental strain BY4741. The results obtained contribute to further characterize acetic acid-induced programmed cell death (PCD), and provide information on new putative targets for its control.

Most of the studies on apoptosis in yeast have been centered in the identification of apoptotic markers, however less is known about the signal transduction pathways that induce apoptosis. Cells possess a network of signal transduction pathways, which allow them to respond to different stimulus, implying several changes in genetic expression. Sfl1p is a transcription factor (TF) involved in repression of flocculation-related genes, and activation of stress responsive genes. We studied, cell death induced by acetic acid in yeast strains deleted in *SFL1* and in genes potentially regulated by Sfl1p (*AQY2, FMP42, FMP45, SUC2, HSP30, HSP104, NNF2, FLO1 FLO8, YMR173W-a, YJR11W* e *YCR006C*). The results obtained suggest that Sfl1p and the genes under its regulation, share a role in the mediation of acetic acid-induced apoptosis. Slf1p harbors 3 domains characteristic of the c-myc oncoprotein, a transcription factor with an important role in apoptosis induction and often found mutated in cancer cells. Our results showing that Sfl1p is also involved in the regulation of apoptosis in yeast suggest that these domains can have a conserved function in apoptosis regulation across kingdoms.

We also studied the involvement of genes regulated by Rlm1p on cell death induced by acetic acid. This TF coordinates an adaptive transcriptional response to the stress induced in the cell wall. Our results show that the genes that confer stability to the cell wall, confers sensitivity to acetic acid, when mutated. On the other hand, the genes involved in the cell wall formation, confers resistance, when mutated.

Identificação de genes e vias de tradução de sinal envolvido na morte celular programada induzida por ácido acético

Resumo

Depois de muitos anos de pesquisa, a *S. cerevisiae* foi aceite como um poderoso modelo que permitiu aumentar a compreensão dos mecanismos subjacentes à apoptose, em organismos mais complexos e menos acessíveis. Assim, para melhor compreensão dos mecanismos apoptóticos, realizámos uma análise funcional, à escala do genoma, com a coleção de mutantes da EUROSCARF. Esta análise revelou 2159 mutantes resistentes e 391 mutantes mais sensíveis à morte induzida por ácido acético do que a estirpe parental BY4741. Os resultados obtidos contribuem para uma melhor caraterização da PCD induzida por ácido acético e fornecem informação sobre hipotéticos alvos para o seu controlo.

A maioria dos estudos sobre apoptose em levedura têm-se centrado na identificação de marcadores apoptóticos, no entanto pouco é conhecido sobre as vias de transdução de sinais que induzem apoptose. As células possuem uma rede de vias de transdução de sinais que lhes permitem responder a diferentes estímulos, implicando grandes mudanças na sua expressão genética. Sfl1p é um fator de transcrição (FT) envolvido na repressão de genes relacionados com a floculação e na ativação de genes de resposta ao stress. Estudamos os genes potencialmente regulados pelo Sfl1p na presença de ácido acético (*AQY2, FMP42, FMP45, SUC2, HSP30, HSP104, NNF2, FLO1 FLO8, YMR173W-a, YJR11W* e *YCR006C*). Os resultados obtidos indicam que Sfl1p, e os seus genes alvo têm um papel na regulação na apoptose induzida por ácido acético. A proteína Slf1 contém 3 domínios característicos da oncoproteína c-myc, um fator de transcrição com um papel importante na indução de apoptose e muitas vezes alterado em células cancerígenas. Os nossos resultados sugerem que estes domínios podem ter uma função conservada na regulação da apoptose em leveduras.

Estudámos também o envolvimento dos genes regulados pelo Rlm1p, na morte celular induzida por ácido acético. Este FT coordena uma resposta de transcrição adaptativa, ao stress provocado na parede celular. Os nossos resultados mostram que os genes que conferem estabilidade à parede celular, quando mutados, conferem sensibilidade ao acido acético. Por outro lado, os genes envolvidos na formação da parede celular, quando mutados, conferem resistência.

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Abbreviations

AIF - Apoptosis-inducing Factor

Apaf-1 - Apoptotic Protease Activating Factor-1

ATP - Adenosine Triphosphate

C.F.U. - Colony forming units

c-FLIP - Cellular-FLICE (FADD-like IL-1β-converting enzyme)-inhibitory Protein

DAPI - 4,6-Diamino-2-phenyl-indole dihydrochlorid

DD - Death Domain

DED - Death Effector Domain

DHE - Dihydroethidium

DISC - Death Inducing Signaling Complex

DNA - Deoxyribonucleic Acid

DR - Death Receptors

Endo G - Endonuclease G

ER - Endoplasmatic Reticulum

FADD - Fas-Associated Death Domain

H_2O_2 – Hydrogen Peroxide

HtrA2/Omi - High Temperature Requirement Protein A2

IAPs - Inhibitors of Apoptosis Proteins

MAPK - Mitogen-activated Protein Kinases

MOMP - Mitochondrial Outer Membrane Permeabilization

PCD – Programmed Cell Death

PI - Propidium Iodide

ROS - Reactive Oxygen Species

SC Gal - Synthetic Complete Galactose medium

Smac/Diablo - Second Mitochondria-derived Activator of Caspases/Direct Inhibitor of Apoptosis Protein (IAP)-Binding Protein With Low Pi

TNF-R - Tumor-Necrosis Factor Receptor

TRADD - TNF-R-Associated Death Domain

TRAIL-R - TNF-related Apoptosis-Inducing Ligand Receptor

1. Introduction

1.1. Cell death

Cell death plays an important role in the maintenance of tissue homeostasis and in the development of organisms (Judah *et al*., 1965). There are different types of cell death, and their classification has undergone significant evolution. The first descriptions of programmed cell death (PCD) mechanisms date back to the mid-1960s, but the term was first used by Lockshin and Williams to describe a type of cell death that was not accidental (Lockshin and Williams, 1965). In 1972, Kerr and coworkers implemented the term apoptosis to define a new pattern of cell death, a genetically controlled sequence of steps that lead to specific morphological and biochemical changes (Kerr *et al.,* 1972). Apoptosis was later considered a synonym of PCD and cell death classified into apoptosis and necrosis. For a long time, necrosis has been considered an accidental cell death mechanism. It is now clear that necrosis can occur in a regulated manner, and that necrotic cell death has a prominent role in multiple physiological and pathological settings. The term 'necroptosis' has recently been used as a synonym of regulated necrosis. However, since necrosis may also be regulated and other forms of cell death exist, this classification was abandoned. Recently, the Nomenclature Committee on Cell Death proposed a functional classification of cell death which includes extrinsic apoptosis, caspase-dependent or -independent intrinsic apoptosis, regulated necrosis, autophagic cell death and mitotic catastrophe (Galluzzi *et al,* 2012).

1.2. Apoptosis

Apoptosis is the best characterized form of programmed cell death. It was originally defined based on morphological and biochemical features found in mammalian cells. The morphological appearance includes chromatin condensation, nuclear fragmentation and cell shrinkage. Biochemical features include high molecular weight DNA fragmentation, phosphatidyl serine externalization and proteolytic cleavage of a number of intracellular substrates (Cohen *et a.l,* 1994; Martin and Green, 1995). The process of apoptosis ensures the quick removal of cells without rupture of the plasma membrane,

thus preventing inflammation (Ballard and Holt, 1968; Bertolaccini and Olivero, 2002). Several diseases associated with severe human pathologies (cancer and neurodegenerative disorders) can be linked to poor regulation of apoptosis. In human adults, 50 to 70 billion cells are eradicated by this process every day (Matsuyama *et al.,* 1999), and therefore it is not surprising that apoptosis de-regulation can contribute to several diseases. The identification of components of the different apoptotic pathways and understanding the mechanisms underlying their regulation is critical to the development of new strategies of prevention and treatment against those diseases.

Apoptosis is mediated by intrinsic and extrinsic mechanisms (Hengartner, 2000). The extrinsic pathway or death receptor pathway (such as TNF receptor-1) is defined as mitochondria-independent, although mitochondria can be involved in the amplification of the death signal. This pathway involves the activation of receptors in the plasma membrane through binding of ligands that trigger a proteolytic process. The second mechanism, the intrinsic or mitochondrial pathway, involves the permeabilization of the mitochondrial outer membrane allowing the release of proapoptotic proteins into the cytosol. The two pathways differ in the initiator caspases that transmit the signal, but later converge at the level of activation of the same caspases. These proteases are responsible for morphological and biochemical alteration typical of apoptosis, and for the rapid clearance of the dying cell (Leist and Jäättelä, 2001; Riedl and Salvesen, 2007; Ow *et al.,* 2008).

1.2.1. The extrinsic apoptotic pathway

The extrinsic pathway involves the activation of receptors in the membrane through binding of ligands that trigger a proteolytic cascade responsible for the characteristic morphological features of apoptosis. Surface death receptors (DR) are characterized by the presence of an intracellular death domain (DD), a stretch of approximately 80 amino acids (Boldin *et al.,* 1995; Chinnaiyan *et al.,* 1995). To date, six human DD-containing receptors have been identified: TNF-R1 (p55/p60 TNF-R), CD95 (Fas, APO- 1), death receptor

3 (DR3, TRAMP), TRAIL-R1 (DR4), TRAIL-R2 (DR5), and DR6 (TNFRSF21). These receptors are activated by their respective ligands: TNF, CD95L (FasL/APO-1L), TL1A, TRAIL (Apo2L) (Friesen *et al.,* 1996). The DD plays a crucial role in signaling induced by these receptors, as it enables the recruitment of proteins that themselves contain DDs (Ashkenazi and Dixit, 1999).

The most prominent and decisive integrators of death receptor signaling are the proteins known as Fas-associated DD (FADD or MORT1) and TNFR-associated DD (TRADD) (Ashkenazi and Dixit, 1998; Yeh *et al.,* 1998). FADD and TRADD do not exert any enzymatic function, but form a bridge between proteins, in this case between receptor and signaling effector proteins. These have their own DD and are recruited to the DD of the activated death receptors. These adapter proteins also have a death-effector domain (DED), with which the DED of procaspase-8 can interact to form the Death Inducing Signaling Complex (DISC). The DISC is formed by FADD and caspase-8. Homotypic interaction of the DEDs of FADD and caspase-8 (Caspase-8 is present in the cytosol as a proenzyme) results in dimerization of caspase- 8, inducing a conformational change that allows caspase-8 to become enzymatically active. It then proteolytically activates the downstream effector caspase-3 (Leist and Jäättelä, 2001). The process leading to the activation of caspase-8 it is identical to that of caspase-10. This pathway is illustrated Figure 1.

The proteolysis of effector caspase substrates is responsible for the characteristic biochemical and morphological hallmarks of apoptosis, proteolysis of vital cellular proteins, including structural components, but also of other proteins such as the inhibitor of caspase-activated DNAse, and cleavage of nuclear DNA (Ding and Yin, 2004).

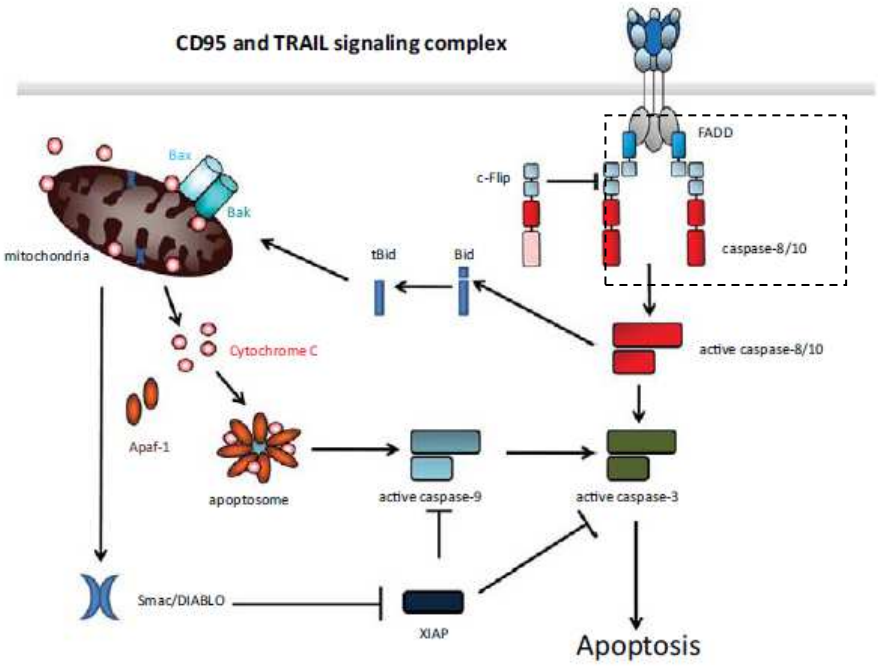


Figure 1 - Schematic representation the extrinsic apoptotic pathway. Binding of CD95 or TRAIL to their respective receptors leads to receptor trimerization and formation of DISC. The FADD is recruited to the DISC where the DD of both interact. Subsequently, procaspases -8 and -10 are recruited to interact with FADD via the DEDs. cFLIP can compete with caspase-8 for binding to FADD. DISC-activated caspase-8 and -10 starts a caspase cascade by cleavage of caspase-3, and also initiate the mitochondrial apoptosis pathway (adapted from Kantari and Walczak, 2011).

DISC can be inhibited by the antiapoptotic factor FLICE-like inhibitory protein (cFLIP), a caspase-8 inhibitor, leading to inactivation of DISC (Hengartner, 1997; Lawen, 2003). cFLIP is structurally similar to caspase-8 and -10, and contains two N-terminal DEDs. However, unlike cysteine proteases, it lacks a cysteine in what otherwise would be its active center, and thus cFLIP lacks enzymatic activity as a protease. Three different splice variants of cFLIP may exert apotosis inhibitory effects: cFLIPL, cFLIPS, and cFLIPR (Irmler *el al,* 1997; Van Parijs *et al,* 1999).

1.2.2. The intrinsic apoptotic pathway

The intrinsic pathway is activated mainly by non-receptor stimuli, such as DNA damage, endoplasmic reticulum stress, metabolic stress, UV radiation or

growth-factor deprivation. As mentioned, the central event in the intrinsic pathway is mitochondrial outer membrane permeabilization (MOMP), which allows the release of proapoptotic proteins from the mitochondrial intermembrane space into the cytosol, such as cytochrome *c* (cyt *c*), Apoptosis-inducing factor (AIF), Second Mitochondria-derived Activator of Caspases/Direct Inhibitor of Apoptosis Protein (IAP)-Binding Protein With Low Pi (Smac/Diablo) and High Temperature Requirement protein A2 (HtrA2/Omi) (Gulbins *et al.,* 2003). In the cytosol, cyt *c* binds to apoptotic protease-activating factor-1 (Apaf-1) and ATP/dATP, forming a large complex known as the apoptosome, a molecular platform which promotes the proteolytic maturation of caspase-9 (Cain *et al.,* 2002). When caspase-9 is activated, it activates caspases-3 and -7. These are subject to a number of controls, for example from proteins that bind and inactivate caspases (Inhibitors of Apoptosis, IAPS). Smac/DIABLO and HtrA2/Omi relieve caspase inhibition.

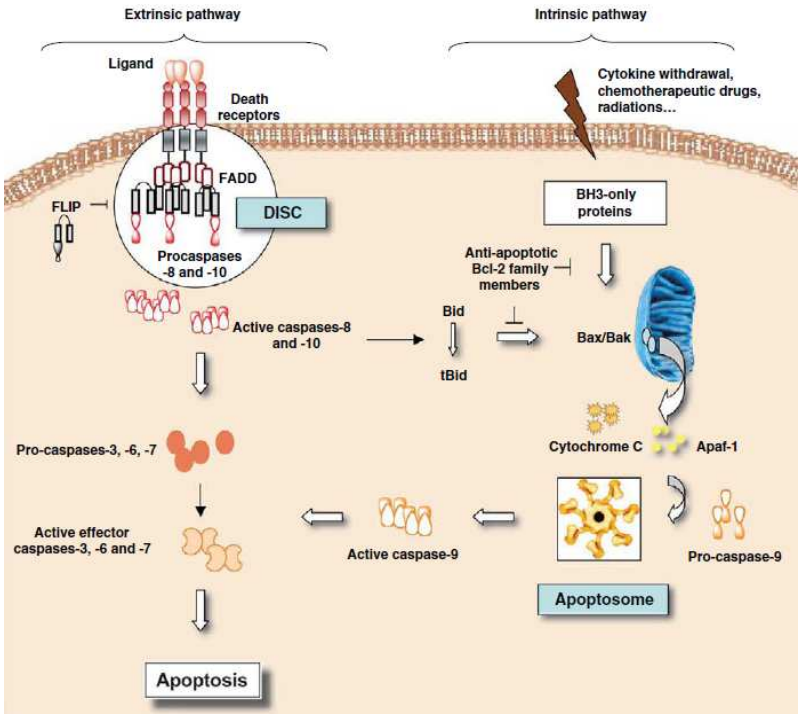


Figure 2 - Schematic representation, of two signaling pathways leading to apoptosis (extrinsic and intrinsic pathways) in mammalian cells (Reed and Green, 2011).

The intrinsic and extrinsic pathways are not completely independent; in some cells activation of caspase 8 results in activation of the mitochondrial

pathway (figure 2). In this case, among other things, caspase 8 cleaves the BH3-only protein BID, generating a truncated fragment known as truncated BID (tBID) that can permeabilize the mitochondrion resulting in MOMP (Favaloro *et al.,* 2012).

1.2.2.1. The BCL-2 family

BCL-2 (B-cell leukemia/lymphoma-2) was the first protein of this family to be discovered and thus lends its name to the entire protein family. Since then, all the proteins of the family that have been discovered are related to BCL-2 by sequence homology, containing at least one BCL-2 homology (BH) domain in their structure as well as an involvement in apoptosis control (Tsujimoto, 1998). This family contains proteins that induce or prevent MOMP and consequently apoptosis. It is sub-divided into anti-apoptotic proteins (which contain all four BH domains (BCL-2, BCL-XL, BCL-W, MCL-1, and A1)), pro-apoptotic multidomain proteins (BAX and BAK) and pro-apoptotic BH3-only proteins (BID, PUMA, NOXA, BIM, BAD, and BIK), represented in figure 4 (these are called BH3-only proteins because of Bcl-2 homology regions, they share only the third) (Fletcher and Huang, 2006; Willis *et al,* 2007; Youle and Strasser, 2008; Brenner and Mak, 2009).

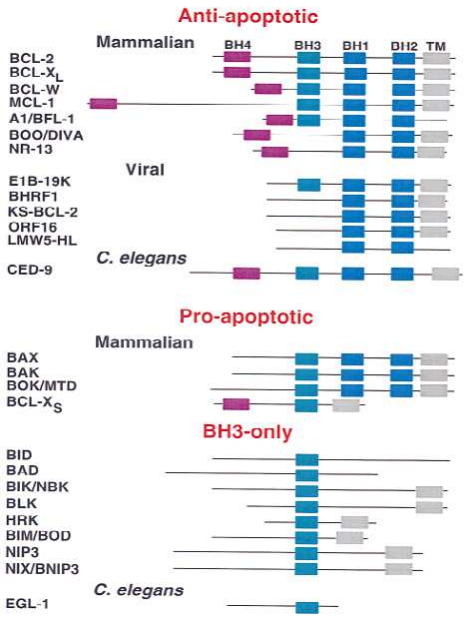


Figure 3 - Bcl-2 family members can be subdivided into three categories according to their function and structure: anti-apoptotic, such as BCL-2, BCL-XL, BCL-W, MCL-1, and A1 (BFL-1); pro-apoptotic, such as BAX, BAK, and BOK (Mtd); and the BH3-only proteins, Bid, Bad, and Bim (Gross A. *et al,* 1999).

Pro-apoptotic proteins, BAX and BAK, are essential effectors of apoptotic signaling in the mitochondrion, when their activated form induces MOMP, which allows the release of IMS proteins (Mcdonnell *et al.,* 1999). The main steps for BAX activation are translocation to the mitochondrion, conformational change, insertion into the mitochondrial membrane, oligomerization and pore formation. To date, two models describing the interaction between BCL-2 proteins that lead to BAX and BAK activation have been reported: in the indirect model, BAX and BAK are sequestered and held inactive by anti-apoptotic BCL-2 proteins. The binding of pro-apoptotic BH3-only proteins to these anti-apoptotic BCL-2 proteins triggers the release of BAX and BAK. The direct model proposes that BAX and BAK are activated by direct binding of pro-apoptotic BH3-only proteins, called activators, such as BID, BIM or PUMA (Willis et al., 2007; Brenner and Mak, 2009). BID is activated by proteolytic cleavage to generate t-BID, which translocates to mitochondria (Mcdonnell *et al.,* 1999; Yin, 2006; Zaltsman *et al.,* 2010). BIM and BAD are activated by dephosphorylation, whereas PUMA and NOXA are transcriptionally regulated by p53 (Youle and Strasser, 2008). Studies using models of combined deletion of BAX and BAK show that there is no MOMP in the absence of both proteins.

Expression of the anti-apoptotic BCL-2 family proteins allows the cell to survive a wide variety of attacks that might induce apoptosis. These proteins inhibit cell death by binding to pro-apoptotic proteins inhibiting the processes described previously. Perhaps their most important function is to bind and sequester the activator BH3-only proteins to prevent their interaction and activation of BAX and BAK (Letai *et al.,* 2002). The fate of the cell is therefore determined by the balance between the intracellular levels and/or activities of the anti-apoptotic BCL-2 family members and the pro-apoptotic BH3-only proteins (Brenner and Mak, 2009).

1.2.2.2. Pro-apoptotic proteins released from mitochondria

Mitochondria are essential organelles that exist in dynamic networks, and often change their localization and shape during stress conditions (Giannattasio

et al., 2005). The action of pro-apoptotic proteins, BAX and BAK, when in the activated form induces MOMP, which allows the release of IMS proteins, such as cyt *c*, Smac/DIABLO, HtrA2/Omi and AIF to the cytosol (Gulbins *et al.,* 2003; Armstrong, 2006).

Cyt *c* was the first mitochondrial protein with an apoptotic function identified, and established the general importance of mitochondria in apoptosis, represented schematicly in figure 5 (Liu *et al.,* 1996; Cai *et al.,* 1998). When in the cytosol, cyt *c* binds to Apaf-1 and forms the apoptosome together with deoxyadenosine triphosphate (dATP) (Zou *et al.,* 1997). The apoptosome activates caspase-9, (Ow *et al.,* 2008) which mediates activation of caspase-3 and -7 and the execution of apoptosis (Zou *et al.,* 1999; Acehan *et al.,* 2002).

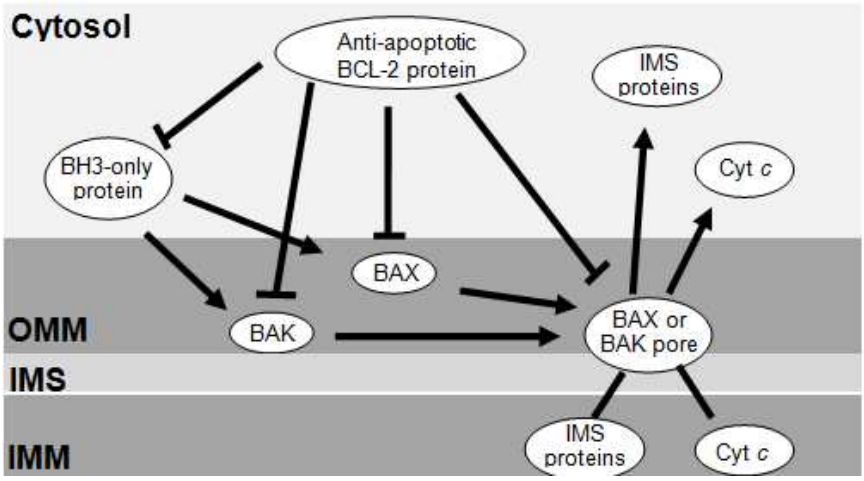


Figure 4 - Schematic representation of the actions of BAX and BAK on the OMM. These cause the permeability of the mitochondria membrane and induce the release of the proteins from the mitochondrial intermembrane space.

Other proteins that accompany cyt *c* during MOMP include SMAC/Diablo and Omi/HtrA2, both of which assist in caspase activation by antagonizing IAPs. This will be discussed in detail in the next section (Wu *et al.,* 2000; Du *et al.*, 2000; Verhagen *et al.,* 2000).

AIF exists in the IMM and appears to play a role in mitochondrial complex I assembly or function. Once MOMP has occurred in response to apoptotic stimuli, AIF is also released from mitochondria and is translocated to the nucleus. When translocated into the nucleus, AIF induces DNA fragmentation

and chromatin condensation (Candé *et al.,* 2002). The contribution of AIF to cell death depends on the cell-type and apoptotic stimulus, and is only seen when caspases are inhibited or not activated, because it functions in a caspase-independent manner (Wissing *et al.,* 2004).

1.2.2.3. Caspases

In 1992, two groups identified a human protease responsible for activating the precursor of interleukin-1β (interleukin-1β converting enzyme) (ICE). Later, it was found that one of the key genes that regulate apoptosis in *C. elegans* (CED3) shows homology with ICE (Alnemri *et al.,* 1996). These publications initiated a search over the ensuing years for mammalian ICE homologs that should govern cell death. Today these proteases are known as caspases (standing for cysteine dependent aspartate-specific protease) (Thornberry *et al.,* 1992).

Of the eleven caspases in humans, seven are known to be involved in apoptosis, three are involved primarily in pro-inflammatory cytokine activation and one is involved in keratinocyte differentiation, figure 5. Thus caspases can be divided into initiators caspases (caspases-2, -8, -9 and -10) and executioners caspases (caspases-3, -6, and -7) (Budihardjo *et al.,* 1999).

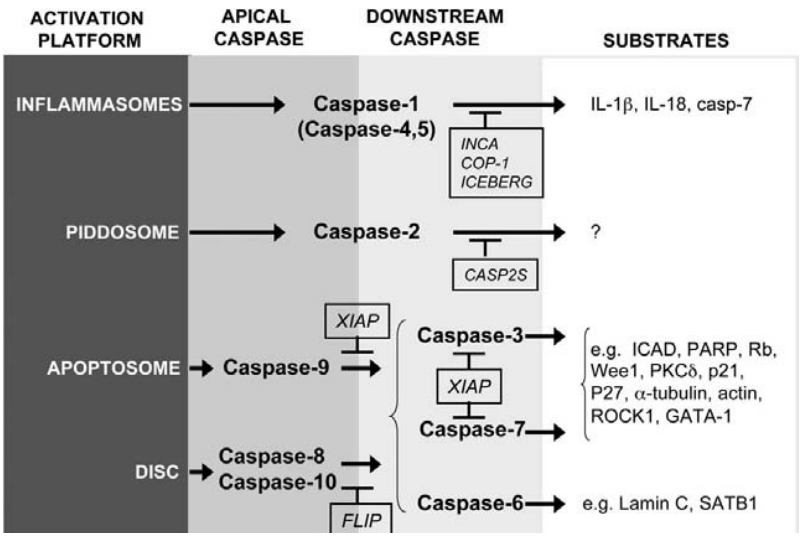


Figure 5 - Schematic representation of human caspases: activation, specificity, and regulation (Pop and Salvesen, 2009).

Caspase-9 is activated by the apoptosome, which then activates caspase-3 and -7. These proteases are responsible for the cleavage of many cellular proteins, which results in the phenotypic hallmarks of apoptosis (Stennicke and Salvesen, 1998). These hallmarks include cutting of DNA into small fragments, condensation of chromatin in the nucleus, dissipation of the mitochondrial membrane potential, and redistribution of phosphatidylserine (Liu *et al.,* 1997; Enari *et al.,* 1998).

Cells also contain natural inhibitors of caspases. IAPs were first identified in baculovirus but were subsequently found in human cells (XIAP, c-IAP1, and c-IAP2) (Deveraux and Reed, 1999; Miller, 1999). IAPs are a family of apoptosis-suppressing proteins that contain at least one copy of a conserved domain called baculoviral IAP repeat (BIR), which represents the defining characteristic of the family (LaCasse *et al.,* 1998; Miller, 1999). This family of proteins inhibits caspases directly, blocking apoptosis. The best-characterized endogenous caspase inhibitor is the X-linked inhibitor of apoptosis protein (XIAP) (Deveraux *el al.,* 1999). Activated caspases-3, -7 and -9 are potently inhibited by XIAP (figure 6) (Fuentes-Prior and Salvesen, 2004; Salvesen and Riedl, 2007; Ow *et al.,* 2008), but this inhibition can be relieved by the action of IAP antagonists, like SMAC/Diablo and serine protease HtrA2/Omi, through the IAP-binding motif (IBM) that disrupts IAP (Suzuki *et al.,* 2004; Brenner and Mak, 2009). Thus XIAP operates both within the intrinsic pathway, downstream of Apaf-1 and at the point of convergence of several apoptosis pathways, where caspases-3 and -7 operate as executioners of the cell death program. In cells that express high levels of XIAP, direct activation of caspase-3 by caspase-8 is blocked so that these cells require the mitochondrial changes induced by cleavage of BID and its pro-apoptotic activity.

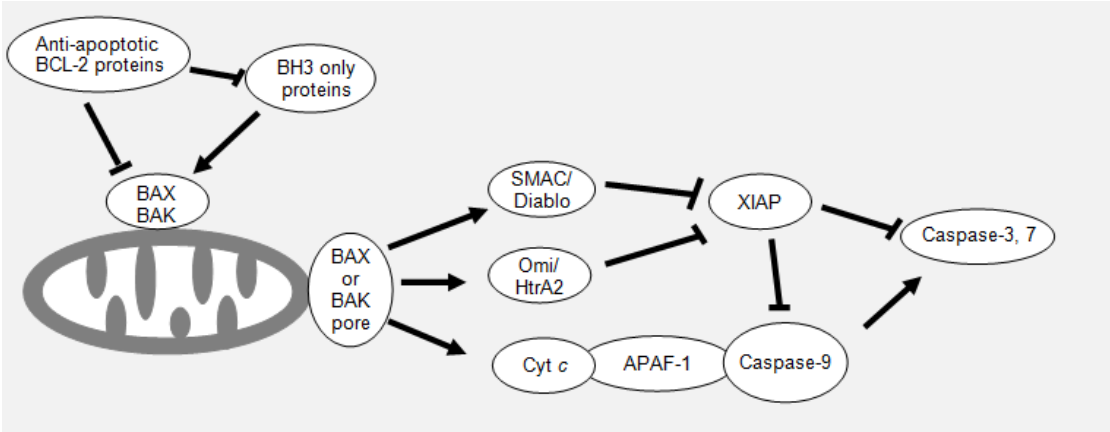


Figure 6 - Events that occur downstream of mitochondrial outer membrane permeabilization and their effects on cytosolic components.

1.3. Apoptosis and diseases

Based on its role in maintaining tissue homeostasis, it is not surprising that alterations in apoptosis play an important role in diseases development. Alterations in the upstream regulators of these pathways are the most common alterations in cancer cells. Disruption of the balance between cell death and proliferation is considered a major factor in the growth of tumors or their regression during therapy. This balance can be disrupted in two ways: by increasing proliferation and/or decreasing apoptosis.

A variety of alterations in the different BCL-2 family members have been described, illustrating the importance of these proteins in cancer development. BCL-2 has been found overexpressed in a variety of cancers. BAX and BAK mutations are frequent in tumours. Various BH3 protein alterations have also been implicated in cancer development; as an example, Bid-deficient mice are prone to develop a form of chronic myelomonocytic leukemia (Zinkel *et al.,* 2005), as well as diffuse large B-cell lymphoma. The possibility to target Bcl-2 family member proteins to induce apoptosis in cancer cells has been studied, and particular attention has been given to BH3 only proteins in the design of drugs that would mimic their pro-apoptotic functions. Some of these are currently being tested in phase I/II clinical trials (Esposti, 2010; Placzek *et al.,* 2010; Kelly and Strasser, 2011). Antisense oligonucleotides targeting BCL-2

have also been developed and in one case have reached the phase III clinical trial for patients with chronic lymphocytic leukemia.

Caspases are the final effectors of both extrinsic and intrinsic apoptosis; interfering with their function impairs these pathways, leading to a survival advantage for cancer cells. Caspase alterations are frequent in a variety of tumours (Olsson and Zhivotovsky, 2011). The altered caspase function can also be a consequence of modified expression of their specific inhibitors. As an example, cFLIP which competes with caspase 8 for FADD binding, thus preventing its activation, is often elevated in tumours, while its down-regulation can sensitize tumour cells to therapy. Among caspase inhibitors, an important role is played by IAPs. Indeed alterations of IAPs also are found in a variety of human cancers (Favaloro *et al.,* 2012).

1.4. The *S. cerevisiae* model

In 1996, *S. cerevisiae* became the first eukaryotic organism to have a fully sequenced genome (Dujon, 1996; Goffeau *et al.,* 1996), thus leading to the creation of several widely accessible databases. After the complete sequencing of the genome, a search for homologies in databases to uncover potential regulators of apoptosis was performed. It was questioned why yeast, an organism composed of a single cell, would undergo a suicide program. Several authors argue that despite the fact that yeast is a unicellular organism, apoptosis could provide an evolutionary advantage at the colony level. Yeasts in the wild exist in multicellular colonies and not as individuals, in which apoptosis may be a mechanism that saves and releases nutrients to the healthier cells, and apoptosis is like a mechanism of self-preservation of the colony as a whole (Gourlay and Ayscough, 2006). So the possibility of a single cell organism to undergo a programmed death program is becoming widely accepted. Indeed, during the last 13 years, many studies have reported the existence of programmed cell death in yeast.

The recognition of a mitochondria-mediated apoptotic pathway in yeast, showing similarities with the mammalian intrinsic pathway was of particular interest. *S. cerevisiae* has characteristics like a short generation time (90-120 min), simple and inexpensive culturing, ease and safety of handling, ability to grow at different temperatures, easy manipulation of mitochondrial respiration, a good characterization of many of its genes (thanks to its responsiveness deletion genes), gene marking or mutations and easy genetic manipulation. Another very important characteristic is its distinctive ability to survive without mitochondrial respiration, which makes them a powerful model to study the involvement of mitochondria in cell death (Pereira *et al.,* 2008). Because of these and other advantageous features, *S. cerevisiae* proved to be a valuable model organism in which several intracellular processes could be characterized in great detail. Thus the *S. cerevisiae* model has become one of the most studied models systems to many researchers in the field of molecular and cellular biology.

1.5. Apoptosis in the yeast *S. cerevisiae*

The apoptosis pathways described previously are from vertebrate organisms; however, other pathways with homologous proteins exist in invertebrates. *S. cerevisiae* PCD shares many morphological and biochemical features with apoptosis in mammalian cells, although there are some differences (figure 7). The first observation that there is apoptosis mechanism in *S. cerevisiae* was made in the $Cdc48^{S565G}$ mutant, a temperature-sensitive mutant. When incubated above the restrictive temperature, these cells showed an apoptotic phenotype with characteristics like DNA damage, phosphatidylserine exposure on the outer leaflet of the plasma membrane, chromatin condensation and fragmentation, ROS production and release of cyt *c* (Madeo *et al.,* 1997; Braun *et al.,* 2006). Several genetic studies contributed to understand the mechanisms of cell death in yeast. Some genes involved in metazoan cell death have been confirmed as apoptotic regulators in yeast. Key events of apoptosis in mammalian cells also occur in *S. cerevisiae*; for example, cyt *c* is also translocated from yeast mitochondria into the cytosol. The yeast

genome also harbors a gene, called *NMA111*, homologous to vertebrate HtrA2/Omi mitochondrial serine protease (Vande *et al.,* 2008). Nuc1p is the yeast homolog of metazoan endonuclease G (EndoG), exerting its pro-death action upon exit from mitochondria and translocation to the nucleus (Büttner *et al.,* 2007). The AIF is a highly conserved protein from yeast to human, which after apoptosis induction translocates to the nucleus, where it participates in apoptotic chromatinolysis.

As in mammalian cells, a IAP has been identified in yeast, termed Bir1p (Uren *et al.,* 1999). Yeast orthologues of mammalian ANT and VDAC have also been identified, *AAC1/2/3* and *POR1,* respectively (Wissing *et al.,* 2004; Ludovico *et al.,* 2005), as has a nuclease (TAT-D) that is apparently involved in DNA degradation during apoptosis. Recently, yeast homologues of mitochondrial fission factors such as Dnm1p (Drp-1 homologue), Mdv1/Net2 and Fis1p were reported to also regulate yeast PCD. Yeast suicide proteins 1, -2 (Ysp1p and Ysp2p), are also required for mitochondrial fragmentation induced by PCD (Pozniakovsky *et al.,* 2005; Sokolov *et al.,* 2006). Mitochondrial fragmentation has been described in yeast, after acetic acid treatment, leading to the formation of the typical punctate pattern (Fannjiang *et al.,* 2004). Yeast internal NADH dehydrogenase (*NDI1*) is the homolog of metazoan AMID, and seems to also be involved in apoptosis.

The yeast protein Yor197w, with structural homology with mammalian caspases, is called Yeast Caspase-1 (*YCA1*) (Madeo *et al.,* 2002). Overexpression of *YCA1* in combination with oxidative stress efficiently triggered yeast cell death, accompanied by common apoptotic features. *YCA1* belongs to the family of metacaspases, proteases that have a caspase-like fold (Uren *et al.,* 2000). Yca1p overexpression enhances apoptotic-like death of the cells, whereas its knockout reduces cell death in response to several stimuli (Madeo *et al.,* 2002b; Silva *et al.,* 2005).

Regulators such as Apaf-1 and most members of the Bcl-2 family of proteins seem to be absent in yeast (Jin and Reed, 2002; Leist and Jäättelä, 2001). Moreover, only a yeast BH3-only protein was identified so far, Ybh3p. Ybh3p translocates to the mitochondria and is capable of mediating the

mitochondrial apoptosis pathway (Büttner *et al.,* 2011). Although the yeast genome does not appear to contain very evident orthologs of the mammalian BCL-2 family genes, expression of pro-apoptotic BAX in yeast leads to apoptotic cell death (Ligr *et al.,* 1998; Priault *et al.,* 1999). This can be prevented by co-expression of antiapoptotic BCL-2 and BCL-XL, suggesting that the function of Bcl-2 family proteins is potentially conserved in yeast and that it can function in yeast in an analogous manner to its role in mammals (Hanada *et al.,* 1995).

In addition to the release of mitochondrial proteins, dissipation of the mitochondrial membrane potential also causes the loss of cell homeostasis via generation of reactive oxygen species (ROS). In *S. cerevisiae*, ROS accumulation is evident in almost every apoptotic scenario. Various studies have identified mitochondria as the major site of ROS production, and implicate ROS as a component of the apoptotic cascade. A Rho^0 strain (lacking mitochondrial DNA) has been shown to display an increased resistance to many apoptotic stimuli. For some stimuli, the higher resistance was accompanied by a decrease in ROS levels (Pereira *et al.,* 2008). Therefore, like in mammalian cells, mitochondria in yeasts play a key role in the apoptotic process.

Several assays for apoptosis detection are routinely used in yeast, and include assessment of viability (CFU), ROS accumulation (DHE), cell integrity (Propidium iodide (PI) staining), chromatin condensation (DAPI staining), DNA fragmentation (TUNEL assay -Terminal dUTP nick-end labeling) and exposure of phosphatidylserine at the outer surface of the plasma membrane (Annexin-V staining) (Carmona-Gutierrez *et al.,* 2010).

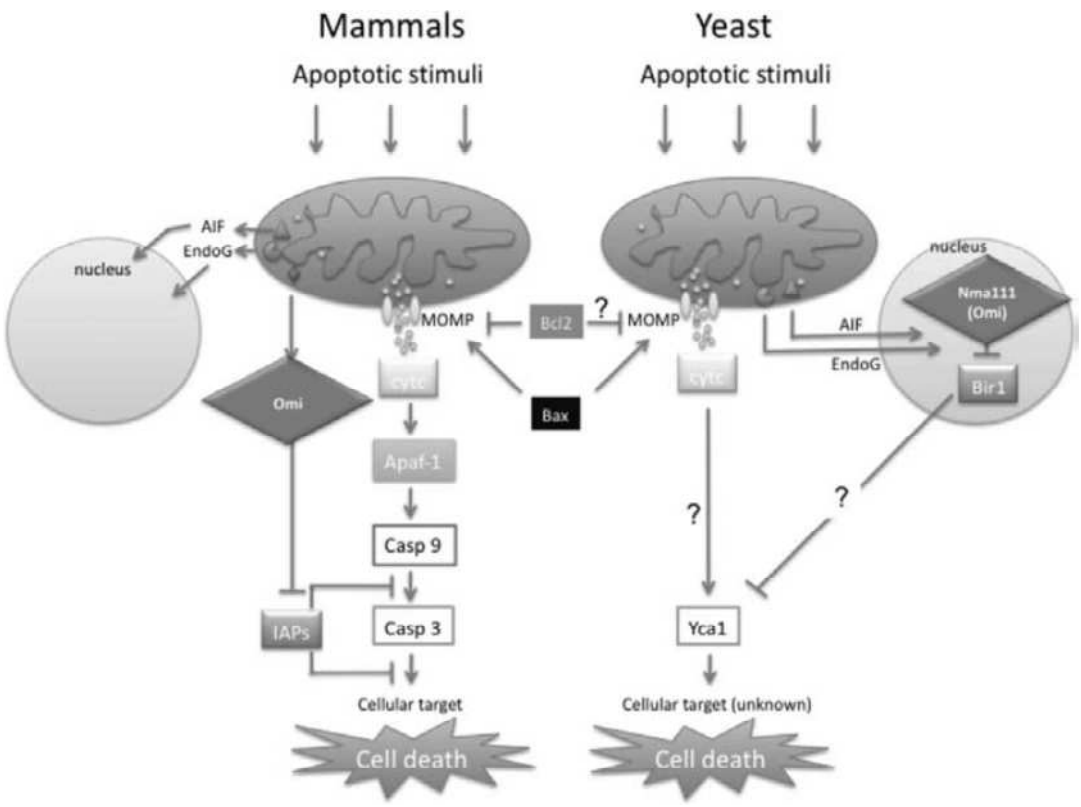


Figure 7 - Components of apoptotic pathways are conserved from yeast to mammals (Reed and Green, 2011).

1.6. Acetic Acid

Apoptosis in yeast can be induced by a variety of compounds and conditions, including hydrogen peroxide, acetic acid, amiodarone, hyperosmotic stress, and aging. Ludovico et al., in 2001, showed that acetic acid in low concentrations (20–80 mM) induces PCD in *S. cerevisiae* cells, which display chromatin condensation and DNA fragmentation. At higher concentrations (above 120 mM), acetic acid induces cell morphological changes typical of necrosis (Ludovico *et al.,* 2001). Later in 2002, Ludovico and collaborators showed a mitochondria-dependent pathway implicated in cell death induced by acetic acid. Translocation of cyt *c* to the cytosol and ROS production was also observed in yeast cells treated with acetic acid (Ludovico *et al.,* 2002).

When an inhibitory concentration of a weak acid is added to an exponentially growing yeast culture, this acid enters the cell in the undissociated

form by simple diffusion. In a growth medium with a pH equal or below pKa, the acetic acid (pKa=4.7) in the undissociated form (RCOOH) prevails. An undissociated form enters the yeast cells by simple diffusion through the plasma membrane (Casal *et al.,* 1996). When inside the cell (where the pH is usually close to neutrality), the chemical dissociation of the weak acid occurs, leading to the release of protons (H+) and of the respective counter ion (RCOO-) and accumulation of protons and acetate in the cell interior figure 8. The *S. cerevisiae* response to weak acids depends on the side chain of R group (R-COOH) (Mira *et al*, 2010).

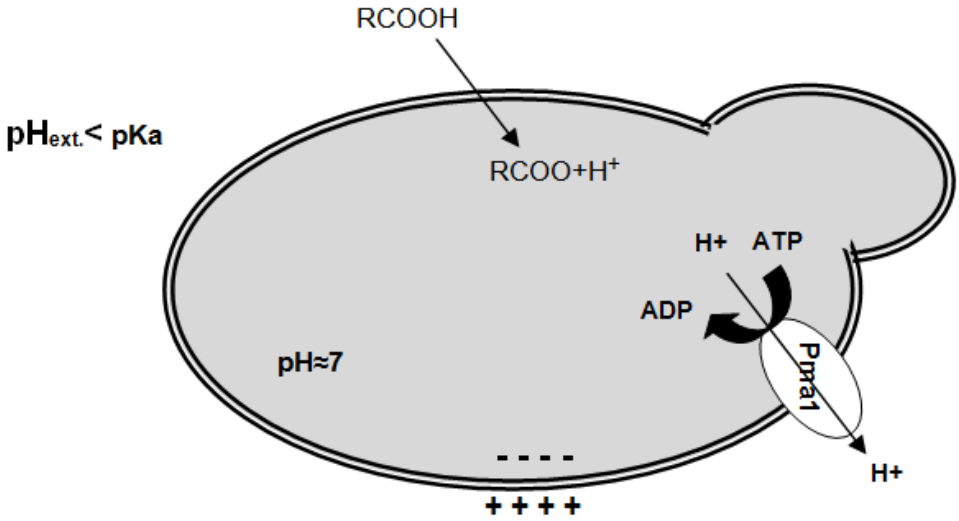


Figure 8 - Recovery of intracellular pH requires stimulation of the activity of plasma membrane, which couples ATP hydrolysis with proton extrusion.

This undissociated form of the acid, due to its electric charge, is not able to cross the hydrophobic lipid plasma membrane bilayer and accumulates in the cell interior. This will lead to intracellular acidification, anion accumulation and inhibition of cell metabolic activity. It also has an impact on the lipid organization and function of cellular membranes, consistent with its strong propensity to become more inhibitory as it becomes more hydrophobic (Stratford and Anslow 1996; Piper *et al.,* 1998).

It has also been described that acetic acid also enters the cell in the undissociated form by simple diffusion, mediated by the aquaglyceroporin

Fps1p. Studies demonstrated that deletion of *FPS1* (gene that encodes an aquaglyceroporin channel) abolishes the accumulation of undissociated acetic acid in the cell and leads to resistance to acetic acid (Mollapour and Piper, 2007). The recovery of intracellular pH requires the stimulation of the activity of plasma membrane Pma1p (PM-H+-ATPase), which couples ATP hydrolysis with proton extrusion, figure 9. Acetic acid has been extensively used as an inducer of apoptosis. In yeast, acetic acid-induced apoptosis is among the best-characterized apoptotic pathways.

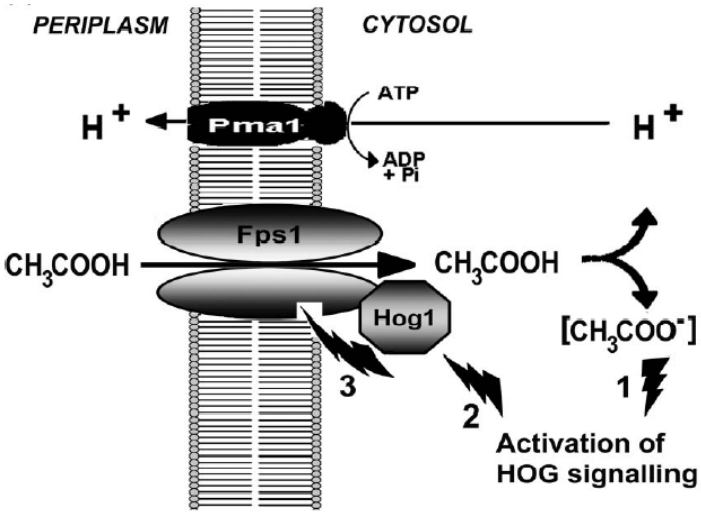


Figure 9 - Entry of undissociated acid into the cell through the Fps1p channel (Mollapour and Piper, 2008).

1.7. Genetic expression of transcription factors involved in apoptosis

To date, most studies regarding yeast apoptosis have focused on the identification of apoptotic markers. However, little is known about the signal transduction pathways that induce apoptosis. Cells possess a network of signal transduction pathways that enable them to respond to different stimuli, which implies strong changes in gene expression. Signal integration occurs at several levels of transduction, including transcriptional control of gene expression, translational regulation, and posttranslational modifications.

Initiation of transcription is arguably the most important control point to regulate gene expression. Transcription initiation begins with recruitment of RNA polymerase to a specific locus upstream of the gene known as the promoter. Transcription factors (TFs) are proteins that bind to the promoter and can activate or repress transcription depending where they bind relatively to the transcription start site of the target gene, and are thus classified as activators or repressors (Fulton *et al.,* 2009). This regulation of transcription initiation can activate or repress the transcription of target genes typically in response to an environmental or cellular trigger (Browning *et al.,* 2004).

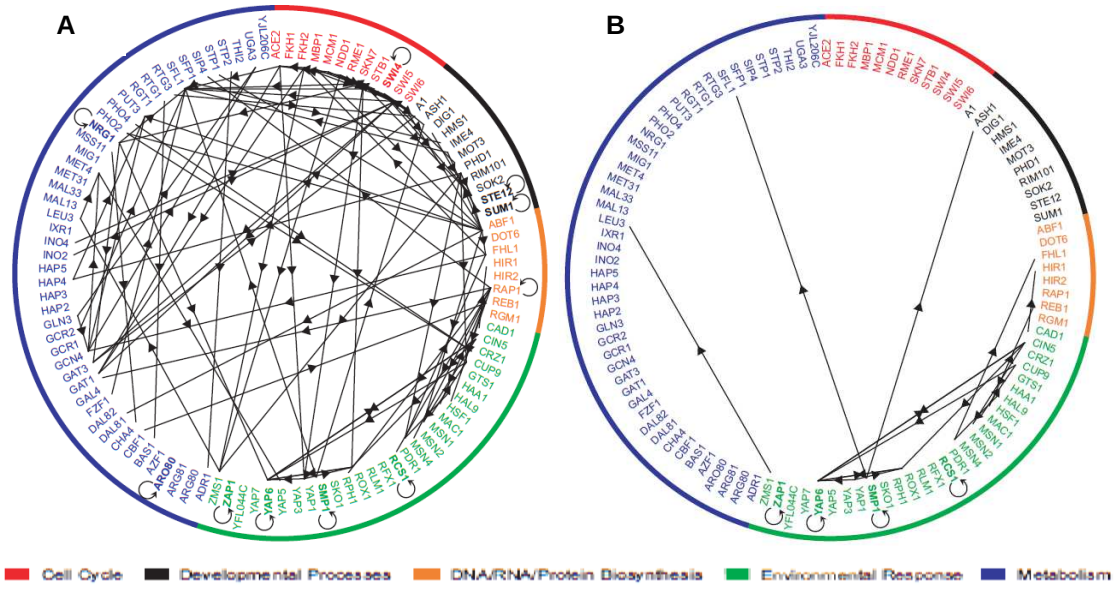


Figure 10 - Various studies in *S. cerevisiae* have led to the identification of genes which are differentially induced in response to different stresses. Representation network of transcriptional regulators binding to genes encoding other transcriptional regulators. Lines with arrows depict binding of a regulator to the gene encoding another regulator. Circles with arrows depict binding of a regulator to the promoter region of its own gene. (A) Circle divided into functional categories based on the functions of the target gene. (B) Representative cycle of transcription factors involved in stress response (Lee *et al.,* 2002).

The basic principles of transcriptional regulation are similar between prokaryotes and eukaryotes (Lee et al, 2002). Despite these similarities, transcription initiation in eukaryotes is considerably more complex, and is related to the genome size as it forms an impediment to the binding of TFs (Kornberg, 1974; Richmond *et al.,* 1984). Prokaryotic repressor proteins bind to promoter DNA sequences and inhibit transcription by steric hindrance of RNA

polymerase. In eukaryotic cells, repression does not occur simply by binding of repressor proteins. Eukaryotic DNA is tightly wrapped around histones, forming nucleosomes, the basic units of chromatin, which becomes limiting for the binding of TFs (Richmond *et al*., 2003). Chromatin modifier complexes are required that either displace or evict nucleosomes or covalently modify histones to loosen their interactions with DNA (Galeote *et al.,* 2007). TFs overcome the chromatin barrier to access DNA through interactions with a host of coregulators that modify the chromatin state.

It is believed that transcriptional activity is in some cases correlated with histone acetylation. Thus, chromatin modifiers can also function as co-repressors by effecting a more closed chromatin conformation. Given the drastic changes in the integrity of DNA and the state of chromatin compaction during apoptosis, histone modifications may play a functional role in promoting these changes. Methylation of sequences in promoter regions is commonly observed during tumor progression to inactivate genes whose products are important for processes such as DNA repair, cell-cycle regulation, cell adhesion, angiogenesis and apoptosis (Miranda *et al.,* 2007). The stress transcription factors are interesting models, and their characterization can lead to the identification of new components of stress signaling pathways in yeast.

1.7.1. The transcription factor SFL1p

In the yeast *S. cerevisiae*, the global transcriptional regulator Ssn6 (Cyc8)-Tup1 was the first transcriptional co-repressor to be described (Keleher *et al.,* 1992; Tzamarias and Struhl, 1994). Ssn6(Cyc8)-Tup1 is recruited to promoters via interactions with DNA-binding proteins, each of which represses genes in a specific biological pathway and inhibits the transcription of a diverse set of genes under a variety of stress conditions (Keleher *et al.,* 1992). This complex is composed of one Cyc8 subunit and four Tup1 subunits (Tzamarias *et al.,* 1994). Tup1 bears the transcriptional repression activity of the co-repressor complex, exerting its function via two distinct mechanisms. One model suggests that Tup1 controls nucleosome positioning so as to mask DNA targets for

activators or transcription factors (Grunstein, 1990; Edmondson *et al.,* 1996; Watson *et al.,* 2000). A second model suggests that Tup1 directly inhibits the function by interacting with subunits of the RNA polymerase II holoenzyme, such as Sin4, Srb10/11, Med3, Hrs1 and Srb7 (Kuchin *et al.,* 1998; Gromoller 2000; Papamichos-Chronakis *et al.,* 2000). Studies show that mutations in components of Pol II holoenzyme alleviate the repression by Tup1 (Balciunas and Ronne, 1995).

Sfl1p was first described as a transcriptional repressor but it can also act as an activator; it is, involved in repression of flocculation-related genes, and activation of stress responsive genes. Steven Conlan and Dimitris Tzamarias in 2001 showed that Sfl1p interacts directly with Ssn6p. In vivo repression data suggest that Sfl1p inhibits transcription by recruiting Ssn6p-Tup1p via a specific domain in the Sfl1 protein. Components of specific RNA polymerase II sub-complexes, Sin4p and Srb10p, are necessary for the Ssn6p-Tup1p repression activity of Sfl1 function. Sfl1p interacts with Tpk2p, a cAMP-dependent subunit that negatively regulates Slf1p function. This interaction of Sfl1p with DNA is thus regulated by Tpk2p, which is involved in the regulation of Sfl1p recruitment to some Ssn6p-regulated genes (Conlan et al., 2001).

1.7.1.1. Regulation of SFL1p

In eukaryotic cells, the secondary messenger cyclic adenosine monophosphate (cAMP) is produced in response to extracellular stimuli (D'Souza and Heitman, 2001). The central role of this signaling pathway in *S. cerevisiae* is nutrient sensing and regulation of diverse biological processes including growth, metabolism, stress resistance, and entry into either meiosis or pseudohyphal diferentiation (D'Souza and Heitman, 2001). The cyclic AMP-dependent signaling transduction pathway is a multienzyme cascade that regulates diverse biological processes. Specific connection of appropriate G-protein receptors followed by adenylate cyclase activation leads to the production of cyclic AMP. Cyclic AMP then binds to cytoplasmic protein kinase

A (PKA), which consists of a single regulatory subunit encoded by the *BCY1* gene and three catalytic subunits, Tpk1p, Tpk2p and Tpk3p, figure 11.

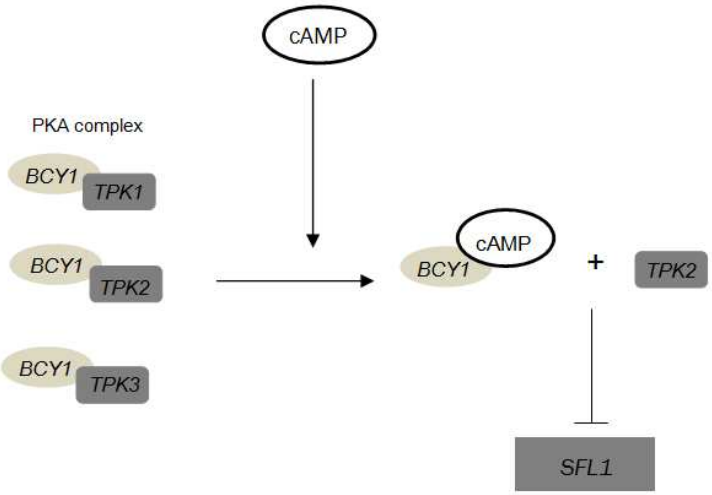


Figure 11 - Schematic representation Sfl1p repression by isoform Tpk2p.

When cAMP levels increase, it binds to the regulatory subunits and induces a conformational change that causes dissociation of the tetramer into dimeric regulatory subunits. These catalytic subunits are enzymatically active and phosphorylate target substrates that include metabolic enzymes and transcription factors (Sfl1p), which elicit alterations in cell cycle progression and stress responses (D'Souza and Heitman, 2001). Tpk1p has been implicated in the branched chain amino acid biosynthesis pathway, mitochondrial iron homeostasis and mtDNA stability. Tpk2p has been shown to influence iron uptake, trehalase synthesis, water homeostasis, pseudohyphal growth and negative regulation of Sfl1 protein (figure 11). Tpk3p is a regulator of mitochondrial function. Its overexpression has been shown to inhibit growth, and deletion of *TPK3* is sufficient to prevent the production of ROS, as this PKA subunit regulates mitochondrial function (Leadsham *et al.,* 2010).

Previous studies in yeast have established links between Ras signaling and mitochondrial function, via cAMP/PKA. The cAMP pathway is the most explored signaling pathway controlled by Ras proteins; it affects a large number of genes, some of which are important for the defence against oxidative stress. In yeast, Ras/cAMP/PKA signaling also controls cellular processes that include

cell growth and proliferation, making this pathway a good candidate to integrate environmental signaling with mitochondrial regulation (Hlavata *et al.,* 2008).

1.7.1.2. Sfl1p homology in mammals

Sfl1p encodes a 767-amino acid transcription factor, which has two domains significantly homologous to Myc protein (Fujita *et al.,* 1989). The proto-oncogene c-Myc encodes a transcription factor that plays a biological role through the modulation of genes in multiple cellular processes like cell growth, proliferation, differentiation and apoptosis (Askew *et al,.* 1991; Evan *et al.,* 1992). Deregulated expression of this oncogene is associated with a wide range of human cancers. This deregulated expression causes uncontrolled cell proliferation, which characterizes most, if not all, human cancer cells (Klefstrom *et al.,* 2002).

Expression of *c-MYC* sensitizes cells to mechanistically diverse pro-apoptotic insults, including DNA damage, death receptor signaling, hypoxia, genotoxic stress, and nutrient deprivation (Askew *et al.,* 1991; Evan *et al.,* 1992; Klefstrom *et al.,* 1994; Alarcon *et al.,* 1996; Hueber *et al.,* 1997). There are two discrete pro-apoptotic effector pathways mediating this sensitization: stabilization of p53 through the ARF (Active Response Factor)/MDM2 (Mouse Double Minute-2) pathway, which serves as a sentinel for genotoxic damage, and release of cyt *c* from mitochondria into the cytosol, possibly through activation of the pro-apoptotic molecule BAX by a mechanism that is independent of both Fas-FasL and DNA damage pro-apoptotic pathways (figure 12) (Juin *et al.,* 1999). Studies investigated a possible physical interaction between c-Myc protein and the Bax promoter using an immunoprecipitation assay. It was found that *c-MYC* strongly binds to the BAX promoter region, contributing to BAX expression. *c-MYC* is a transactivator of BAX, based on the presence of four CACGTG motifs located in the BAX gene (Mitchell *et al.,* 2000). Activated BAX within the mitochondrial membrane leads to apoptosis, through the mechanisms described previously. It remains to be established if there are other mitochondrial factors, such as AIF, released during c-MYC-induced apoptosis, and their involvement in this process.

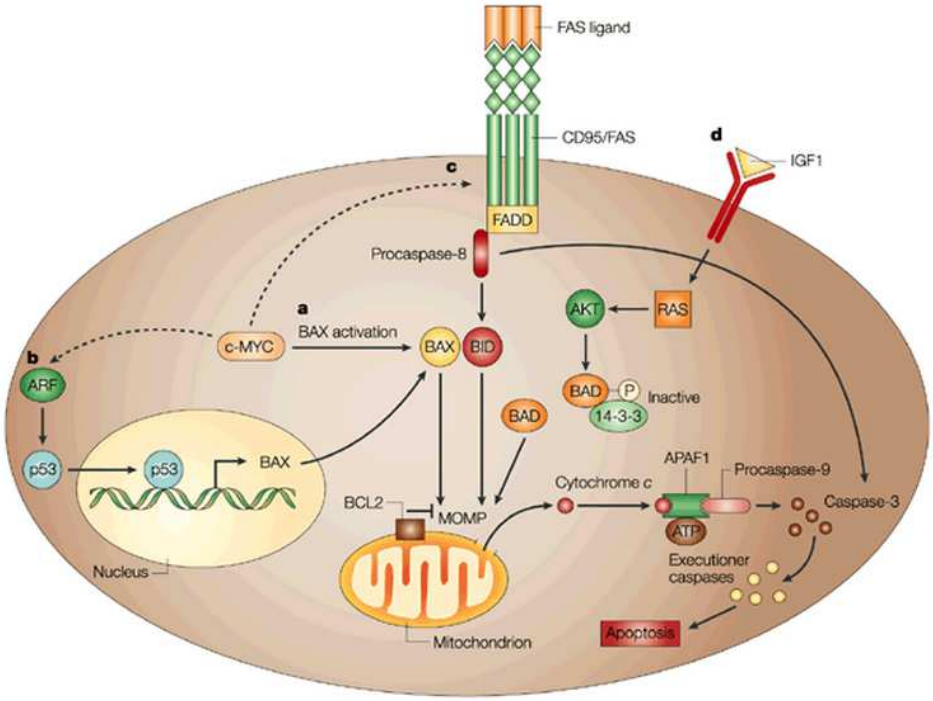


Figure 12 - Expression of *c-MYC* triggers two discrete proapoptotic effector pathways: the stabilization of p53 through the ARF/MDM2 pathway, which serves as a sentinel for genotoxic damage, and the triggers the release of cyt *c* from mitochondria into the cytosol, possibly through activation of the expression of the pro-apoptotic molecule BAX (Pelengaris et al., 2002).

Survival signals, which serve to block *c-MYC*, include signaling via IGF1R (Insulin-like Growth Factor-1 Receptor). Activation of the IGF-1 receptor triggers a survival-signal, routing through Ras, PI3-kinase, serine/threonine kinase PKB/Akt and subsequent phosphorylation of the pro-apoptotic protein BAD. Phosphorylated BAD is sequestered and inactivated by cytosolic 14-3-3 proteins. Functionally, this inactivates BAD, which cannot antagonize BCL-2 (Kauffmann-Zeh *et al.,* 1997; Evan and Littlewood 1998).

1.7.2. The transcription factor Rlm1p

Four essential mitogen-activated protein kinase (MAPK) cascades respond to different external signals in yeast (figure 13). The mating pathway is activated by pheromones and induces cell-cycle arrest and the morphological

changes required for mating (Gustin *et al.,* 1998). The Kss1 vegetative growth pathway may be activated by cell wall stress or changes in osmolarity (Lee and Elion, 1999; Cullen *et al.,* 2000). The invasive growth pathway is activated by starvation. The high osmolarity glycerol (HOG) pathway increases intracellular glycerol levels in response to hypertonic stress. The cell wall integrity pathway (CWI) is activated by hypotonic stress, heat shock, or impaired cell wall synthesis.

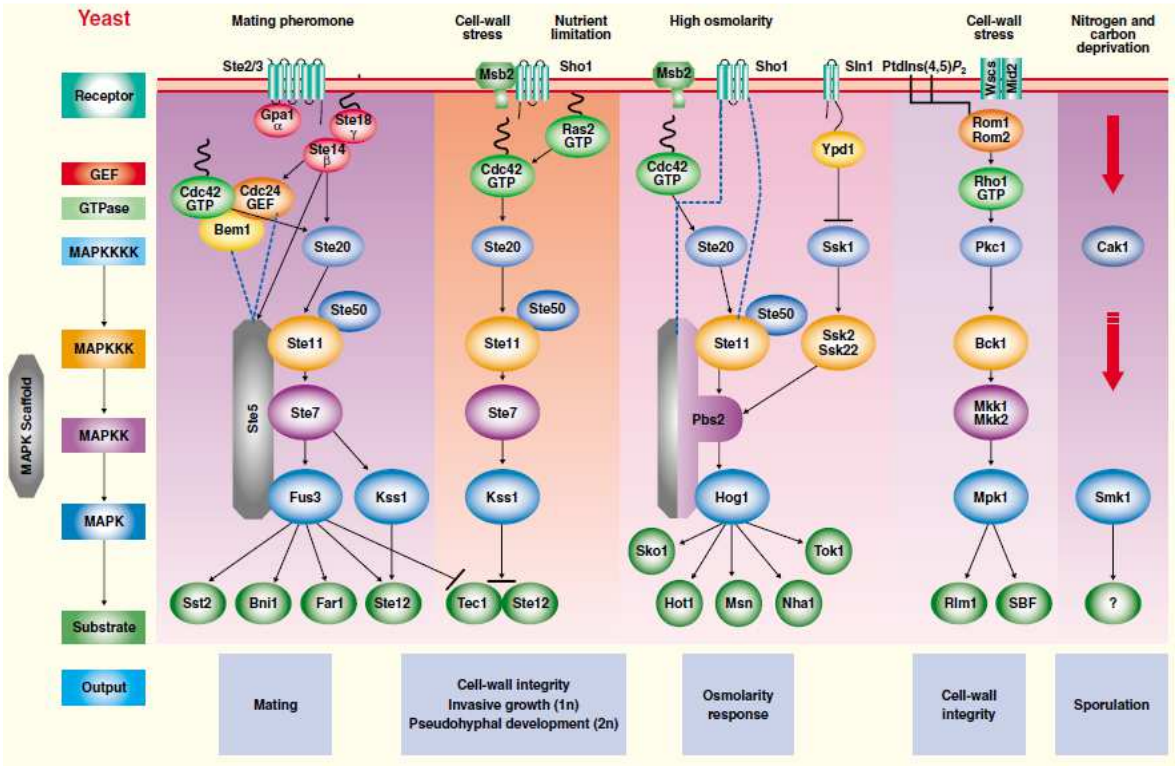


Figure 13 - Overview of the MAPKinase pathways in yeast (Qi M. and Elion EA. 2005).

The cell wall of *S. cerevisiae* is an external envelope that protects it against different environmental conditions. The adaptive response of yeast to cell wall stress is mainly mediated by the CWI pathway (Levin *et al.,* 2005; Fuchs and Mylonakis, 2009; Kim and Levin, 2011).

Two membrane proteins, namely Mid2 and Wsc1, act as the main sensors of the CWI pathway. These, when activated, interact with the guanine nucleotide exchange factor Rom2, activating the GTPase Rho1, which then interacts and activates Pkc1. Pkc1 then activates a downstream MAP kinase

cascade comprising three protein kinases, MAPKKK (Bck1), MAPKK (Mkk1/ Mkk2), and finally the MAPK (Mpk1/Slt2) (figure 14) (Kim and Levin, 2011).

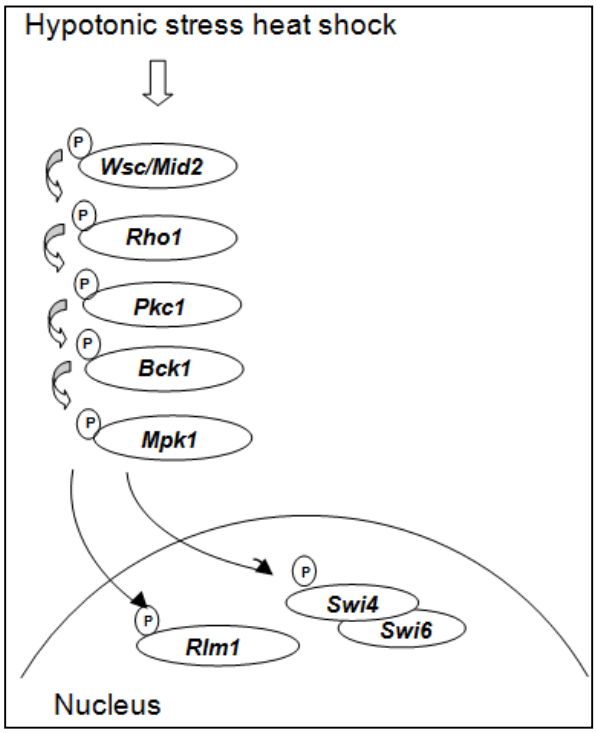


Figure 14 - Schematic overview of the cell wall integrity pathway.

Mpk1/Slt2 targets the transcription factor complex SBF (SCB-binding factor) and Rlm1p. SBF is a complex of two proteins, Swi4 and Swi6, which is involved in the regulation of the yeast cell cycle and polarized growth, especially during the transition from G1 to S phase, via transcriptional activation of genes such as *CLN1, CLN2, PCL1* and *PCL2* (Fong *et al.,* 2008; Chiu *et al.,* 2011). Rlm1p is a MADS-box transcription factor that promotes the expression of cell wall maintenance proteins (Watanabe *et al.,* 1997; Heinisch *et al.,* 1999; Jung *et al.,* 2002; Garcia *et al.,* 2004; Fuchs and Mylonakis, 2009). Thus the final consequence of the activation of the CWI pathway by cell wall stress is the induction of an adaptive transcriptional response (Jung and Levin, 1999; Lagorce *et al.,* 2003; García *et al.,* 2009). The elements of the yeast transcriptional machinery working in concert with Rlm1p for transcriptional activation upon cell wall stress and the molecular mechanisms involved in this process are completely unknown. Under cell wall stress conditions, Slt2p phosphorylates Rlm1p and the SWI/SNF recruited complex is targeted to the

promoters of CWI-responsive genes, altering the nucleosome positioning at the promoter, facilitating the binding of Rlm1p to sites previously occluded by nucleosomes (Kasten *et al.,* 2011). Finally, binding of Pol II stimulates transcription initiation. *S. cerevisiae* SWI/SNF has 11 subunits: Arp7, Arp9, Snf2, Snf5, Snf6, Snf11, Snf12, Swi1, Swi3, Swp82 and Taf14 (Yudkovsky *et al.,* 1999).

2. Objetives

This work aimed to identify genes involved in the regulation of acetic acid-induced apoptosis, and involved three approaches:

The first part of this work aimed to identify, at a whole-genome scale, the genes required for sensitivity/resistance phenotypes under acetic acid-induced apoptotic conditions (400 mM acetic acid, at pH 3.0) in *S. cerevisiae,* by screening the EUROSCARF haploid mutant collection (http://web.unifrankfurt.de/fb15/mikro/euroscarf/). A set of genes involved in resistant and sensitive phenotypes were clustered according to biological function (MIPS Functional Catalogue) and known physical and genetic interactions (STRING Protein-Protein Interactions).

In the second part, the aim was to identify regulators and downstream targets of Sfl1p involved in acetic acid-induced apoptosis. Deletion mutants in genes regulated by Sfl1p were tested for their sensitivity/resistance to acetic acid-induced cell death and cell death markers were assessed in mutants displaying altered resistance.

The third part was identification the downstream targets of Rlm1p involved in acetic acid-induced apoptosis. Deletion mutants defective in genes regulated by Rlm1p were tested for their sensitivity/resistance to acetic acid.

3. Material and methods

3.1. Yeast strains

In this study microorganisms used were the parental strain of *S. cerevisiae* BY4741 (MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0) and the respective EUROSCARF collection of derived deletion mutant strains, containing all the non-essential open reading frames replaced by the KanMX cassette.

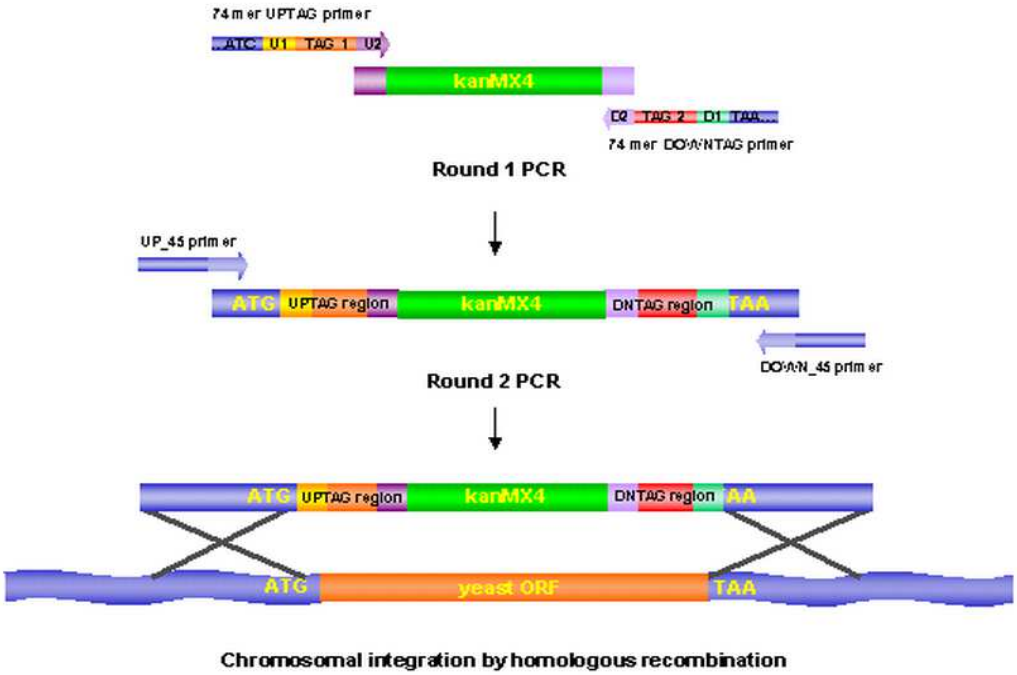


Figure 15 - Strategy for deletion of genes used in the construction of the EUROSCARF mutant collection (Saccharomyces Genome Deletion Project).

3.2. Growth conditions and treatments

Yeast cells were grown on YPDA medium (1% yeast extract, 1% Bacto-peptone, 2% glucose and 2% agar) plates for 2 days at 30 ºC. After growth on YPDA, cells were then inoculated in 10 ml of YPD medium until early exponential phase (OD_{640nm} = 0.5 - 0.7) at 30°C in a shaker at 200 rpm. Thereafter cells were harvested, suspended in Erlenmeyers with 10 ml YPD medium adjusted to pH 3.0 with HCl and 120mM of acetic acid (with a ratio of flask volume/medium of 5:1), and incubated for up to 220 min at 30°C, with agitation (200 rpm). At specific time intervals (0, 60, 120, 180, 200 and 220 min), 100µl of cells were collected, ressuspended in water, and serial dilutions were plated on YPDA. After 2 days of incubation at 30 °C, cell viability was

measured as a percentage of Colony Forming Units (CFU). The percentage of viable cells was estimated, considering 100% survival the number of CFU obtained in time 0, by the formula:

$$\%\ of\ viable\ cells = \frac{number\ of\ colonies\ in\ time\ X\ (\min)}{number\ of\ colonies\ in\ time\ 0} \times 100$$

For semi-quantitative viability assays, 10µL of cell suspensions in water with the dilution rate of 10-1 were spotted onto YPDA plates. After 2 days of incubation at 30 °C, photographs of the plates were taken with ChemiDoc XRS (BioRad).

Cell viability assays were also performed in galactose medium. In thses assays, after growth on YPDA the strains regulated by *RLM1* were inoculated into 10 ml of Synthetic Complete Galactose medium (SC Gal- 2% galactose, 0.67% yeast nitrogen base without aminoacids, 0.14% Dropout mixture lacking 0.008% histidine, 0.04% leucine, 0.008% tryptophan and 0.008% Uracil). SC Gal treatment medium was adjusted to pH 3.0 with HCl and contained 100 mM acetic acid.

3.3. Analysis of apoptotic markers

3.3.1. PI staining

Detection of the integrity of the cell plasma membrane was assessed by flow cytometry using propidium iodide (PI). Cells were treated with acetic acid as described above, and, after specific time intervals (0, 60, 120, 180, 200 and 220 minutes), 100 ul of cells were collected by centrifugation, washed in deionised water, resuspended in 500 uL of phosphate buffered saline (PBS) (80 mM Na_2HPO_4, 20 mM NaH_2PO_4 and 100 mM NaCl) and stained with PI (1 µg/ml) (Sigma). Afterwards, samples were incubated for 10 min at room temperature in the dark. Finally, fluorescence was detected in an Epics® XL™ (Beckman Coulter) flow cytometer, where 30,000 cells from each sample were analyzed. Cells with red fluorescence (FL-3 (488/620 nm)) were considered to contain plasma membrane disruption.

3.3.2. ROS Production

To visualize the accumulation of ROS, cells were treated with acetic acid as described above, and, after specific time intervals (0, 60, 120, 180, 200 and 220 minutes), 100 ul of cells were collected by centrifugation, washed in deionised water, resuspended in 500 uL PBS and incubated with 1 µg/mL DHE (Dihydroethidium) (Molecular Probes) for 20 minutes at room temperature in the dark. To quantify the number of cells displaying high ROS levels, 30,000 cells were counted in an Epics® XL™ (Beckman Coulter) flow cytometer. Cells with fluorescence detection (FL-4 (488/675 nm)), were considered to contain superoxide anion or mitochondrial ROS.

3.3.3. DAPI / Chromatin Condensation

Chromatin condensation was assessed by DAPI (4,6-diamino-2-phenylindole dihydrochloride) (Sigma). Cells were treated as described above, after specific time intervals (0, 60, 120, 180, 200 and 220 minutes) 100 ul of cells were collected by centrifugation, washed in deionised water, fixed in 500 uL of PBS and 500 uL of 99% (v/v) ethanol, and afterwards stained with DAPI (1 µg/ml) for 5 min at room temperature in the dark. Cells were visualized in a Leica Microsystems DM-5000B epifluorescence microscope with appropriate filter settings, using a 100x oil-immersion objective. Images were acquired with a Leica DCF350FX digital camera, and at least 200 cells were counted per sample.

3.4. Screening of the EUROSCARF deletion mutant collection

To identify a higher number of the genes potentially involved in the regulation of acetic acid-induced apoptosis, the strains were cultured in spotted 96-dot arrays in rich solid medium YPDA for 48 hours at 30 °C. Then, using a 96-pin replicator, strains were transferred into 96-well plates with YPD, and grown for an additional 24 hours at 30 °C (no agitation). After this time, the

inoculum was diluted 100 fold using a multichannel pipette (because the assay revealed to be extremely sensitive to differences in cell density during acetic acid treatment). Afterwards, cells were incubated in YPD liquid medium, adjusted to pH 3.0 with HCl, and acetic acid was added to a final concentration of 400 mM. The 2 M stock solution of acetic acid used was prepared with distilled water and the pH adjusted to 3 with NaOH. At different times of incubation (100, 200, 300 and 400 minutes), cells were replicated into 96-well plates containing YPD medium, and the plates were incubated at 30 °C for 24 hours. For the detection of mutants with higher resistance or sensitivity to acetic acid-induced cell death, all ODs were compared with that of the wild type strain. Mutants whose growth was be reduced compared to the wild-type strain and mutants that still grew at a time point where the control strain did not grow were considered sensitive and resistant, respectively. It was possible to determine the biomass in each well through optical density of 640 nm using a microplate reader (Molecular Devices SpectraMax Plus).

Cell viability assays of the strains regulated by Rlm1p were performed as above with the following modifications: cells were grown on YPDA during 24 hours, and then transferred into 96-well plates with SC Gal. After, cells were incubated in SC Gal medium adjusted to pH 3.0 with HCl, and acetic acid added at a final concentration of 250 mM.

4. Results

4.1. Part I

Functional screening of the EUROSCARF mutant collection for the identification of determinants of resistance and sensitivity to acetic acid-induced apoptosis

S. cerevisiae is a powerful model system that enabled the increase in our understanding of the mechanisms underlying apoptotic cell death, which may be extended to the more complex and less accessible multicellular organisms. To uncover the genes involved in acetic acid-induced apoptosis, we performed a genome-wide screening of the EUROSCARF haploid mutant collection, for altered sensitivity to a short exposure to acetic acid. Resistance and sensitivity to acetic acid-induced cell death was based on the comparison of the susceptibility of the mutants of the EUROSCARF haploid collection with the parental strain BY4741 to 400 mM acetic acid, in glucose medium at pH 3.0. The screening protocol was optimized and started in a previous study (Marlene Sousa, Masters Thesis, 2012) and completed in the present work. The analysis of the mutant collection revealed 2159 resistant strains and 391 susceptible mutants when compared with the parental strain (800 mutants were screened previously and 5000 in this work).

All genes whose deletion caused sensitivity/resistance to acetic acid were grouped based on their function, according to the MIPS functional catalogue (http://mips.helmholtz-muenchen.de/proj/funcatDB/). The frequency of each functional class was compared in our dataset and in the yeast genome. The Figures 16 and 17 show the functional classes that were significantly enriched (p-value below 0.01).

4.1.1. Genes whose deletion causes sensitivity to acetic acid-induced cell death

Clustering of the genes whose deletion causes sensitivity to acetic acid-induced cell death based on their function revealed that the functional categories most significantly enriched are: "Protein fate", "Biogenesis of cellular components", "Transcriptional control", "C-compound and carbohydrate metabolism", "Respiration", "Ribosomal proteins", "Ion transport" and "Homeostasis of cations". The “Protein fate” class is essentially composed of genes coding for proteins involved in folding, stabilization, targeting, sorting and translocation of proteins, modifications, as with sugar residues (e.g.

glycosylation, deglycosylation), and protein/peptide degradation. Many of the mitochondrial proteins found in the screen are involved in respiration and some play a role in the electron transport chain, such as Aac3, Atp2, Coq9, Cox16, Cox18, Cyt1, Oar1, Por1 and Rip1; their deletion might increase ROS production in the presence of acetic acid, leading to the cell death (Ludovico et al., 2002). Other genes are involved in the transfer of electrons to the respiratory chain (*SDH4*). Proteins like Cox11, Cox16, Cox17, Cox20 and Sco1 are essential for the assembly of the multi subunit enzyme cyt *c* oxidase, which catalyzes the terminal step in the electron transport chain of cellular respiration. Deletion of genes coding for proteins required for cyt *c* oxidase assembly resulted in sensitivity to acetic acid. Studies have previously demonstrated that COX activity is affected when cells are exposed to acetic acid and ROS production increases (Ludovico et al., 2002), suggesting deficient COX assembly may potentiate this effect. These results showed the importance of inducing apoptosis when the respiratory chain is deregulated and high production of ROS may occur. Other genes whose deletion confered sensitivity to acetic acid, like *IMP1, MDL1, MDM12, MDM20, POR1* and *UGO1* are involved on the stability and permeability of mitochondrial membranes. Deletion of these genes may facilitate translocation of pro-apoptotic proteins from mitochondria into the cytosol. Our results further reinforce that the normal function of the mitochondria is essential for the yeast tolerance to acetic acid-induced cell death.

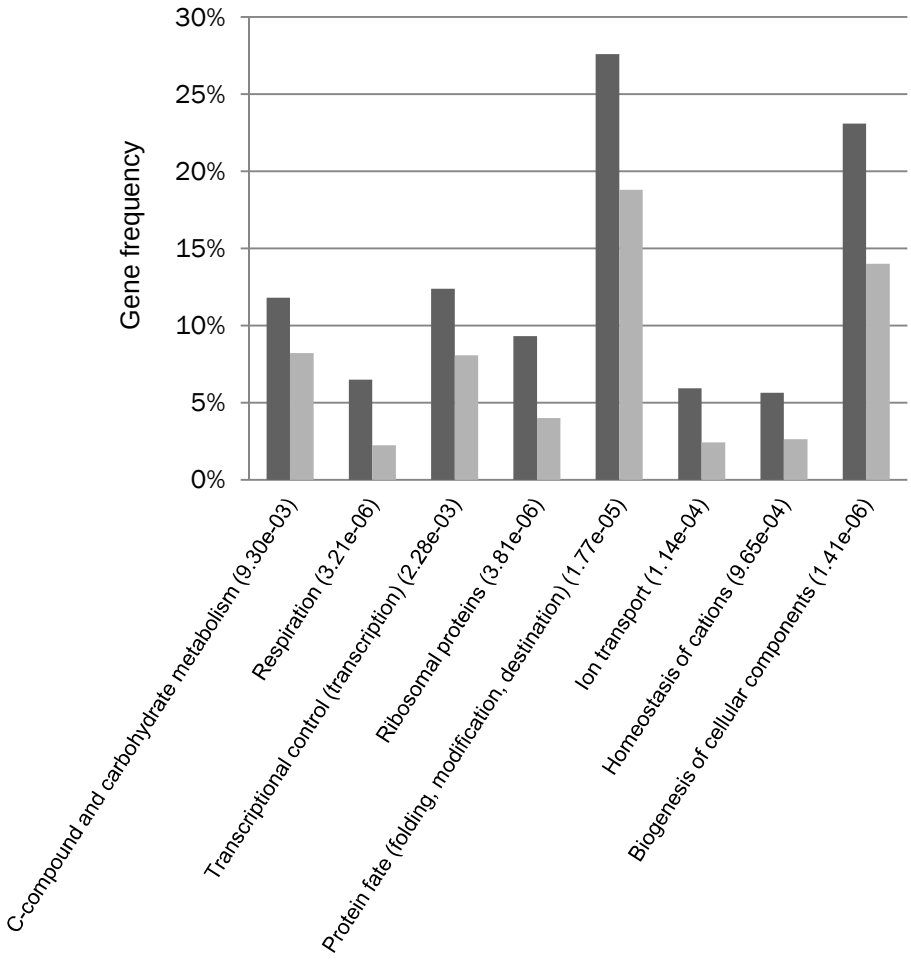


Figure 16 - Functional categories significantly enriched in the set of genes whose deletion renders cells sensitive to acetic acid-induced cell death. The frequency in our dataset (dark grey) is compared with the frequency in the whole yeast genome (light grey).

Other determinants of sensibility to acetic acid clustered in the "Carbohydrate metabolism" functional category. The function of some genes from this class is related with the synthesis of cell wall polysaccharides. A high percentage of genes from our sensitivity data set encode ribosome proteins (*RPL1B, RPL17B, RPL20A, RPL21A, RPL27A, RPL2A, RPL39* and *RPL41B*). It has been demonstrated that certain pathways of cell death are accompanied by the destruction of nucleic acids. For example, in metazoan apoptosis there is irreversible DNA damage, which is considered an apoptotic hallmark. However, specific cleavage of several RNA species is also involved. It was proposed that rRNA degradation could contribute to cell auto-destruction, and that degradation of mRNAs for anti-apoptotic factors would accelerate apoptosis (Seweryn

Mroczek and Joanna Kufe., 2008). A large numer of genes from our sensitivity data set are rRNA helicases (*MRH4, DBP7*) and proteins involved in pre-rRNA processing (*Rex4p*), in RNA synthesis (*CAF120, CTK2, HOS1, ITC1, NAT4, RTF1, SDS3, SIF2, SOH1, STB2* and *TSR2*), and in mRNA processing (*EDC1, FIR1, HIR2, LEA1, LSM6, MUD2, NPL3, PML1* and *XRN1*). This indicates that RNA processing and degradation may have a role in acetic acid-induced cell death.

Other genes whose deletion also confers sensitivity to acetic acid-induced cell death are involved in "Ion transport" and “Homeostasis”. These classes include a number of genes related to proton homeostasis, in other words the assembly and regulation of the plasma membrane H-ATPase (PM-H-ATPase), of vacuolar H-ATPase (V-ATPase) and of mitochondrial F1F0 ATP synthase. To avoid the dissipation of plasma membrane potential and to maintain the internal pH within physiological values, yeast cells rely on the activity of the PM-H+-ATPase. Deletion of *NHA1* and *MCH2* (encoding an antiporter involved in sodium and potassium efflux and a protein involved in the transport of monocarboxylic, respectively) increased sensitivity to acetic acid-induced cell death.

Deletion of genes involved in vacuolar V -ATPase function, as *VMA3 VMA16, VMA22, VMA4, VMA7* and *VMA8* increased acetic acid-induced cell death, supporting the idea that the V-ATPase present in the vacuolar membrane is crucial for pH homeostasis when cells are exposed to weak acid stress. Sequestering the exceeding protons present in the cytosol in the vacuole lumen of acetic acid-challenged cells may aid in the recovery of the cytosolic pH to more physiological values. These results are in agreement with earlier evidence showing that V-ATPase activity is important to maintain vacuolar pH and ensuring the normal operation of several vacuolar processes, which are necessary for growth in the presence of a weak acid (Mira et al., 2010; Kawahata et al., 2006; Makrantoni et al., 2007; Mira et al., 2009). Other genes related with vacuole function also increased acetic acid-induced cell death when deleted, such as *VPS1, VPS16, VPS28, VPS33, VPS72, VPS73* and *VPS8,*

which encode proteins belonging to the family of the vacuolar sorting proteins responsible for vesicle-mediated transport.

Deletion of genes involved in F1F0 ATP synthase function, as *ATP1, ATP11, ATP12, ATP17, ATP2* and *ATP4* increased acetic acid-induced cell death. *Pmr1p* is the major Golgi membrane P-type ATPase ion pump responsible for transporting calcium and manganese ions into the Golgi complex. Thus, Pmr1p provides a major route for cellular detoxification. Excess levels of cytosolic calcium and manganese ions are transported into the Golgi complex and then exit the cell via secretory vesicles. In our study, deletion of *PMR1* sensitized cells to acetic acid, showing that this detoxification of the cytosol is important to protect cells from acetic acid-induced cell death. This is in accordance with the increase in calcium concentration observed in cells undergoing acetic acid-induced cell death (Pereira et al 2008). Other genes found in our data set of sensitive strains were *KHA1* and *MDL1*. Kha1p is involved in intracellular cation (K+/H+) homeostasis localized to Golgi vesicles and Mdl1p mediates the export of peptides generated upon proteolysis of mitochondrial proteins and plays a role in the regulation of cellular resistance to oxidative stress. Our results suggest that these peptides may have a pro-apoptotic role.

Strains deleted in genes encoding proteins related with ion import were also found to be susceptible to acetic acid-induced cell death, suggesting that the uptake of these ions plays a crucial role in the yeast response to acetic acid. A previous study had shown potassium uptake is increased in response to acetic acid stress, possibly to compensate the stimulation in the activity of Pma1p (extrusion H+) occurring in these cells, thus keeping the electrical balance across the plasma membrane (Mira et al., 2010). A similar adaptive response is proposed to occur in the presence of other ions.

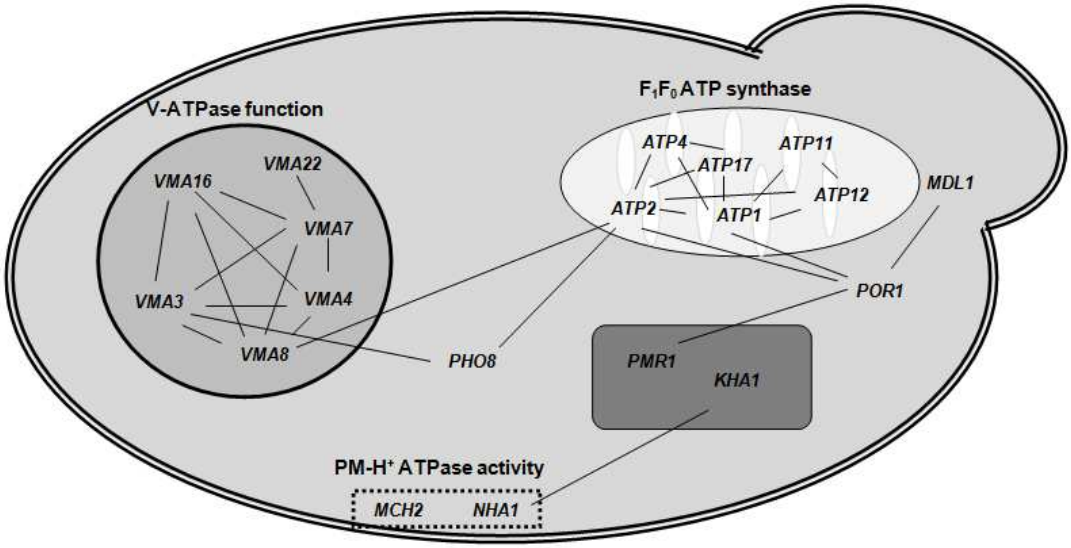


Figure 17 - Schematic representation of the interaction network of genes whose deletion renders cells sensitive to acetic acid-induced cell death, and that were clustered in the ion transport functional class, using STRING (Protein-Protein Interactions). In the depicted map, a line represents an interaction.

4.1.2. Genes whose deleted causes resistance to acetic acid-induced cell death

The functional categories most significantly enriched in the data set of genes whose deletion confers resistance to acetic acid-induced cell death are: "Transcriptional control", "Stress response", "Phosphate metabolism", "Cell type differentiation", "Amino acid metabolism", "Cell wall", "Meiosis", "Protein kinase" and "Detoxification" (figure18).

The “Amino acid metabolism” class is basically composed of genes encoding proteins involved in assimilation of ammonia, metabolism and biosynthesis of glutamate and arginine, metabolism of the urea cycle, and metabolism of several amino acids (of the aspartate family, threonine, methionine, cysteine aromatic group, serine, phenylalanine, tryptophan, of the pyruvate family (alanine, isoleucine, leucine, valine), D-alanine and leucine). The economy in energy and resources resulting from blocking amino acid

biosynthetic pathways might improve the cellular response to a toxic concentration of acetic acid, and explain these results. (Li and Yuan, 2010; Almeida et al., 2009).

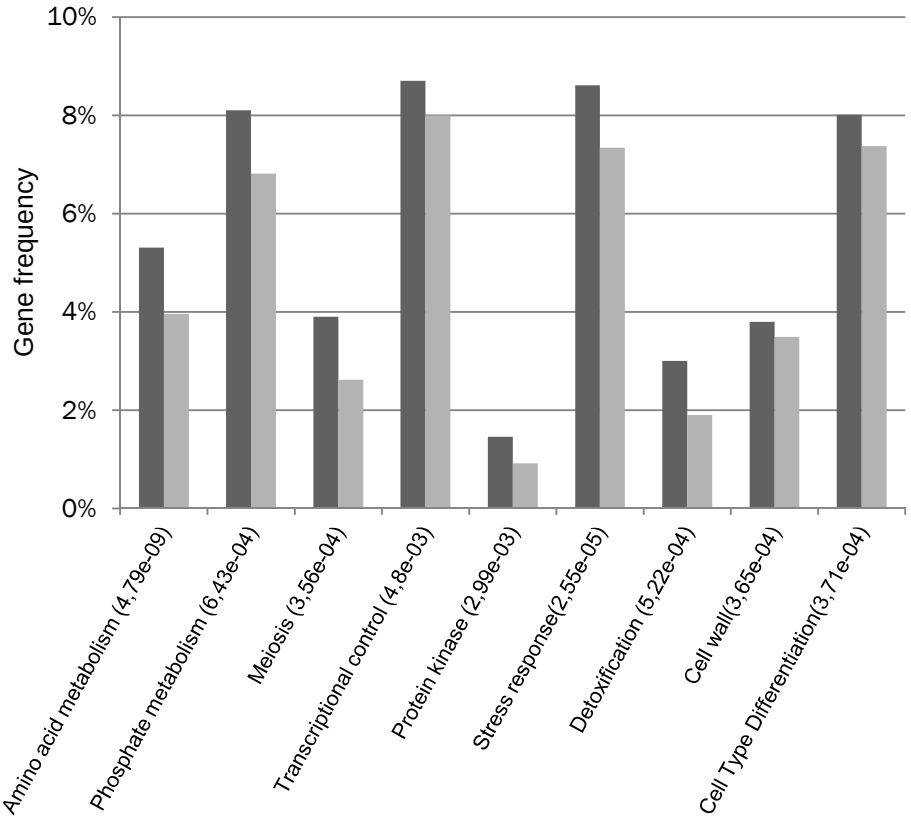


Figure 18 - Functional categories significantly enriched in the data set of genes whose deletion renders cells resistance to acetic acid-induced cell death. The frequency in our dataset (dark grey) is compared with the frequency in the whole yeast genome (light grey).

Reinforcement of the cell wall structure is a general response mechanism to weak acid-induced toxicity. The acidic condition affects cell-wall architecture, which might be a reflexion of changes in the expression of cell wall-related genes under these conditions. We found that depletion of cell-wall components encoded by *SED1, SCW11, SUN4, TOS6, EAF3, EAF7, MNN2, MNN9, MNN11, ANP1, VMR1* and *GON7* increased the resistance to acetic acid, indicating that hindering these cell changes can protect cells from death.

It is important to refer that, within the "cell type differentiation" category, there were several genes that belong to the invasive and pseudohyphal growth pathway, like *HMS1*. Absence of other genes involved in the cell wall integrity MAP kinase cascade (*PKH3, MKK2, PKH1, BCK1* and *MKK1*) also protected cells from acetic acid-induced cell death.

Our combined screening uncovered 204 genes in the "transcriptional control" class important for acetic acid-induced cell death (M. Sousa 2012, this study). The absence of 160 transcription factors conferred resistance to acetic acid, whereas absence of 44 transcription factors conferred increased sensitivity. For some of these genes, a role in the response to acetic acid stress had been described before; others are described here for the first time.

Factors that when mutated confer resistance to acetic acid include *SPT10, HFI1 and SDS3*, involved in de-acetylation and *RPD3*, *HOS3*, *HOS1*, *HTA1*, *NHP6B*, *SAS5*, *SPT2* and *ECM11*, involved in acetylation. It is believed that transcriptional activity is correlated with histone acetylation in some cases. Given the drastic changes in the integrity of DNA and the state of chromatin compaction during apoptosis, histone modifications may play a functional role in promoting these changes. Thus, chromatin modifiers can also function as co-repressors by promoting a more closed chromatin conformation. Methylation of sequences in promoter regions is commonly observed during tumor progression to inactivate genes whose products are important for processes such as DNA repair, cell-cycle regulation, cell adhesion, angiogenesis and apoptosis (Miranda TB, et al, 2007).

Genes encoding transcription factors are of particular interest because their increased expression may result in the simultaneous induction of a set of acetic acid-resistance and sensitivity genes under their control. Cells possess a network of signal transduction pathways that enable them to respond to different stimuli, which implies strong changes in gene expression. The stress transcription factors are thus interesting models, and their characterization can lead to the identification of new components of the stress signaling pathway in yeast.

4.2. Part II

The role of Sfl1p in acetic acid-induced apoptosis

4.2.1. Sfl1p and the response to acetic acid

In a previous study, it had been shown that *SFL1* deletion leads to increased resistance to acetic acid-induced cell death (Marlene Sousa, 2012). In this work, we aimed to study the role of Sfl1p in the mitochondrial-dependent apoptotic process induced by this acid in *S. cerevisiae* cells. We first characterized the cell death process induced by acetic acid in the *sfl1Δ* mutant. Cells were cultivated in YPD medium, exposed to 120 mM acetic acid, pH 3.0, for 220 min and cell viability and apoptotic markers were assessed over time. Viability was assessed by spotting serial dilutions of the cultures (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) onto YPD plates (figure 19A) and quantified by colony forming units (c.f.u) (figure 19B).

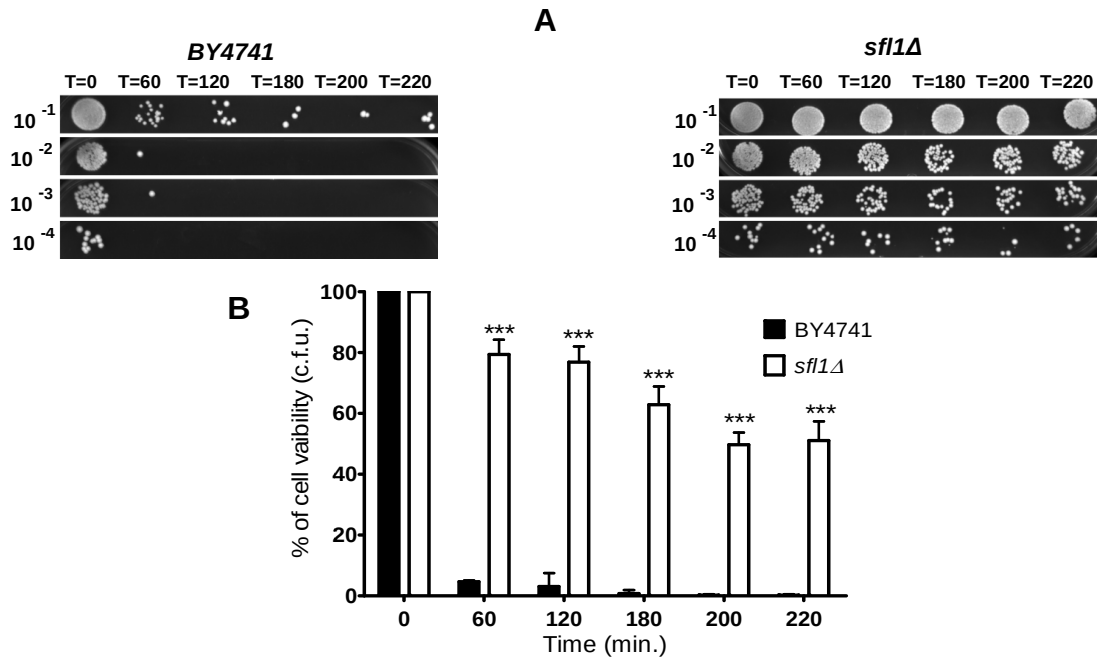


Figure 19 - Relative cell survival of wild type and *sfl1Δ* strains after exposure to 120 mM of acetic acid. Cells were grown at 30 °C and samples were taken after 0, 60, 120, 180, 200 and 220 minutes. (A) Serial dilutions (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) were spotted onto YPD plates. B) Cell viability was determined by C.F.U. counts.. Values represent means and standard deviations of 3 independent experiments. Values significantly different between BY4741 and *sfl1Δ* strain - *** P<0.001, Statistical analysis was performed using a two-way ANOVA test.

Exposure of the *sfl1Δ* mutant to acetic acid under the conditions referred above induced loss of viability only in about half of the cells in culture after 220 minutes of incubation. Under the same conditions, only 1% of the wild-type cells were viable, thus confirming that deletion of the *SFL1* gene increases resistance to acetic acid–induced cell death.

To characterize the nature of cell death in *sfl1*Δ mutant cells, we studied several apoptotic markers, namely ROS production, chromatin condensation and fragmentation, and plasma membrane integrity. To determine the levels of ROS, *sfl1*Δ mutant cells treated with acetic acid were labeled with DHE. DHE can penetrate the membrane of living cells and intercalate into DNA after it is dehydrogenated (oxidized by superoxide anions generated in mitochondria). Intracellular ROS were assessed by flow cytometry and the results expressed as a percentage of ROS-positive cells (Figure 20A). After 220 minutes of treatment with acetic acid, only 18.5±11% of the *sfl1*Δ cells stained positive with DHE, showing that the mutant had a much lower accumulation of ROS than the wt cells (91%). These results are in agreement with its resistance phenotype. In addition, membrane integrity was measured by propidium iodide (PI) staining, under the same conditions. The results show that after 220 minutes of acetic acid-treatment, 19.9±2.4% of the *sfl1*Δ mutant cells are PI-positive (figure 20B). The percentage of PI positive cells over the time of treatment is low, showing that there is no significant loss of plasma membrane integrity. In contrast, 94% of wild-type cells had lost plasma membrane integrity after 220 min.

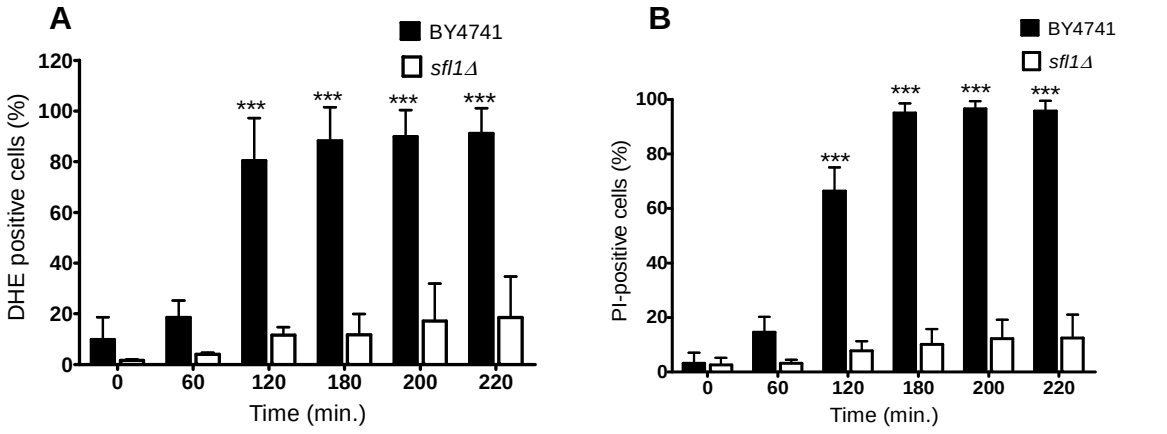
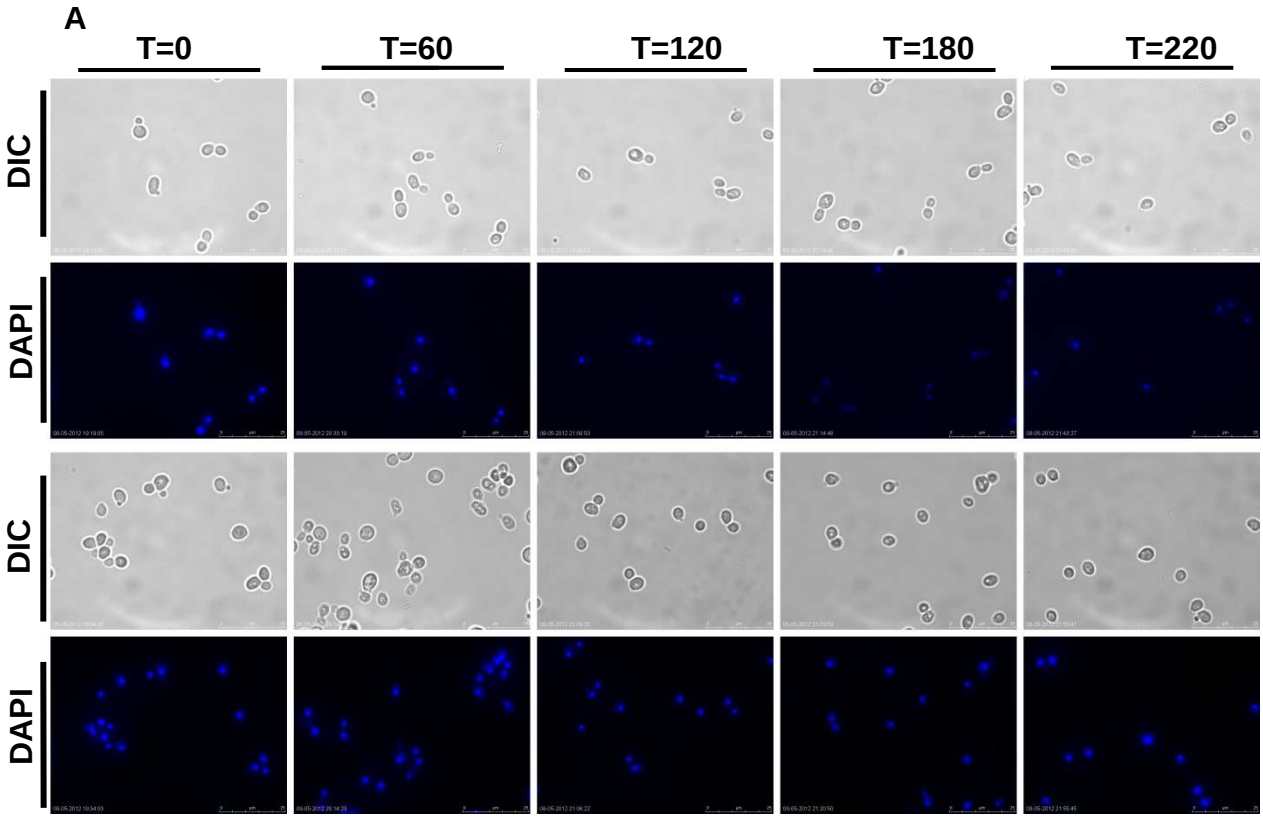


Figure 20 - Superoxide anion accumulation (A) and loss membrane integrity (B) in wt and *sfl1Δ* strains incubated with acetic acid. Samples were collected after 0, 120, 180,

240 and 300 minutes at 30°C, stained with DHE (1µg/mL) and PI (1µg/mL) respectively, and the fluorescence measured by flow cytometry. Percentages of cells stained with DHE and PI are shown. Values represent means and standard deviations of 3 independent experiments. Values significantly different between BY4741 and *sfl1*Δ strain: *** P<0.001, Statistical analysis was performed using a two-way ANOVA test.

To determine if exposure to acetic acid leads to chromatin condensation in *sfl1*Δ cells, nuclear DNA was stained with DAPI and cells were observed by fluorescence microscopy (Figure 21A). After 220 minutes of the treatment with acetic acid, only a few apoptotic nuclei were observed in *sfl1*Δ (17%) compared to the wild type strain (88%) (Figure 21B).



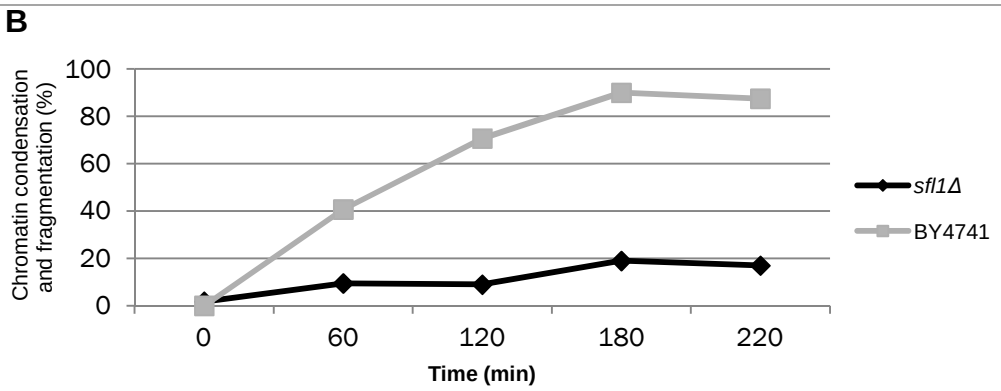


Figure 21 - Chromatin condensation and fragmentation in wt and *sfl1Δ* strains incubated with acetic acid. Exponential cultures of *sfl1Δ* and wt strains were treated with 120mM acetic acid. Samples were collected after 0, 60, 120, 180 and 220 minutes, stained with DAPI (2µg/mL) and observed by fluorescence microscopy. (A) Photomicrographs of fluorescence and DIC (differential interference contrast) images (B) Quantification of apoptotic nuclei.

Taken together, the results show that deficiency in Sfl1p abrogates the appearance of all the cellular changes induced by acetic acid assessed, indicating a role for Sfl1p in the induction of acetic acid-induced apoptosis.

4.2.2. Role of the catalytic subunits of PKA in acetic acid-induced cell death

The investigators Conian and Tzamarias demonstrated that the interaction of Sfl1p with DNA is regulated by PKA, and that the yeast PKA isoform Tpk2p is involved in the regulation of Sfl1p recruitment to Ssn6p, thus showing that Sfl1p is negatively regulated by the Tpk2p isoform of protein kinase A (Conlan and Tzamarias, 2001).

TPK1, TPK2 and *TPK3* encode the catalytic subunits of PKA in *S. cerevisiae* (Toda *et al.,* 1987). Tpk1p was described as regulating genes involved in respiration, in the maintenance of iron levels, DNA stability in mitochondria, and derepression of branched chain amino acid biosynthesis genes (Gourlay and Ayscongh, 2006). Tpk2p, besides its involvment in the negative regulation of Sfl1p (Conlan *et al.,* 2001), is also responsible for the

repression of transcription of genes involved in iron uptake, trehalose breakdown, water homeostasis, flocculation and pseudohyphal development. On the other hand, Tpk3p is involved in negative regulation of pseudohyphal growth and in flocculation (Robertson and Fink, 2000). It has been shown that loss of Tpk3p leads to the reduction of respiratory functions and that this protein is also involved in the regulation of mitochondrial enzyme content (Chevtzoff *et al.,* 2005).

We next assessed the role of individual PKA isoforms in acetic acid-induced cell death using strains deleted in each of the three genes. The cell viability of the three mutants was determined after exposure to 120 mM of acetic acid along for up to 220 minutes (figure 22).

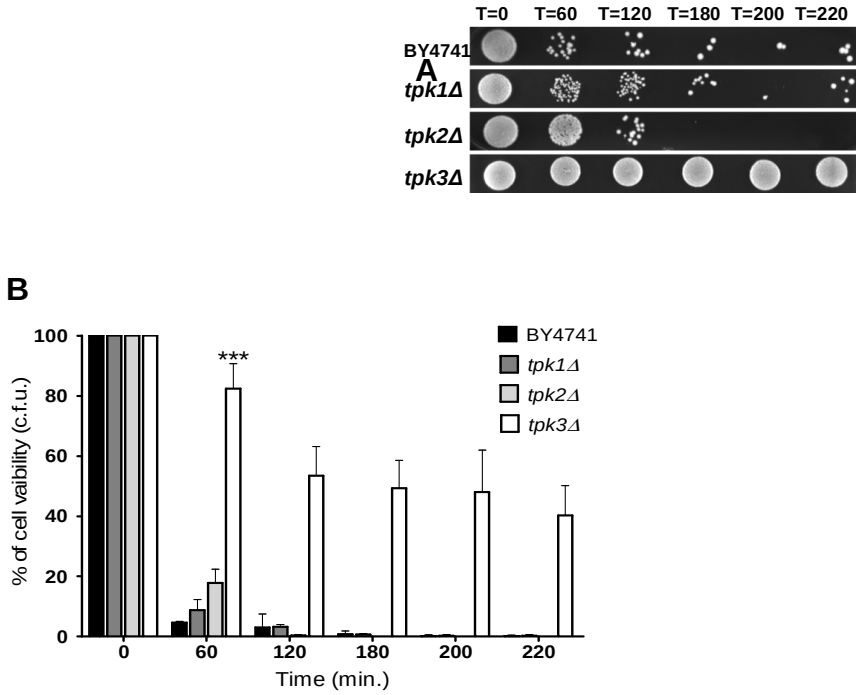


Figure 22 – **Relative cell survival of wild, *tpk1Δ*, *tpk2Δ* and *tpk3Δ* strains after exposure to 120 mM acetic acid.** Cells were grown at 30 °C and samples were taken after 0, 60, 120, 180, 200 and 220 minutes. (A) Serial dilutions (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) were spotted onto YPD plates. (B) Cell viability was determined by C.F.U. counts. Values represent means and standard deviations of 3 independent experiments. Values significantly different between BY4741 and *tpk3Δ* strains - *** P<0.001, Statistical analysis was performed using a two-way ANOVA test.

To determine the levels of ROS in cells treated with acetic acid, cells were labeled with DHE and fluorescence was analyzed by flow cytometry. The strains *tpk1Δ*, *tpk2Δ* and *tpk3Δ* were incubated with 120 mM of acetic acid, at pH 3 for 220 min, and the percentage of ROS-positive cells determined over time (Figure 23A). The *tpk3Δ* mutant had the lowest percentage of cells with mitochondrial ROS, only 10.2±4.9% after acetic acid treatment for 220 mim. The *tpk1Δ* and *tpk2Δ* mutants displayed higher levels of superoxide anion, 92.3±3% and 64.4±6.6%, respectively, under the same treatment conditions. Again, the results are in agreement with their phenotypes, since *tpk1Δ* and *tpk2Δ* mutants are more sensitive to acetic acid than mutant *tpk3Δ*. This effect might be related with a lower respiration level in *tpk3Δ*, and as a consequence lower ROS accumulation.

Cells were exposed to acetic acid, under the same previous conditions, and stained with PI to measure the integrity of plasma membrane. Fluorescence levels were measured by flow cytometry. For the *tpk3Δ* mutant, there was only a residual percentage of PI positive cells of 5.9±4.9% (figure 23B).

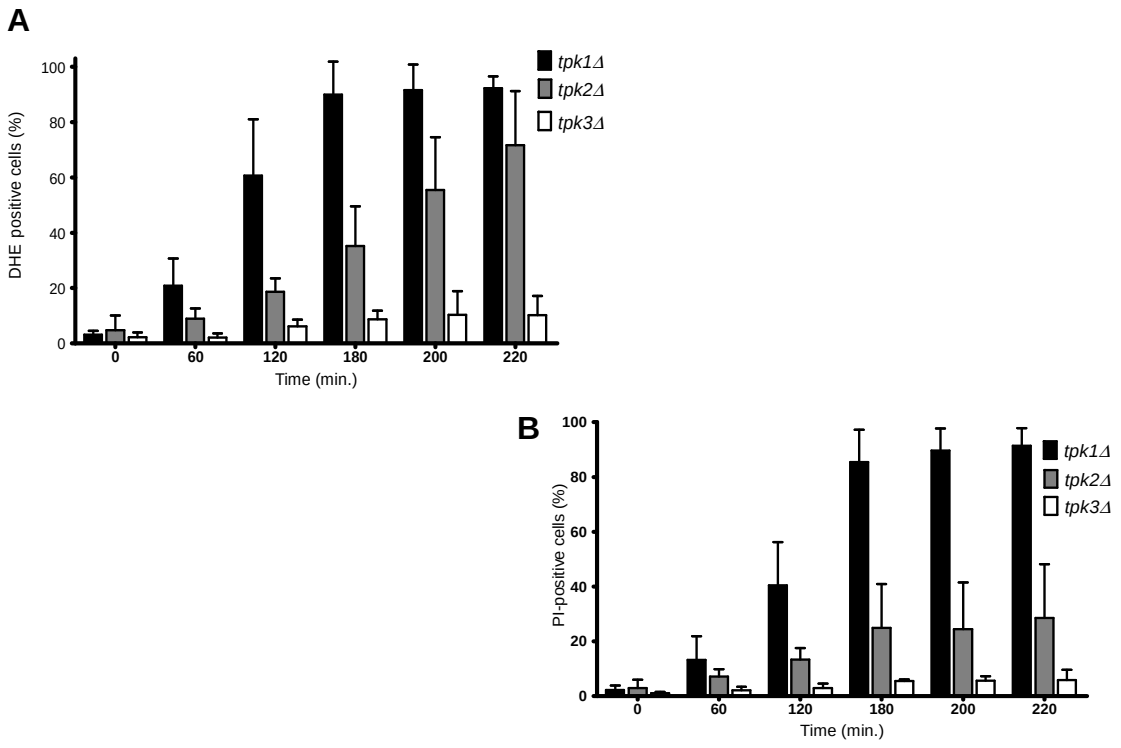


Figure 23 – Superoxide anion accumulation (A) and loss of membrane integrity (B) in *tpk1Δ, tpk2* and *tpk3Δ* strains incubated with acetic acid. Samples were collected after 0,

120, 180, 240 and 300 minutes at 30°C, stained with DHE (1µg/mL) and PI (1µg/mL) respectively, and the fluorescence measured by flow cytometry. Percentages of cells stained with DHE and PI are shown. Values represent means and standard deviations of 3 independent experiments.

The nuclear DNA of the *tpk3Δ* mutant was stained with DAPI and observed by fluorescence microscopy (Figure 24A). Observations after 220 minutes of the treatment with acetic acid showed that only 13% of the cells had abnormal nuclei and chromatin condensation, in comparison with 88% of the wild-type cells (figure 24B).

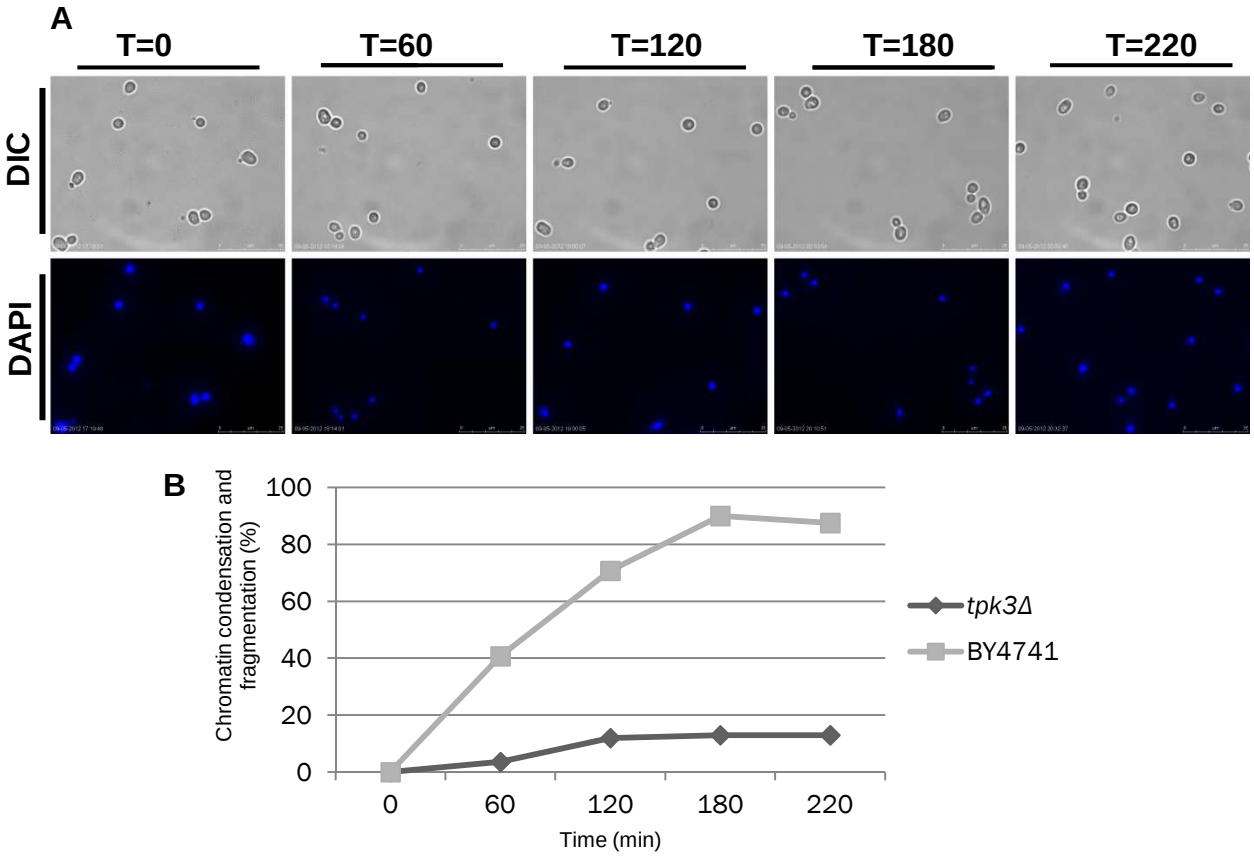


Figure 24 - Chromatin condensation and fragmentation in wt and tpk3Δ strains incubated with acetic acid. Exponential cultures of *tpk3Δ* and wt strains were trated with 120mM acetic acid. Samples were collected after 0, 60, 120, 180 and 220 minutes, stained with DAPI (2µg/mL) and were observed by fluorescence microscopy. (A) Photomicrographs of fluorescence and DIC images (B) Quantification of apoptotic nuclei.

The results indicate that, from the three PKA isoforms, Tpk3 has the most relevant role in the induction of apoptosis triggered by acetic acid. *TPK3* deletion, besides decreasing the loss of cell viability, also abrogated the appearance of the other cell death markers evaluated. On the other hand, the results obtained with *tpk2Δ* are not consistent with a role of this isoform in the regulation of Sfl1p in acetic acid induced cell death.

4.2.3. Identification of the downstream targets of Sfl1p involved in programmed cell death

To further assess the role of Sfl1p in acetic acid-induced apoptosis, we next studied the genes that are described as being regulated by this transcription factor. Using the database http://www.yeastract.com/, we identified the genes listed in table 1. This table also shows the function of each gene, its location, the phenotype in our genome-wide screen and the genes that have been described as repressed or activated by Sfl1p experimentally. These genes were grouped by function using the database MIPS functional catalog. Most of these genes are located in the nucleus (34%), in the endoplasmic reticulum (13%), integral membrane/endomembranes (10%), vacuole and outer membrane of mitochondria (9% and 6% respectively).

In our genome-wide screen, genes potentially regulated by Sfl1p were identified as involved in acetic acid-induced cell death. Of the 48 genes described as being regulated by Sfl1p, deletion of only two of these genes resulted in increased sensitivity to acetic acid (*NNF2* and *TAF14*), deletion of one gene had no phenotype (*TPK1*), and deletion of all the others resulted in increased resistance to acetic acid-induced cell death. Taf14p is a component of different complexes, like the SWI/SNF chromatin remodeling complex, and is involved in DNA replication, stress response and transcription. It binds non-specifically to DNA, altering nucleosome structure to facilitate the binding of transcription factors. Its sub-unit TFIID is also a transcription factor complex that is required for RNAPII-mediated transcription of protein-coding genes and some small nuclear RNAs. This could explain the sensitivity of *taf14Δ* to acetic acid.

taf14Δ mutants are viable, but grow slowly on rich media and display decreased transcription, defects in actin organization, increased osmosensitivity, heat sensitivity and sensitivity to caffeine, hydroxyurea, UV, and methyl methanesulfonate. The same happens with the mutant *nnf2Δ*, deficient in a protein that exhibits physical and genetic interactions with Rpb8p, which is a subunit of RNA polymerases I, II, and III, and so also involved in transcription. Thus, the results indicate that most of the genes regulated by Sfl1p are involved in mediation of cell death, and their deletion seems to be beneficial during acetic acid-induced cell death.

Table 1 - Genes that are described as regulated by Sfl1p and respective function, location, phenotype in our genome-wide screen and whether they have been described as repressed or activated by Sfl1p.

Gene	Function protein	Location	Genome-wide screening	Regulated by SFL1p
ADE2 (YOR128C)	Encodes phosphoribosylaminoimidazole carboxylase, which catalyzes the sixth step in the biosynthesis of purine nucleotides (Som I, et al. 2005).	Cytoplasm	Resistant	
AGP3 (YFL055W)	Plays a role in regulating Ty1 transposition (Nyswaner KM, et al. 2008).	Integral membrane	Resistant	
APT2 (YDR441C)	Is a gene with similarity to adenine phosphoribosyltransferase (APRT), not expressed under normal physiological conditions in yeast (Alfonzo JD, et al. 1999).	Cytoplasm	Resistant	
AQY2 (YLL052C)	Water channel that mediates the transport of water and small uncharged molecules across cell membranes, controlled by osmotic signals, may be involved in freeze tolerance (Laize V, et al. 2000).	ER membrane and plasma membrane	Resistant	**Repressed** (stress osmotic) (Furukawa et al., 2009; Carbrey et al., 2001).
AHC2 (YCR082W)	Protein of unknown function, putative transcriptional regulator, proposed to be a histone acetyltransferase complex component (Lee KK, *et al.* 2011).	Cytoplasm e nucleus	Resistant	
BRF1 (YGR246C)	BRF1 is an gene encodes is a initiation factor for one of three subunits of RNA polymerase III, which transcribes tRNAs, most small nuclear RNAs, and 5S rRNA (Alexander DE, *et al.* 2004).	Nucleus	Resistant	
COG4 (YPR105C)	Essential component of the Golgi complex, a cytosolic complex that functions in protein trafficking to mediate fusion of transport vesicles to Golgi compartments (Loh E and Hong W 2004).	Golgi transport complex	Resistant	
FLO8 (YER109C)	Transcription factor required for flocculation, diploid filamentous growth, and invasive growth (Liu H, *et al.* 1996).	Nucleus and cytoplasm	Resistant	**Repressed** (Kim et al., 2004)
FLO1 (YAR050W)	Gene involved in flocculation, cell wall protein that binds to mannose chains on the surface of other cells, confers floc-forming ability that is chymotrypsin sensitive and heat resistant (Stratford M 1989).	Cell Wall	Resistant	**Repressed** (Shen et al., 2006)
FMP42 (YMR221C)	Is detected in highly purified mitochondria, physical interaction with Atg27p suggests a possible role in autophagy (Tarassov K, et al. 2008).	Mitochondria and vacuole	Resistant	
FDC1 (YDR539W)	Essential for the decarboxylation of aromatic carboxylic acids to the corresponding vinyl derivatives Mukai N, et al. 2010).	Cytoplasm	Resistant	
FMP45 (YDL222C)	Gene encodes an integral membrane protein localized in mitochondria, which is required for sporulation and sphingolipid metabolism (Young ME, et al. 2002).	Cytoplasm and mitochondria	Resistant	**Repressed** (stress ethanol) (Galeote et al., 2007)
HEM1 (YDR232W)	Encodes the enzyme 5-aminolevulinate synthase, which catalyzes the first step in heme biosynthesis and is also involved in regulating the transcription of genes involved in iron and copper transport (Urban-Grimal D, *et al.* (1986).	Mitochondrial matrix	Resistant	
HSP104 (YLL026W)	Encodes a general anti-stress chaperone of the HSP100 gene family. Hsp104p, in conjunction with the chaperone and co-chaperone Ssa1p and Ydj1p, helps to disassemble protein aggregates that have accumulated due to stress (Chernoff YO, et al. 1995).	Cytoplasm and nucleus	Resistant	
HSP30 (YCR021C)	Stress-responsive protein that negatively regulates the H(+)-ATPase Pma1p, is induced by several stresses, during exposure to a variety of stress conditions including heat shock, exposure to weak organic acids, hyper-osmotic stress, oxidative stress, glucose limitation, exposure to alcohol and entry into stationary phase (Meena RC, *et al.* 2011).	Plasma membrane	Resistant	**Activated** (stress ethanol) (Galeote et al., 2007)

MGA1 (YGR249W)	Protein similar to heat shock transcription factor, suppressor of pseudohyphal growth defects of ammonium permease mutants (Lorenz MC and Heitman J., 1998).	Nucleus	Resistant	
MUC1 (YIR019C)	GPI-anchored cell surface glycoprotein (flocculin), required for pseudohyphal formation, invasive growth, flocculation, and biofilms. Transcriptionally regulated by the MAPK pathway and the cAMP pathway (Douglas LM, et al., 2007).	Cell Wall	Resistant	
NNF2 (YGR089W)	Protein that exhibits physical and genetic interactions with Rpb8p, which is a subunit of RNA polymerases I, II, and III (Briand JF, *et al.,* 2001).	Cytoplasm and ER	Sensitive	
PTI1 (YGR156W)	Is a component of CPF (cleavage and polyadenylation factor); involved in 3' end formation of snoRNA and mRNA; interacts directly with Pta1p **(**Dheur S, et al., 2003**)**.	Cytoplasm and nucleus	Resistant	
PET309 (YLR067C)	PET309 encodes protein that is required for two steps in the expression of COX1, the encoding subunit 1 of cty c oxidase, also influences stability of intron-containing COX1 primary transcripts **(**Tavares-Carreon F, *et al*., 2008).	Mitochondrial inner membrane	Resistant	
RPN2 (YIL075C)	Subunit of the 26S proteasome, substrate of the N-acetyltransferase Nat1p (Kimura Y, et al., 2003**)**.	ER membrane, cytoplasm and nuclear envelope	Resistant	
SSA4 (YER103W)	Member of the HSP70 family, heat shock protein that is highly induced upon stress (Chughtai ZS, *et al.,* 2001).	Cytoplasmic and nucleus	Resistant	**Repressed** (stress temperature) (Kryndushkin *et al.*, 2002)
STA1	Glucoamylase (glucan 1,4-alpha-glucosidase) **(**Yamashita I, et al., 1985).	Cellular component unknown	Resistant	**Repressed** (stress temperature) (Galeote *et al*., 2007).
SUC2 (YIL162W)	Invertase, sucrose hydrolyzing enzyme, glycosylated form is regulated by glucose repression, and an intracellular, nonglycosylated enzyme is produced constitutively **(**Lutfiyya LL and Johnston M., 1996**)**.	Mitochondria	Resistant	**Repressed** (stress ethanol) (Conlan R. S. and Tzamarias D., 2001)
SPI1 (YER150W)	GPI-anchored cell wall protein involved in weak acid resistance, expression is induced under conditions of stress and during the diauxic shift (Simoes T, et al., 2006**)**.	Cytoplasm and vacuole	Resistant	**Repressed** (stress ethanol) (Galeote et al., 2007)
SOR2 (YDL246C)	Protein of unknown function; protein sequence is 99% identical to the Sor1p sorbitol dehydrogenase, computational analysis also suggests a role in fructose or mannose metabolism (Gonzalez E, *et al.* 2000**)**.	Cellular component unknown	Resistant	
STE13 (YOR219C)	Dipeptidyl aminopeptidase, Golgi integral membrane protein that cleaves on the carboxyl side of repeating -X-Ala- sequences, required for maturation of alpha (Nothwehr SF, *et al.,* 1993**)**.	Golgi	Resistant	
SMI1 (YGR229C)	Protein involved in the regulation of cell wall synthesis; proposed to be involved in coordinating cell cycle progression with cell wall integrity (Martin-Yken H, et al., 2003).	Golgi-vacuole transport vesicles, nucleus and cytoplasm	Resistant	
TL(CAA)G1	Leucine tRNA (tRNA-Leu), predicted by tRNAscan-SE analysis (Chan PP and Lowe TM., 2009).	Cytosol	Resistant	
TPK1 (YJL164C)	Promotes vegetative growth in response to nutrients via the Ras-cAMP signaling pathway, inhibited by regulatory subunit Bcy1p in the absence of cAMP (Robertson LS, et al., 2000).	Nucleus	No phenotype	

TAF14 (YPL129W)	Taf14p is a component of subunit of TFIID, TFIIF, INO80, SWI/SNF, and NuA3 complexes, involved in RNA polymerase II transcription initiation and in chromatin modification (Tora L., 2002).	Nucleus	Sensitive	
VTC4 (YJL012C)	Vacuolar membrane polyphosphate polymerase, subunit of the vacuolar transporter chaperone complex involved in synthesis and transfer of polyP to the vacuole, regulates membrane trafficking, role in non-autophagic vacuolar fusion (Uttenweiler A, et al., 2007).	Cytoplasm and vacuolar membrane	Resistant	
YLR125W	Unknown function.	Cellular component unknown	Resistant	
YIA6 (YIL006W)	Mitochondrial NAD+ transporter, involved in the transport of NAD+ into the mitochondria, member of the mitochondrial carrier subfamily; disputed role as a pyruvate transporter (Todisco S, *et al.*, 2006**)**.	Mitochondria	Resistant	
YAR023c	Putative integral membrane protein, member of DUP240 gene family (Poirey R, et al. 2002).	Cellular component unknown	Resistant	
YCR006c	Unknown function.	Cellular component unknown	Resistant	**Activated** (stress ethanol) (Galeote et al., 2007)
YJR115w	Unknown function.	Cytoplasm	Resistant	**Repressor** (stress ethanol) (Galeote et al., 2007)
YEL010w	Unknown function.	Cellular component unknown	Resistant	
YCL074w	Pseudogene: encodes fragment of Ty Pol protein (Kim JM, et al., 1998).	Cellular component unknown	Resistant	
YDL196w	Unknown function.	Cellular component unknown	Resistant	
YFR054c	Unknown function.	Cellular component unknown	Resistant	
YLR352w	Unknown function.	Cellular component unknown	Resistant	
YOR051c	Nuclear protein that inhibits replication of Brome mosaic virus in *S. cerevisiae*, which is a model system for studying replication of positive-strand RNA viruses in their natural hosts (Henri J, et al., 2010).	nucleus	Resistant	
YGR045c	Unknown function.	Cellular component unknown	Resistant	
YOR105w	Unknown function.	Cellular component unknown	Resistant	
YCR087c-a	Unknown function.	Nucleus	Resistant	
YMR173w-a	Unknown function.	Cellular component unknown	Resistant	**Repressed** (stress ethanol) (Galeote et al., 2007)

In the literature, a few of these genes have been described as repressed or activated by Sfl1p under different stress conditions, as supported by experimental evidence. Galeote and his collaborators, in 2007, showed that Sfl1p activates the transcription of the HSP30 gene during growth on glucose as well as under various stress conditions, including ethanol stress, heat shock, or limitation of carbon source. They also showed that Sfl1p activates the expression of the YCR006C gene and represses the genes *YJR115W*, *FMP45*, *YMR173W-a* and *SPI1* in response to ethanol stress (Galeote *et al.,* 2007). Kim and collaborators demonstrated that glucose-dependent repression of *STA1* is imposed by Sfl1p, and Sfl1p also represses *FLO8* expression (Kim *et al.,* 2004). *FLO1*, another gene involved in yeast flocculation (Stratford and Assinder, 1992), also seems to be repressed by Sfl1p (Shen *et al.,* 2006). Another study showed that *SUC2*, a gene involved in sucrose catabolism, is also partially regulated by Sfl1p, which is required for regulation of *SUC2* expression only when glucose levels are near depletion (Conlan and Tzamarias, 2001). *AQY2* also seems to be repressed by Sfl1p. Aqy2p is a water channel involved in the transport of water across the cell membrane, and is thus implicated in controlling cell surface properties (Furukawa *et al.,* 2009; Carbrey *et al.,* 2001). *SSA4* is a member of the Hsp70 family and its expression seems to be induced under stress conditions and repressed by Sfl1p (Kryndushkin *et al.,* 2002).

4.2.3.1. Characterization of the role of genes under Sfl1p regulation in acetic acid-induced cell death

To confirm the involvement the genes under Sfl1p regulation in acetic acid induced cell death, we individually tested the deletion mutants in some of these genes for cell viability and apoptotic markers. For this purpose, we selected deletion mutants in genes that were identified as being regulation by Sfl1p by experimental evidence. Several assays for apoptosis detection are routinely used in yeast (Carmona-Gutierrez *et al.,* 2010). They include determination of viability (assessed by CFU), ROS accumulation, chromatin condensation and cell membrane integrity.

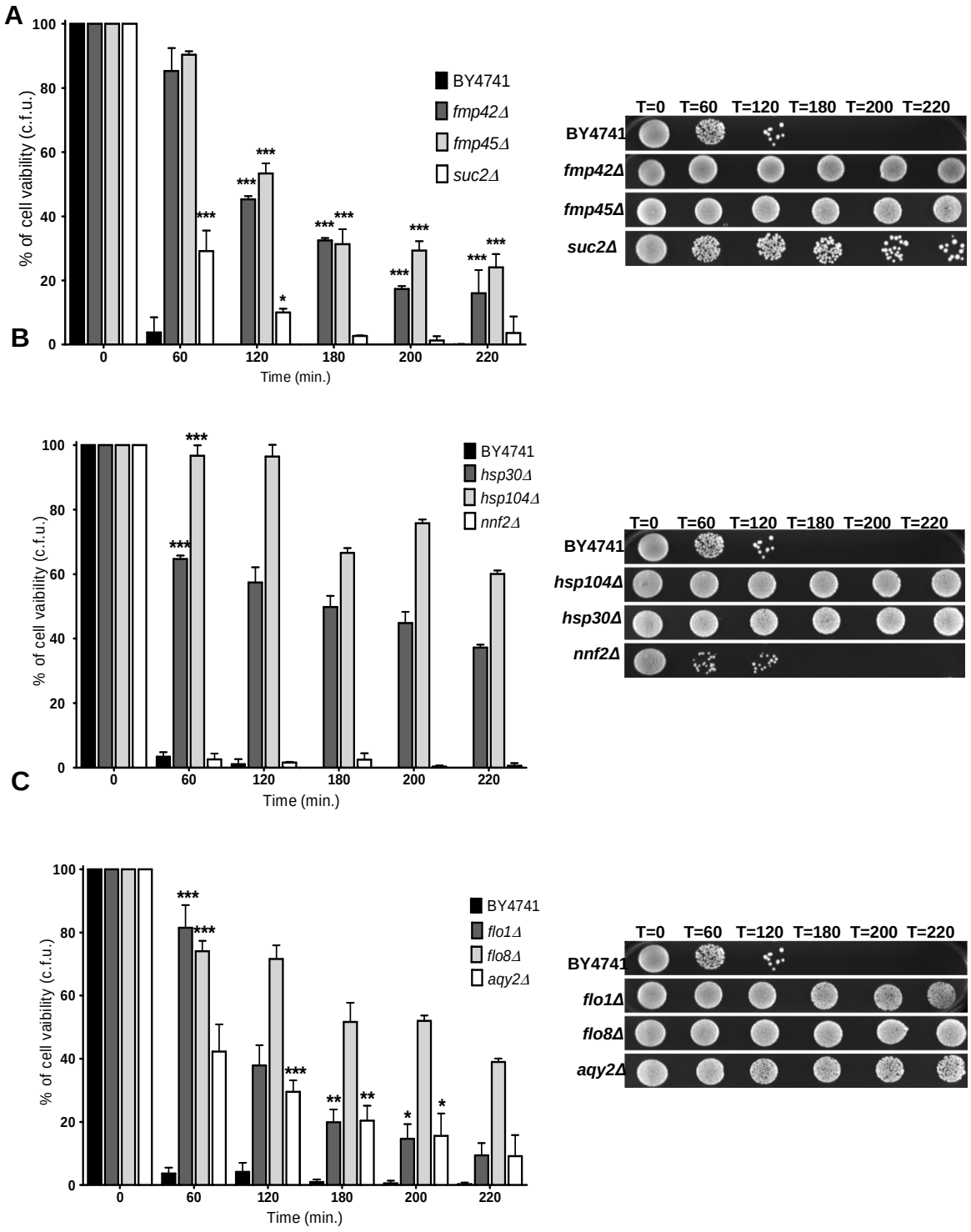
As seen in Figure 25A, the percentage of viable cells of *fmp42*Δ and *fmp45*Δ mutants was significantly higher that that of the wild type (wt) cells (0%), with 24.1±2.91% of *fmp45*Δ and 16±5.1% of *fmp42*Δ cells remaining viable after 220 minutes of exposure to the acid. These results indicate that deletion of these genes seems to be beneficial during acetic acid-induced cell death. On the other hand, *suc2Δ* shows only slightly higher resistance when compared to wt cells, presenting 3.6±3.6% viable cells at 220 minutes. The differences between the parental and deletion strains treated with acetic acid are statistically significant for *fmp42*Δ and *fmp45*Δ from 60 min to 220 minutes (***P<0.001), and for *suc2*Δ at 60 minutes (***P<0.001) and 120 minutes (*P<0.05).

The *nnf2*Δ mutant strain behaved similarly to wild type, since no significant change in sensitivity to acetic acid-induced cell death was observed. On the contrary, the *hsp30*Δ and *hsp104*Δ mutants exhibited a phenotype of resistance with a percentage of cell viability of 37.2±0.62% and 60±0.73%, respectively figure 25B). The differences between the parental and deletion strains treated with acetic acid are statistically significant for *hsp30*Δ and *hsp104*Δ from 60 minutes to 220 (***P<0.001).

The *flo1*Δ, *flo8*Δ and *aqy2*Δ deletion strains were also more resistant to acetic acid (figure 25C). However, *flo8*Δ was considerable more resistant than the other mutants, with 9.5±4.1% of *flo1*Δ, 39±0.8% of *flo8*Δ and 9.2±4.7% of *aqy2*Δ cells viable after 220 minutes. The differences between the parental and deletion strains treated with acetic acid are statistically significant for *flo1*Δ and *aqy2*Δ at 60 (***P<0.001), 120 (***P<0.001), 180 (**P<0.01), and 200 (*P<0.05) minutes, and for *flo8*Δ from 60 minutes to 220 minutes (***P<0.001). So, deficiency in these three mutants seems to be beneficial during acetic acid-induced cell death, but Flo8p has a more relevant role in the mediation of cell death.

The results obtained with ymr173-a, yJR115w and ycr006c strains showed that cell viability was higher than that of the wild type strain (Figure 25D). The difference between the parental and deletion strains treated with acetic acid are

statistically significant for ymr173w-a at 60 minutes (**P<0.01), for yJR115w at 60 minutes (***P<0.001), and for ycr006c from 60 to 220 minutes (***P<0.001).



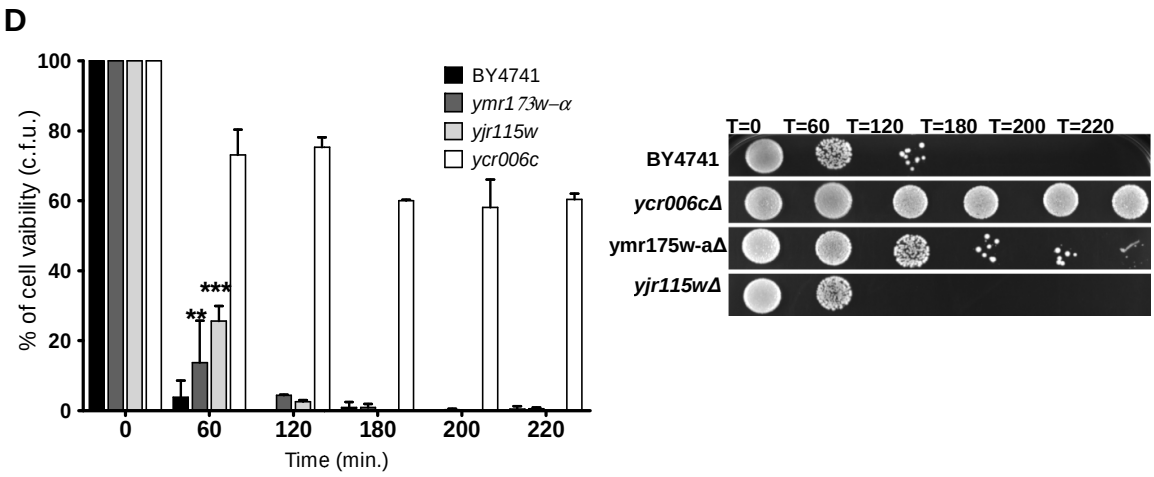


Figure 25 - Relative cell survival of wild, *fmp45Δ, fmp42Δ, suc2Δ, flo1Δ, flo842Δ, aqy2Δ, hsp104Δ, hsp30Δ, nnf2Δ,* ymr173-a*Δ, yjr115wΔ and ycr006cΔ* strains after exposure to 120 mM acetic acid. Cells were grown at 30 °C and samples were taken after 0, 60, 120, 180, 200 and 220 minutes. (A) Serial dilutions (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) were spotted onto YPD plates. (B) Cell viability was determined by C.F.U. counts. Values represent means and standard deviations of 3 independent experiments. Statistical analysis was performed using a two-way ANOVA test. The difference between the parental and deletion strain, statistically significant: ***P<0.001, **P<0.01 and *P<0.05.

In summary, the results from the assays where the strains were tested individually confirmed the results previously obtained in the screen, and allowed relative quantitation of the resistance of individual strains.

4.2.3.2. Cell death markers

After assessing the cell viability of the deletion strains exposed to acetic acid, we analyzed the appearance of several cell death markers. The first marker to be analyzed was the integrity of plasma membrane by PI staining, and the fluorescence was evaluated by flow cytometry. The results were in agreement with the resistance phenotypes, and the mutants most resistant to acetic acid had the lowest percentage of PI-positive cells (figure 26)

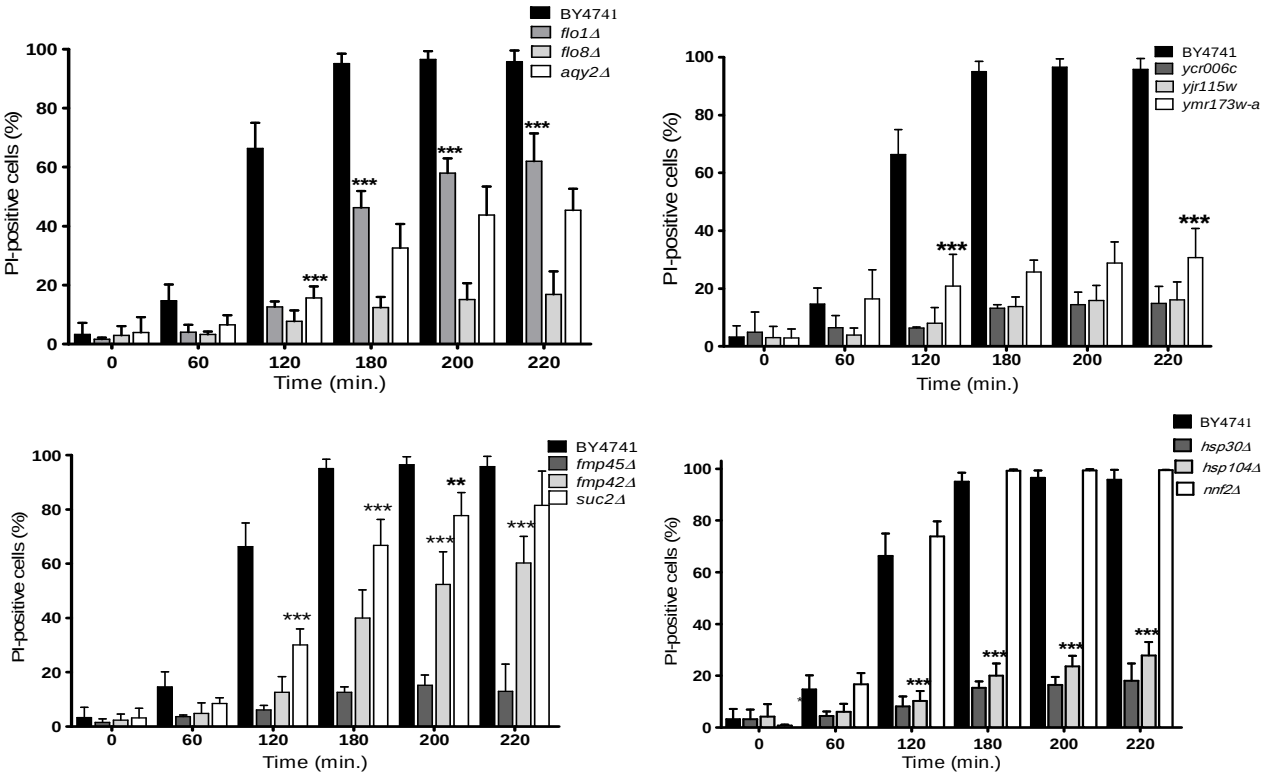


Figure 26 - Membrane integrity loss in wt and *fmp42Δ, fmp45Δ, aqy2Δ , flo1Δ, flo8Δ, nnf2Δ, hsp30Δ, hsp104Δ, suc2Δ ycr006cΔ, yjr115wΔ* and *ymr173w-aΔ* strains incubated with acetic acid. Samples were collected after 0, 120, 180, 240 and 300 minutes at 30°C, stained with PI (1µg/mL), and the fluorescence measured by flow cytometry. Percentages of cells stained with PI are shown. Values represent means and standard deviations of 3 independent experiments. Statistical analysis was performed using a two-way ANOVA test. The difference between the parental and deletion strain, statistically significant: ***P<0.001, **P<0.01 and *P<0.05.

To determine the levels of ROS in mutant cells treated with acetic acid we labeled cells with DHE. The ROS levels were assessed by flow cytometry and results expressed as a percentage of ROS-positive cells. The strains were incubated with 120 mM of acetic acid, for 220 minutes, and stained with DHE (Figure 27).

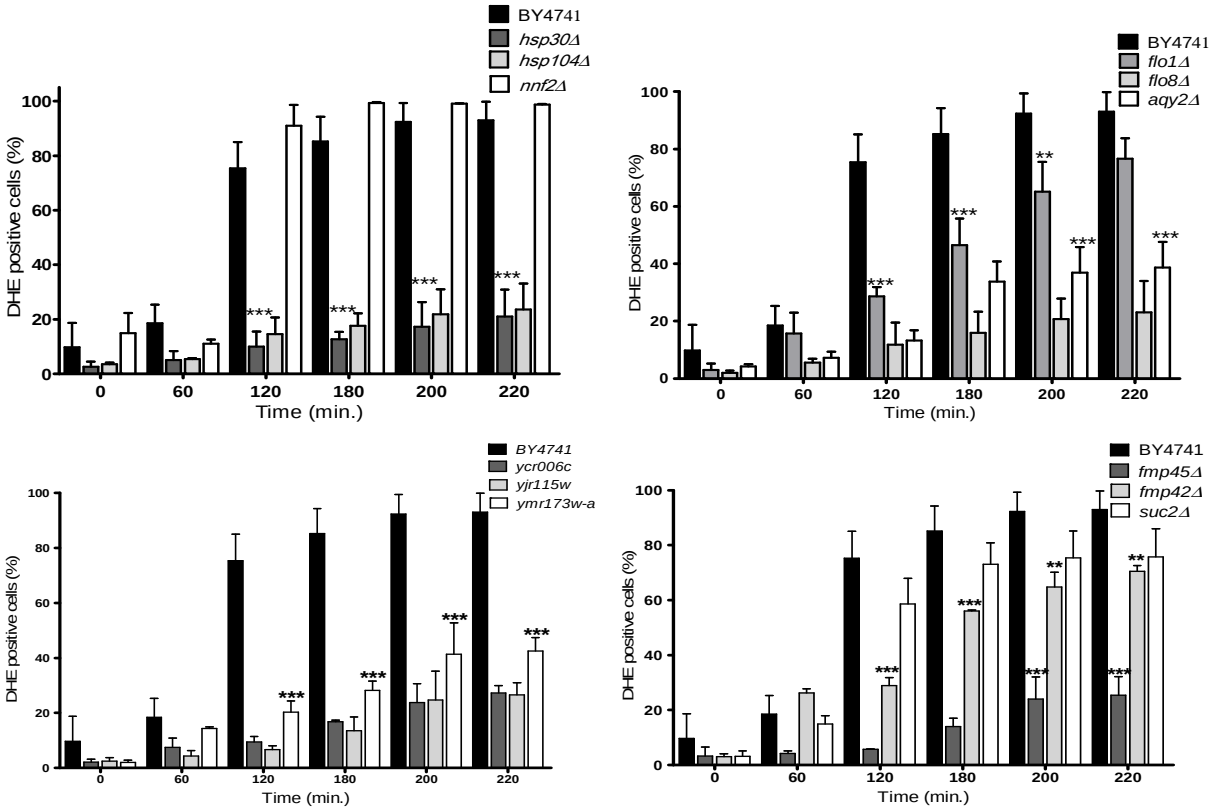
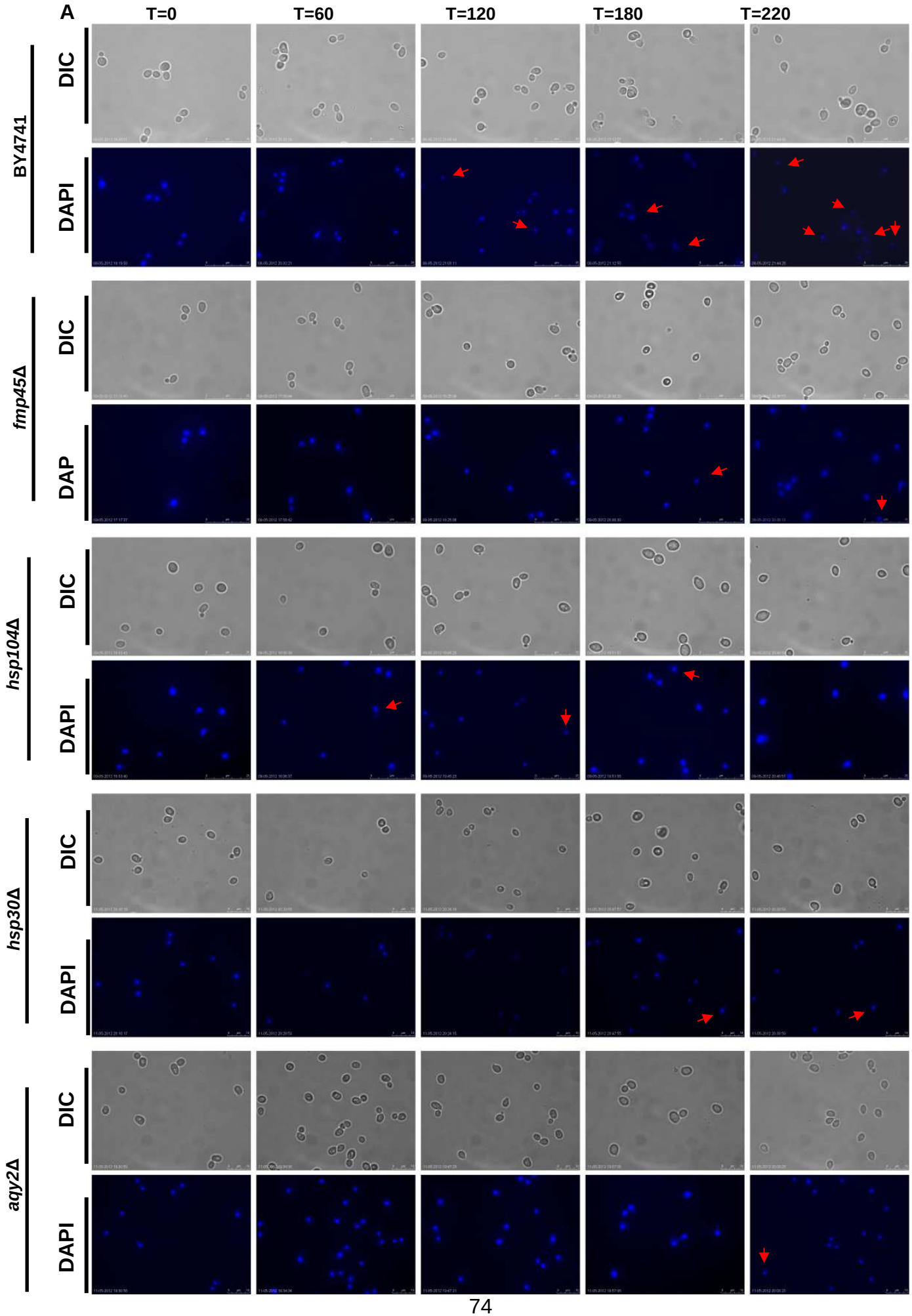


Figure 27 - Superoxide anion accumulation loss in wt and *fmp42Δ, fmp45Δ, aqy2Δ , flo1Δ, flo8Δ, nnf2Δ, hsp30Δ, hsp104Δ, suc2Δ ycr006cΔ, yjr115wΔ* and *ymr173w-aΔ* strains incubated with acetic acid. Samples were collected after 0, 120, 180, 240 and 300 minutes at 30°C, stained with DHE (1µg/mL), and the fluorescence measured by flow cytometry. Percentages of cells stained with DHE are shown. Values represent means and standard deviations of 3 independent experiments. Statistical analysis was performed using a two-way ANOVA test. The difference between the parental and deletion strain, statistically significant: ***P<0.001, **P<0.01 and *P<0.05.

The percentage of wild-type ROS-positive cells after acetic acid treatment was 93±4.8% after 220 min of treatment. The *suc2Δ, flo1Δ* and *nnf2Δ* mutants also showed higher levels of mitochondrial ROS after 220 min of treatment, about 75.8±7.2%, 76.6±5% and 98.8±0.1% respectively. On the other hand, the other mutants had a lower percentage of cells with ROS, after 220 min of treatment. The results were in agreement with their resistance phenotypes, and the mutants most resistant to acetic acid had the lowest levels of ROS.

Additionally, we quantified the appearance of nuclei with chromatin condensation during acetic acid treatment, one of the hallmarks of apoptotic cell death. Nuclear DNA was stained with DAPI, observed by fluorescence microscopy (Figure 28A), and results quantified (Figure 28B). Nuclei with normal chromatin are round and regular shaped, whereas nuclei with chromatin condensation can be visualized in the form of semicircles.


A
T=0
T=60
T=120
T=180
T=220
BY4741
DIC
DAPI
fmp45Δ
DIC
DAP
hsp104Δ
DIC
DAPI
hsp30Δ
DIC
DAPI
aqy2Δ
DIC
DAPI

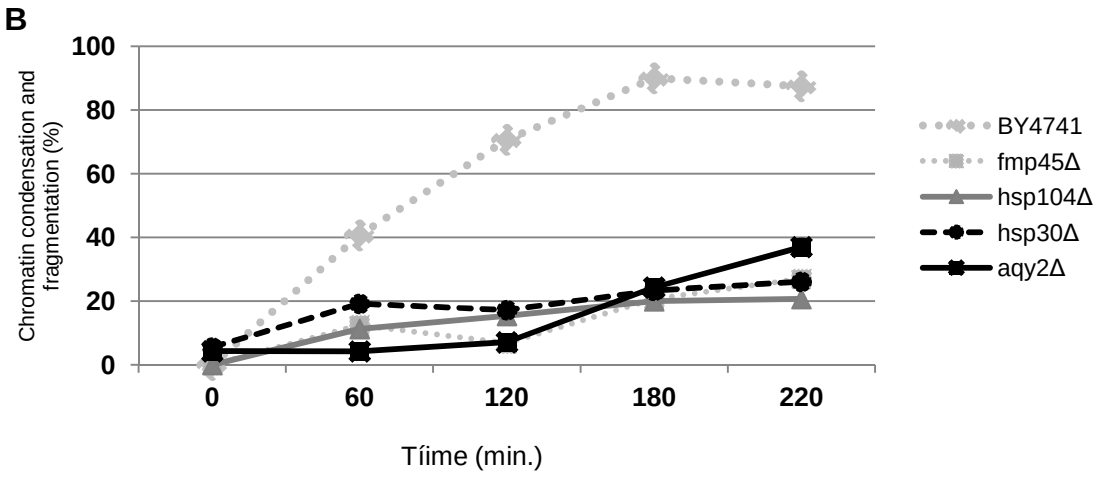


Figure 28 – The effect of acetic acid on chromatin condensation and fragmentation. (A) Samples were collected after 0, 60, 180 and 220 minutes, stained with DAPI (2 µg/mL) and samples were observed by fluorescence microscopy. (B) Quantification of apoptotic nuclei from cells treated with 120 mM acetic acid.

As expected, we observed an increase in the number of wild-type cells exhibiting chromatin condensation, which can be visualized by the fluorescent semicircles formed by chromatin fragments. In the mutants tested, few apoptotic nuclei showing chromatin condensation were observed; instead they show single round fluorescent circles. After 220 minutes of treatment with acetic acid, 27%, 20.7%, 26.1% and 37% of the cells had abnormal nuclei and chromatin condensation in the mutants *fmp45*Δ, *hsp104*Δ, *hsp30*Δ and *aqy2*Δ respectively. 87.5% of wild-type cells exhibited an apoptotic nucleus after this time.

4.2.3.3. Alignment Mycp with Sfl1p

The results presented above show the involvement of Sfl1p in the regulation of acetic acid-induced cell death. From a structural point of view, Slf1p is a protein of 766 amino acids with 3 domains characteristic of the c-myc oncoprotein (similarity of 43% between the domains of these two proteins). In Figure 30, we show the amino acid alignment of Sfl1p and Myc. Some authors have described that c-myc oncoprotein has an important role in apoptosis, since

it activates the protein Bax, which induces the release of cyt *c* into the cytosol. The conserved c-Myc domains are responsible for its function as a transcription factor, but have also been shown to be required for its ability to induce apoptosis. Our results showing that Sfl1p is also involved in the regulation of a mitochondrial-dependent apoptotic process suggest that these domains can have a conserved function not only on transcription regulation but also in apoptosis regulation across kingdoms.

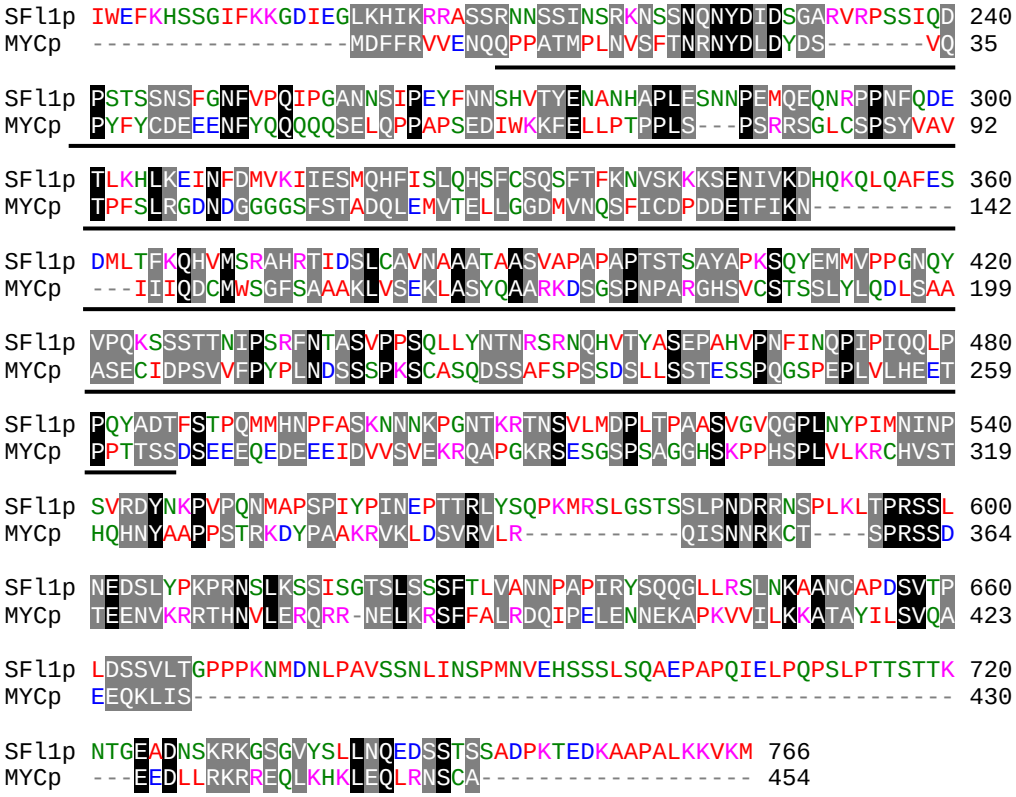


Figure 29 - Alignment of SFL1p amino acid sequences from *S. cerevisiae* with MYCp amino acid sequences from *H. sapiens*.

4.3. Part III

The role of Rlm1p in acetic acid-induced apoptosis

As mentioned previously, the cell wall of *S. cerevisiae* is an external envelope that protects it against environmental conditions. The adaptive response of yeast to cell wall stress is mainly mediated by the CWI pathway. The final consequence of the activation of the CWI pathway by cell wall stress is the induction of an adaptive transcriptional response (Lagorce *et al.,* 2003; García *et al.,* 2004; García *et al.,* 2009, Jung and Levin, 1999). The transcriptional program triggered by cell wall stress is coordinated by Slt2/Mpk1, and is mostly mediated by the transcription factor Rlm1p. Previous results in the lab had shown that under non-repression conditions (galactose grown cells) deletion of *slt2Δ* or of *rlm1Δ* induced resistance to acetic acid (Flávio Azevedo, unpublished results). However, the involvement of downstream components of this signaling pathway in acetic acid-induced apoptosis is not known.

With the aid of bioinformatics tools, in particular with data available in the database YEASTRACT (http://www.yeastract.com/), we could identify 197 genes regulated by *Rlm1p*, of which 29 are essential. To identify genes regulated by *Rlm1p* required for resistance to acetic acid induced-cell death, we screen the strains mutated in all the non-essential genes under Rlm1p control from the EUROSCARF haploid mutant deletion collection (EUROSCARF; http://www.uni-frankfurt.de/fb15/mikro/euroscarf/).

4.3.1. Optimization of screening conditions

First, we optimized treatment parameters in order to obtain the best experimental conditions to detect of mutant strains with higher resistance or sensitivity to acetic acid-induced cell death in galactose medium, when compared with the wild type strain. Several parameters, such as acetic acid concentration (from 200 mM - 300 mM), cell concentration and treatment time were tested. Six control strains (*hog1Δ, rlm1Δ, slt2Δ, swi4Δ, swi6Δ* and wild type) were distributed in triplicate by the plates, as a control, as these strains have different susceptibility to acetic acid in galactose-containing media (Flávio Azevedo, unpublished results). Treatment conditions chosen were exposure of cultures to 250 mM acetic acid, at pH3, for 400 minutes. The stringent assays

reported in this study increased the feasibility of exploring yet unknown pathways of cell death in yeast.

An array of the 168 strains was then patched as 96-dot arrays onto the surface rich solid medium and grown at 30°C for 2 days. Using a 96-pin replicator, strains were transferred into 96-well plates with synthetic complete liquid medium (SC-Gal) and grown for an additional 24 hours at 30 °C, without agitation. Cultures were diluted 100 fold using a multichannel pipette into SC-Gal medium at pH3 containing 250 mM acetic acid, for 400 minutes. At different times of incubation (100, 200, 300 and 400 min.) cells were replicated into 96-well plates containing YPD medium, and the plates were incubated at 30°C for 2 days. Mutants whose growth was reduced compared to the wild-type strain and mutants that still grew at a time point where the control strain did not grow were considered sensitive and resistant, respectively. To further validate our tests, we determined the viability of 50 mutant strains individually and compared the phenotype with the screening results (46 coincident) (table 2).

Table 2 - Genes that are described as regulated by Rlm1p, phenotype in genome-wide screen and viability individually.

Genes	Screening	Individual	Genes	Screening	Individual
AFR1	+		***PRY2***	+	+
AGE1	+		***PST1***	+	
AGP3	+		***PTP2***	+	
ALD3	no phenotype		***PUN1***	-	
AMN1	+		***PXL1***	+	
API2	+		***RAD30***	+	
APQ12	no phenotype		***RAD55***	+	
ARI1	+	+	***RBA50***	not viability	
ASE1	+		***RCK1***	+	
ASK10	no phenotype		***RIM21***	+	
ATG8	+		***RMD1***	-	no phenotype
BER1	no phenotype		***RMD6***	+	
BGL2	+	+	***RML2***	-	
BSC1	+		***RNH203***	+	+
BUD22	+	+	***RPA34***	+	
CCW12	+	+	***RPC10***	not viability	
CCW14	+		***RPI1***	+	
CHS1	no phenotype		***RPL9b***	+	+
CHS3	-	no phenotype	***RTS3***	+	
CHS5	no phenotype		***SBP1***	+	+
CIS3	no phenotype		***SEC59***	not viability	
CLB4	+		***SED1***	no phenotype	
COS9	not viability		***SFG1***	+	
COY1	+		***SGE1***	+	
CRG1	no phenotype	no phenotype	***SIM1***	+	
CRH1	+	+	***SLM5***	+	
CRM1	not viability		***SLT2***	+	
CSN9	no phenotype	no phenotype	***SMF1***	-	
CTT1	-		***SNF11***	+	+
CWP1	-		***SOR1***	not viability	
CWP2	-		***SOR2***	not viability	
DDR48	no phenotype	no phenotype	***SPO77***	no phenotype	
DFG5	+	+	***SPS100***	no phenotype	
DSD1	-		***SPS2***	+	+
DSE2	no phenotype		***SRL3***	+	+
ECM19	+	no phenotype	***SUN4***	+	
ECM38	+		***SUR1***	no phenotype	
EGD2	no phenotype		***SUS1***	-	
EXG1	+		***TAT2***	+	+
EXG2	+	+	***TPO2***	-	
FAA4	+	+	***TSL1***	-	
FBP26	+		***TUB3***	-	
FIT2	+		***UTR2***	+	
FKS1	-		***VPS74***	-	
FLC1	+		***WSC4***	no phenotype	
FLC2	+	+	***YAR053w***	not viability	
FLO10	+	+	***YBR071w***	+	
FMP33	no phenotype	no phenotype	***YCL049C***	+	
FRE6	no phenotype		***YCL065w***	not viability	

FRQ1	not viability		***YCR018c-a***	not viability	
GDS1	+		***YDR042C***	-	
GFA1	not viability		***YDR210c-d***	not viability	
GIC2	+		***YDR417C***	+	
GVP36	-		***YEF1***	+	+
HAL1	no phenotype		***YEL074w***	not viability	
HOG1	-		***YER001W***	+	
HOR7	-		***YER138c***	not viability	
HSP12	+	+	***YGL159W***	+	+
HSP150	-		***YGL260W***	no phenotype	
HXT15	not viability		***YGP1***	+	+
HXT16	not viability		***YGR149W***	+	
ICS2	+	+	***YHL041W***	no phenotype	+
IME2	-		***YHL042W***	+	
INO1	+		***YHR033W***	+	
IPT1	+		***YHR097C***	no phenotype	no phenotype
IRC22	+		***YIL108W***	+	
KAR2	not viability		***YJL105w***	+	
KDX1	+	+	***YJL107C***	no phenotype	
KTR2	+		***YJL160c***	+	+
LHS1	+	+	***YJL171C***	+	+
LYS9	+		***YJR157w***	not viability	
MAK32	+		***YKE4***	+	+
MCH5	-		***YKR104W***	+	
MDM31	+	+	***YLR040c***	+	+
MGA1	+		***YLR111W***	no phenotype	
MID2	no phenotype		***YLR194C***	+	
MLP1	+	+	***YLR463c***	not viability	
MPH2	not viability		***YLR465c***	not viability	
MSB4	+	+	***YMR103C***	+	
MSC1	+	+	***YMR295C***	+	
MSC6	no phenotype		***YMR315w***	+	+
MTF2	-		***YNL010W***	no phenotype	
MUM2	no phenotype		***YNL058C***	+	
NAG1	not viability		***YNL208W***	+	
NFT1	+	+	***YOL159C***	+	
NFU1	+		***YOL160W***	+	
NNF2	-		***YOR314W***	no phenotype	
PAM16	not viability		***YPL088W***	+	+
PAU20	not viability		***YPR195C***	no phenotype	
PCL1	no phenotype		***YPS1***	no phenotype	no phenotype
PCM1	not viability		***YPS3***	no phenotype	
PGK1	not viability		***YPS5***	+	
PIR1	no phenotype		***YPS6***	+	+
PIR3	-		***YRF1-4***	not viability	
POG1	no phenotype	no phenotype	***YRF1-5***	not viability	
PPR1	no phenotype	no phenotype	***YSN1***	no phenotype	
PRM10	no phenotype		***YSP3***	-	
PRM5	+	+	***YSR3***	-	
PRY1	+				

4.3.2. Functional categories significantly enriched in the data set of resistant strains

Of the 168 mutants tested, 102 were resistant to acetic acid-induced cell death. The functions of these genes were determined using the MIPS database (http://mips.helmholtz-muenchen.de/proj/funcatDB/), and are shown in figure 31. One functional class was considered enriched over random whenever the attributed p-value was below 0.01. The description of gene function was complemented using the information available at SGD (http://www.yeastgenome.org).

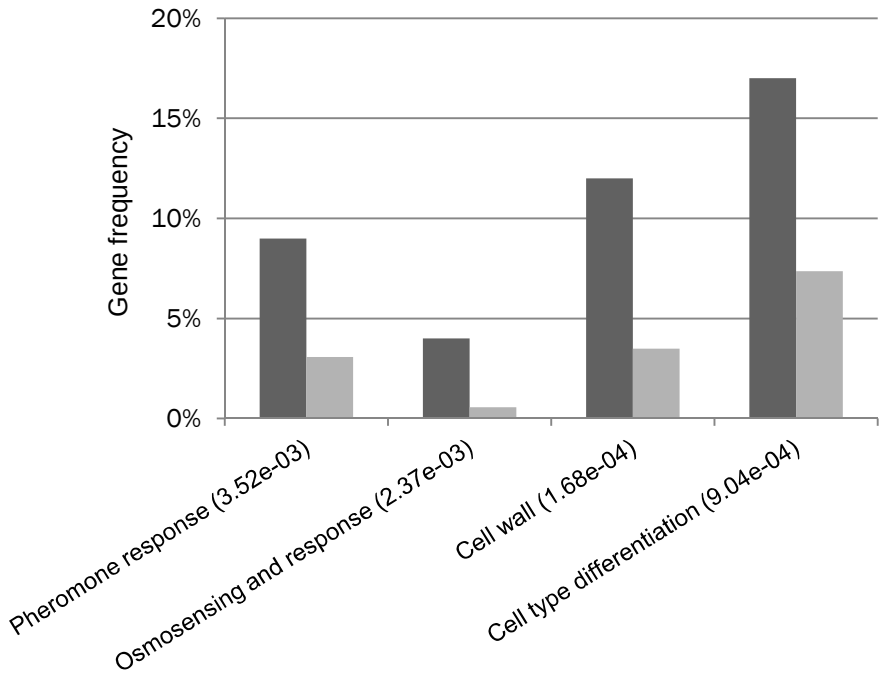


Figure 30 - Functional categories significantly enriched in gene whose deletion renders cells resistant to acetic acid-induced cell death. The frequency in our dataset (dark grey) is compared with the frequency in the whole yeast genome (light grey).

In the data set of resistant strains, the functional categories most significantly enriched are: "Cell type differentiation", "Cell wall", "Pheromone response" and "Osmosensing and response". The “Cell type differentiation” class in basically composed of genes encoding proteins involved in filamentous growth (*DFG5*), hyphae formation (*SFG1*) and sporulation (*SPS2*).

"Osmosensing and response" is composed of genes involved in temperature perception and response (*HSP12*) and osmolarity (*PTP2*). Another important group consists in genes involved in pheromone-regulated proteins like Prm5, Afr1 and Kdx1.

Protein Bgl2, Exg1, Exg2, Flc1, Flc2, Rim21 and Sun4 are involved in the formation of the cell wall. Mutation of their respective genes conferred resistance to acetic acid-induced cell death, confirming that the cell wall is important in the response to this acid and that cell wall remodeling plays a decisive role in the induction of apoptosis.

4.3.3. Functional categories significantly enriched in the data set of sensitive strains

The analysis of the genes whose deletion caused sensibility to acetic acid-induced cell death revealed 27 sensitive strains. The functional categories of these genes also were determined using the MIPS database and are shown in figure 32. A functional class was considered enriched over random whenever the attributed p-value was below 0.01.

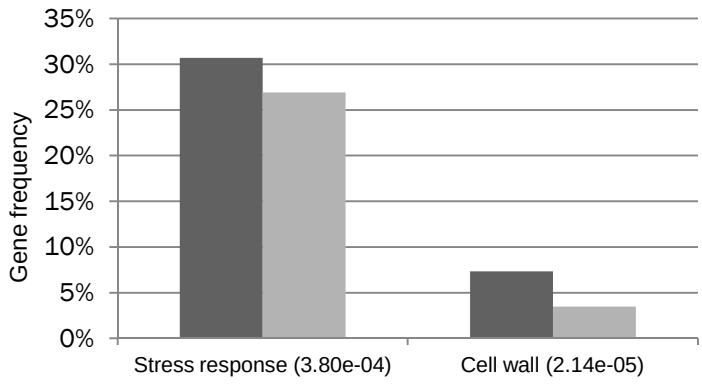


Figure 31 - Functional categories significantly enriched in gene whose deletion renders cells sensitive to acetic acid-induced cell death. The frequency in our dataset (dark grey) is compared with the frequency in the whole yeast genome (light grey).

In the data set of sensitive strains, the functional categories most significantly enriched are: "Stress response" and "Cell wall". The "Stress response" class is composed of genes that code for proteins involved in oxidative stress response (*CTT1* has a role in protection from oxidative damage), osmotic and salt stress response (*HOG1*) and detoxification (*TPO2*). The “Cell wall” class is basically composed of genes encoding proteins involved in the stability of the cell wall, like *PIR3, HSP150, CWP2, FKS1* and *PUN1*. Therefore, proteins regulated by *Rlm1p* that ensure the stability of the cell wall protect cells from acetic acid-induced cell death. Acetic acid can enter the cells by diffusion. One of the mechanisms proposed to reduce the diffusion rate of anions is the reinforcement of the cell wall structure to decrease its porosity. Cell integrity depends on the cell wall, which protects cells against extreme environmental conditions. Stress conditions can alter the cell wall, leading to the activation of a cellular response that allows cells to adapt and survive. The results from our screen seem to indicate that it is the stabilization of the cell wall and not its remodeling that is important for the cell´s ability to cope with acetic acid stress.

5. Discussion

One of the aims of the present work was the identification of genes involved in the acetic acid-induced programmed cell death through a genome-wide analysis. To this end, we utilized the EUROSCARF knock-out mutant collection in BY4741 background. This screening uncovered a set of genes involved in resistant and sensitive phenotypes that were clustered according to their biological function and known physical and genetic interactions using as support STRING and MIPS. The frequency of each functional class in our dataset was compared with the frequency in the whole genome also using the MIPS functional catalogue. Approximately, 2159 strains were found to be resistant and 391 strains were found to be sensitive. The genes whose deletion increased resistant to acetic acid-induced cell death belong to the functional categories, transcriptional control, stress response, phosphate metabolism, cell type differentiation, amino acid metabolism, cell wall, meiosis, protein kinase and detoxification. The stress response and transcriptional control, groups were the functional category most significantly enriched among the resistant strains. The stress response group included genes involved in the oxidative stress response, osmotic and salt, pH, heat shock, cold shock, DNA damage, nutrient starvation and UV. Proteins involved in regulation of transcription that when mutated confer resistance to acetic acid (*Rpd3, hos3, Hos1 Hta1, Nhp6b, Sas5, Spt2* and *Ecm11*) were involved in acetylation, histones complexes and nucleosomes. Thus, chromatin modifiers may function as co-repressors by effecting a more closed chromatin conformation and possibly inactivate genes whose products are important for apoptosis.

The functional categories most significantly enriched in the deletions strains that displayed increased sensitivity to acetic acid-induced cell death were: protein fate, biogenesis of cellular components, transcriptional control, c-compound and carbohydrate metabolism, respiration, ribosomal proteins, ion transport and homeostasis of cations. From this data set of genes, those related to proton homeostasis and ion transport include a number of genes involved in the assembly and/or regulation of the activity of plasma membrane H-ATPase (PM-H-ATPase), of vacuolar H-ATPase (V-ATPase) and of mitochondrial F1F0 ATP synthase. Changes in cytoplasmic H^+ concentration are considered an important signal for the control of cell function. Intracellular pH not only governs

the actual activity of enzymes but also affects the expression of signaling proteins (Capuano and Capasso, 2003; Lehen'kyi, 2011). Therefore, intracellular pH controls essential steps in the cell cycle and is involved in the decision to undergo either proliferation or apoptosis.

Mutants, whose deleted genes encode proteins involved in ion import, were found to be susceptible to acetic acid, suggesting that the uptake of these ions plays a crucial role in yeast response to acetic acid stress. A study, showed an increase in potassium uptake, in response to acetic acid stress, being expected to compensate the stimulation in the activity of extrusion of H^+ occurring in these cells, and thus keeping the electrical balance across the plasma membrane (Mira *et al.,* 2010). A similar adaptive response is proposed to occur, in the presence of other ions. The levels of the other ions as Na^+ and Ca^{2+} represent central determinants in signaling events leading to cell death. Thus the ion fluxes mediated by ion channels are extremely important mechanisms of apoptosis regulation and discovery that the expression of ion channels is not limited solely to the plasma membrane, but also include membranes of internal compartments.

Many of the mitochondrial proteins found in the screen are involved in respiration and some play a role in the electron transport chain such as Aac3, Atp2, Coq9, Cox16, Cox18, Cyt1, Oar1, Por1 and Rip1. Proteins like Cox11, Cox16, Cox17, Cox20 and Sco1 are essential for the assembly of the multi subunit enzyme cyt *c* oxidase, these deletions of these genes triggered sensitivity to acetic acid. These results showed the importance of inducing apoptosis when the respiratory chain is deregulated and high production of ROS may occur.

In this work we also aimed to study the role of Sfl1p in acetic acid-induced apoptosis, since it had been described that deletion of *SFL1* confers a resistance phenotype to this cell death stimulus. Sfl1p is a transcription factor involved in repression of flocculation-related genes, and activation of stress responsive genes, and it was described that Sfl1p is negatively regulated by *TPK2* isoform of protein kinase A (PKA) (Conlan *et al.,* 2001). So cell viability of deletion mutants in the three PKA isoforms *TPK1, TPK2* and *TPK3* were

assessed after exposure to acetic acid at 120 mM. From these three mutants, the strains *tpk1Δ and tpk2Δ* showed a phenotype similar to the parental strain. The only mutant that presented a phenotype of resistance was the strain *tpk3Δ*, this characteristic can be explained by the reduction of the respiratory functions and consequently lower production of ROS (Gourlay and Ayscongh, 2006), that was confirmed by assessment of accumulation of superoxide anion. It is known that Sfl1p leads to a decrease of pseudohyphal and invasive growth, and Tpk3p also inhibits the pseudohyphal development (Robertson and Fink, 1998). Since the deletion of *TPK3* and *SFL1* genes presented higher resistance in the presence of acetic acid, the results suggest that the filamentous growth invasion pathway is important in induction of acetic acid-induced apoptosis. We also studied apoptotic markers in these three isoforms, and as expected the results agreed with the viabilities. It should be also noted that *tpk2Δ* mutant strain also displaying rapid loss of CFUs did not present a high production of ROS and the membrane integrity was not totally lost. Thus, these data suggested that this strain still presented a programmed death.

To further assess the role of Sfl1p in these conditions, the viability of strains deleted in genes reported as being under Sfl1p regulation was tested. We studied a group of genes reported to be repressed (*aqy2Δ, flo1Δ, flo8Δ, fmp45Δ, suc2Δ, yjr115wΔ* and *ymr173-aΔ*) and activated (*hsp30Δ* and *ycr006cΔ*) by Sfl1p under certain stress conditions, not including the acetic acid. Other genes (*fmp42Δ, hsp104Δ* and *nnf2Δ*) also potentially regulated by Sfl1p, but for which repression or activation regulation is unknown, were also studied. The viability of these mutant strains was assessed after exposure to acetic acid (120 mM, pH3.0). Of these strains only *nnf2Δ* did not show a resistant phenotype compared to the parental strain. This phenotype could be explained by the interaction of *NNF2* with RNA polymerase, which could lead to the inhibition of transcription. Through our genome-wide screening we also evaluated the phenotype of the deletion mutants in the remaining genes documented as being regulated by SFL1p. From these, deletion of only two genes showed an increased sensitivity to acetic acid (nnf2Δ and taf14Δ), while the others presented an increased resistance when submitted to acetic acid–induced cell death. Taf14p complex is involved in RNA polymerase II

transcription initiation and in chromatin modification. These mutants are viable, but grow slowly on rich media and displays decreased transcription, defects in actin organization, increased osmosensitivity, heat sensitivity and sensitivity to caffeine, hydroxyurea, UV, and methyl methanesulfonate. This could explain the sensitivity to acetic acid. The same happens with the mutant nnf2Δ, protein that exhibits physical and genetic interactions with Rpb8p, which is a subunit of RNA polymerases I, II, and III. These results evidence the need for an active transcription in the response to acetic acid stress.

Overall, the results indicate that Sfl1p, during acetic acid induced cell death, acts as an activator of these genes, whose deletion gives a resistant phenotype. So the phenotype of sfl1Δ strain can be explained by the coordinate decrease in gene expression of a group of genes that cooperate to promote acetic acid induced-apoptosis.

The stress transcription factors are interesting models, and their characterization can lead to the identification of new components of the stress signaling pathways in yeast. Little is known about the signal transduction pathways that induce apoptosis. Cells possess a network of signal transduction pathways that enable them to respond to different stimuli, which implies strong changes in gene expression. Sfl1p has great similarity in the amino acid sequence with the oncoprotein c-Mycp, a trancription factor also involved in apoptosis regulation. Some studies confirm that this oncoprotein is mutated in many human carcinomas, and when expressed induces apoptosis through the activation of BAX with the consequent release of cty *c* from mitochondria to cytosol.

After these data, that support a role for Sfl1p in acetic acid induced apotosis, it would be interesting to study also the release of cty *c* from mitochondria in the *sfl1Δ* mutant strain, to further evaluate the putative parallelism with the oncoprotein c-myc.

The remodelling of yeast cell wall structure in response to acetic acid is an essential response to reduce the diffusion rate of the undissociated weak acid forms into the cell interior. The cell wall of *S. cerevisiae* is an external envelope

that protects it against environmental conditions. The transcriptional program triggered by cell wall stress is coordinated mostly by transcription factor Rlm1p. Several genes regulated by *RLM1*, which play a role in cell wall, when mutated confer resistance or sensitivity to acetic acid, confirming that the cell wall plays a decisive role in the resistance to acetic acid. While deficiency in proteins Bgl2, Exg1, Exg2, Flc1, Flc2, Rim21 and Sun4, involved in the formation of the cell wall, confer resistance to acetic acid, deletion of one group of genes involved in the stability of the cell wall constituted by *PIR3, HSP150, CWP2, FKS1* and *PUN1*, induced a sensitivity phenotype to acetic acid-induced cell death. So we can say that genes activated by *RLM1* can ensure the stability of the membrane, allowing better response to acetic acid, while remodeling of the cell wall increases cell death.

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