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New method for monitoring the progression of cell death

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STATEMENT OF INTEGRITY

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

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

RESUMO

O equilíbrio entre a divisão celular e a morte celular é extremamente importante para o desenvolvimento e manutenção de organismos multicelulares. A proliferação celular descontrolada pode resultar no desenvolvimento de tumores, ao passo que um nível excessivo de morte celular pode resultar em doenças neurodegenerativas como Alzheimer e Parkinson.

No último século, as leveduras têm servido como ferramenta para a investigação, inclusive no estudo da morte celular. Além de ser o organismo eucariótico mais estudado, contribuiu para o conhecimento atual de diversos mecanismos celulares conservados entre a espécie humana e as leveduras. O ácido acético e o peróxido de hidrogénio estão entre os agentes mais comummente descritos como indutores de um fenótipo apoptótico em leveduras, associado à perda de viabilidade, sem perda da integridade da membrana plasmática. A integridade da membrana plasmática é normalmente avaliada durante a exposição das células a um estímulo e a viabilidade celular apenas alguns dias após o plaqueamento dessas células. Portanto, o número de células com integridade da membrana plasmática e as contagens de unidades formadores de colónias (CFUs) nem sempre se correlacionam num cenário apoptótico. Uma vez que a morte celular ocorre apenas após a quebra irreversível da membrana plasmática, são necessários novos métodos para correlacionar melhor essas características. Assim, o principal objetivo deste trabalho foi desenvolver um método simples, capaz de explorar em que ponto, após um estímulo apoptótico e plaqueamento de células, estas morrem, em consequência de rutura da membrana plasmática.

Após os tratamentos com ácido acético e peróxido de hidrogénio, as células foram lavadas e suspensas em meio fresco para mimetizar o plaqueamento, sendo depois avaliada a integridade da membrana plasmática por marcação com iodeto de propídio. Os nossos resultados mostraram que, após o estímulo ser removido, as células que ainda mantinham a integridade da membrana plasmática continuam o processo de morte celular e perdem a integridade da membrana plasmática em meio fresco e a capacidade de se dividir. Ambos os estímulos induziram a translocação de Nhp6Ap-GFP, caraterística de necrose.

Em conclusão, desenvolvemos com sucesso um método capaz de demonstrar o momento de perda da integridade celular por ácido acético e peróxido de hidrogénio em leveduras, contribuindo para uma melhor caracterização da morte celular em levedura e para estabelecer a ordem cronológica de diferentes eventos, ajudando a caracterizar o processo de necrose secundária após apoptose.

ABSTRACT

The balance between cell division and cell death is extremely important for the development and maintenance of multicellular organisms. Uncontrolled cellular proliferation can result in the development of cancer, whereas an excessive level of cell death can result in Alzheimer's and Parkinson's diseases.

Yeasts have served as a successful research tool for the last century, including in the cell death field. In addition to being the most extensively studied eukaryotic organism, it also contributed to the current knowledge of several cellular mechanisms conserved between the human species and yeast, like death execution machinery. Acetic acid and hydrogen peroxide are among the most common agents that have been reported to induce an apoptotic phenotype in yeast, associated with loss of viability but maintenance of plasma membrane integrity. This concept would at first seem implausible, but one should consider that plasma membrane integrity is assessed during the exposure of cells to a stimuli and cell viability assessed only days after plating those cells. Therefore, the number of cells with plasma membrane integrity and the CFU counts do not always correlate in an apoptotic scenario. Since cell death occurs only upon irreversible plasma membrane breakdown, novel methods to better correlate these hallmarks are needed. So, the main objective of this work was to develop a simple method able to explore at what point after an apoptotic stimuli and plating cells, do cells die as result of plasma membrane disruption.

After acetic acid and hydrogen peroxide treatments, cells were washed and suspended in fresh medium to mimic plating and plasma membrane integrity was analysed by PI staining. Our results showed that after the stimuli is removed, cells that still maintained plasma membrane integrity continue the cell death process and lose plasma membrane integrity in fresh medium and the ability to divide. The point-of-no-return for cells after exposure to hydrogen peroxide occurs much later than in acetic acid treatments, most likely due to a slower transition from apoptosis to secondary necrosis. Both stimuli induced Nhp6Ap-GFP translocation, a hallmark of necrosis.

In conclusion, we successfully developed a method capable of demonstrating the moment of cell integrity loss by acetic acid and hydrogen peroxide in yeast. These results contributed to a better characterization of yeast cell death induced by these stimuli and set some chronological times points for different events helping to better define the process of secondary necrosis after apoptosis.

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CHAPTER I – INTRODUCTION

I.1. Cell death

Balance between cell division and cell death is extremely important for the development and maintenance of multicellular organisms. Uncontrolled cellular proliferation can result in the development of diseases such as cancer, whereas an excessive level of cell death can result in Alzheimer and Parkinson diseases (D'Arcy, 2019). Under these circumstances, cell death serves as an important mechanism for maintain appropriate cell numbers and to eliminate unwanted, aged and potentially dangerous cells.

According to the Nomenclature Committee on Cell Death, cell death occurs only when the point-of-no-return is surpassed, followed by irreversible plasma membrane breakdown or complete cellular fragmentation irrespectively of the subroutine that has been activated (Galluzzi *et al.*, 2014). Other important key concepts, to distinguish from cell death, are cell viability, which reflects the ability of a cell to divide and thus to proliferate, and cell vitality, that reflects the physiological capabilities of a cell and thus its metabolic activity (Galluzzi *et al.*, 2014). Cell death is generally classified into two types: accidental cell death (ACD) and regulated cell death (RCD). ACD is the instantaneous and catastrophic demise of cells exposed to severe insults of physical, chemical, or mechanical nature (Hu *et al.*, 2021). RCD can occur in the absence of any exogenous environmental perturbation in physiological programs for development or tissue turnover (Fuchs & Steller, 2011) or originate from perturbations of the intracellular or extracellular microenvironment, when such perturbations are too intense or prolonged for adaptative responses to cope with stress and restore cellular homeostasis. Unlike to ACD, RCD can be pharmacologically or genetically modulated depending on the progression of the process and subclassified into different types (Galluzzi *et al.*, 2016).

I.1.1. Types of regulated cell death

As the cell death field progressed, novel signaling pathways that orchestrate RCD were characterized, resulting in multiple classifications and types of RCD. The most relevant include the intrinsic and extrinsic apoptosis, mitochondrial permeability transition driven necrosis, lysosome-dependent cell death, autophagy-dependent cell death, necroptosis, ferroptosis, pyroptosis, parthanatos, entosis, netotic cell death, immunogenic cell death, cellular senescence, and mitotic catastrophe (Figure 1).

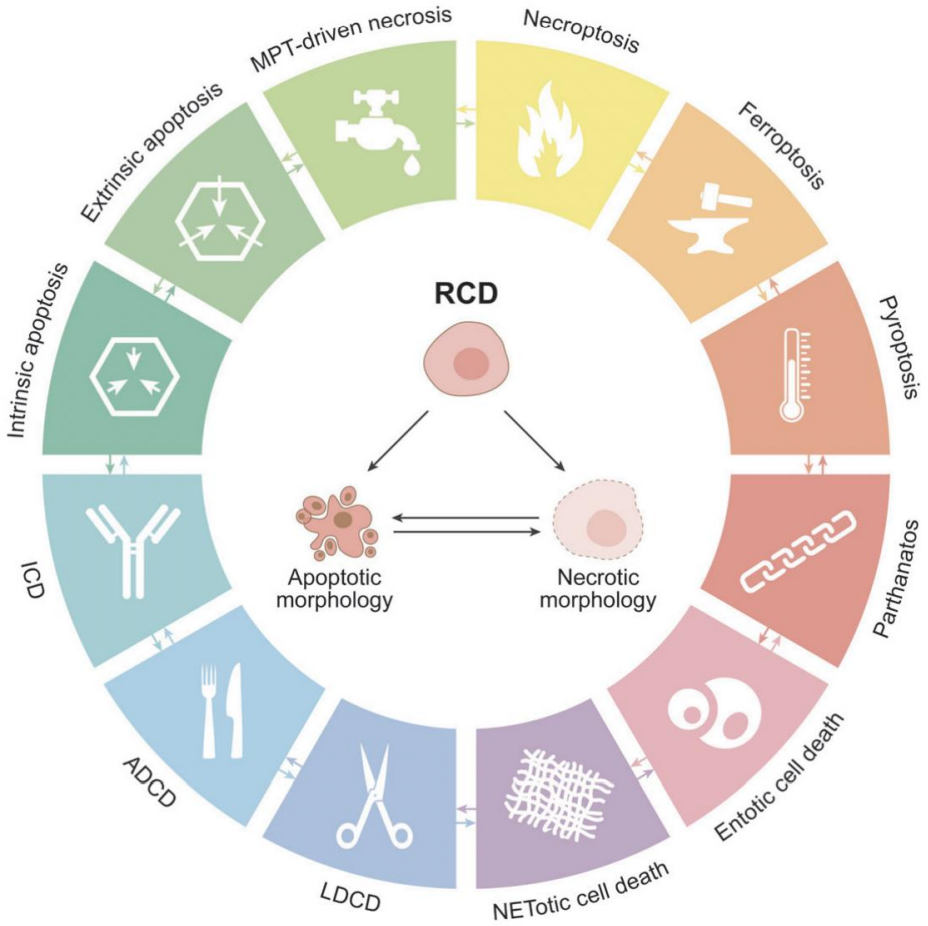

Figure 1. Major cell death types. Necroptosis is a form of RCD initiated by perturbations of the extracellular or intracellular microenvironment detected by specific death receptors (Vercammen *et al.*, 1997). Ferroptosis is initiated by severe lipid peroxidation, which relies on ROS generation and iron availability (Yang & Stockwell, 2016). Pyroptosis is triggered by perturbations of homeostasis related to innate immunity (Jorgensen & Miao, 2015). Parthanatos is driven by the hyperactivation of a specific component of the DNA damage response machinery (Fatokun *et al.*, 2014). Entosis is a form of cell cannibalism that occurs in healthy and malignant mammalian tissues, involving the engulfment of viable cells by non-phagocytic cells (Krishna & Overholtzer, 2016). Entosis is mainly triggered by the detachment of epithelial cells from the extracellular matrix and consequent loss of integrin signaling (Overholtzer *et al.*, 2007). The "netotic cell death" refers to a rather controversial type of RCD initially characterized in neutrophils (Remijsen *et al.*, 2011). Immunogenic cell death (ICD) is a functionally peculiar form of RCD that is sufficient to activate an adaptive immune response specific for endogenous or exogenous antigens expressed by dying cells (Galluzzi *et al.*, 2016). The "cellular senescence" refers to a pathophysiological process by which the cells permanently lose their proliferative capacity while remaining viable and metabolically active (Sharpless & Sherr, 2015). Mitotic catastrophe is a regulated oncosuppressive mechanism that impedes the proliferation and/or survival of cells that are unable to complete mitosis (Castedo *et al.*, 2004).

All the types of RCD can manifest with an entire spectrum of macroscopic morphological features ranging from necrotic to apoptotic. Intrinsic apoptosis is a form of RCD initiated by a variety of microenvironmental perturbations like DNA damage, reactive oxygen species (ROS) overload or replication stress (Brumatti *et al.*, 2010; Roos *et al.*, 2015). Apoptotic cells retain plasma

membrane integrity and metabolic activity as the process proceeds to completion, which allows for the rapid clearance by cells with phagocytic activity (Green *et al.*, 2016). The critical step for intrinsic apoptosis is the irreversible and widespread mitochondrial outer membrane permeabilization (Tait & Green, 2010), that directly promotes the cytosolic release of apoptogenic factors that normally reside in the mitochondrial intermembrane space like the apoptosis-inducing factor (AIF) and cytochrome *c* (Tait & Green, 2013). Extrinsic apoptosis is an RCD modality initiated by perturbations of the extracellular microenvironment (Ashkenazi & Dixit, 1998). Is mostly driven by either of two types of plasma membrane receptors: death receptors, whose activation depends on the binding of the cognate ligands, and dependence receptors, that trigger cell death when unbound but support cell survival after ligand binding (Aggarwal *et al.*, 2012; Gibert & Mehlen, 2015). Mitochondrial permeability transition (MPT)-driven necrosis is a form of RCD initiated by specific perturbations of the intracellular microenvironment such as severe oxidative stress and cytosolic Ca^{2+} overload, which generally manifests with a necrotic morphotype, which is characterized by disruption of the plasma membrane, uncontrolled leakage of deleterious intracellular components that damage the neighboring cells and subsequent inflammatory response. (Izzo *et al.*, 2016). Lysosome-dependent cell death (LDCD) is a subroutine of RCD initiated by perturbations of intracellular homeostasis and demarcated by the permeabilization of lysosomal membranes. Lysosome-dependent cell death is relevant for several pathophysiological conditions, including aging, neurodegeneration and cardiovascular disorders (Aits & Jäättelä, 2013). Autophagy-dependent cell death (ADCD) is a type of RCD that relies on the autophagic machinery or components thereof (Zhang & Baehrecke, 2015), most often operating at the hub of adaptation to stress, hence mediating cytoprotective effects (Galluzzi *et al.*, 2015). Other cell death types are described in figure 1.

I.2. Yeast cell death

Yeasts have served as a successful research tool for the last century, including in the cell death field. In addition to being the most extensively studied eukaryotic organism, it also contributed to the current knowledge of several cellular mechanisms conserved between the human species and yeast, such as cell division, DNA replication, metabolism and protein conformation, as well as intracellular transport (Fields & Johnston, 2005). Genetic manipulations are also facilitated by their

great efficiency, both in terms of transformation and homologous recombination (Goffeau *et al.*, 1996), which makes it relatively easy to insert, delete or mutate any genomic sequence at the chromosomal level (Sugiyama *et al.*, 2009). Yeast was the first eukaryotic organism to be completely sequenced in 1996 (Goffeau *et al.*, 1996), thus playing an important role in the subsequent sequencing of the human genome. The enormous amount and variety of information obtained through computational genomic approaches is well organized and constantly updated in public databases. Therefore, the combination of powerful genetic tools, as well as bioinformatics, and the accumulated knowledge about yeast, have led to the use of yeast to study various human pathologies related to cell death.

Yeast cell death was been associated with several scenarios involving physiological death circumstances (Carmona-Gutierrez *et al.*, 2010). Although in multicellular organisms there is a controlled suicide of single cells that is crucial for development and homeostasis, in yeast arises the question of why a unicellular organism have a suicide program in which the demise of a single cell can be viewed as the death of the whole organism. The explanation comes from the fact that yeast populations should not be interpreted just as a group of partitioned unicellular organisms that do not communicate among each other, but rather as a multicellular community of interacting individuals (Carmona-Gutierrez *et al.*, 2010). Thus, under certain circumstances, the death of unfit or damaged yeast cells promotes the survival of the population with the biological goal of maintaining the genetic information. For example, during ageing of a yeast population, death of older and damaged cells not only preserves resources but also releases nutrients to the fittest cells (Herker *et al.*, 2004). During mating, pheromones trigger cell death to clean the culture from infertile or damaged haploid cells, ensuring the adaptive benefit of the diploid state, which allows meiotic recombination and genetic diversity (Severin & Hyman, 2002). Several other cell death scenarios of the yeast unicellular organism have been described. Yeasts can undergo different forms of cell death in response to external and internal triggers, as observed in mammalian cells. Nevertheless, it has become apparent that, among other cellular processes, the basic cell death execution machinery is conserved in yeast to a degree that makes it a suitable model organism to address pending questions on human cell death and its deregulation (Carmona-Gutierrez *et al.*, 2018).

I.2.1. Types of yeast cell death

Yeast cells can die either via accidental cell death (ACD) or via regulated cell death (RCD). While ACD manifests with a necrotic morphotype, RCD can result from multiple signalling pathways, including regulated necrosis or apoptosis. Programmed cell death (PCD), represents a specific type of RCD related to physiological scenarios. There is also a possible role of autophagy as a cell death pathway in yeast, but it remains elusive (Figure 2) (Carmona-Gutierrez *et al.*, 2018).

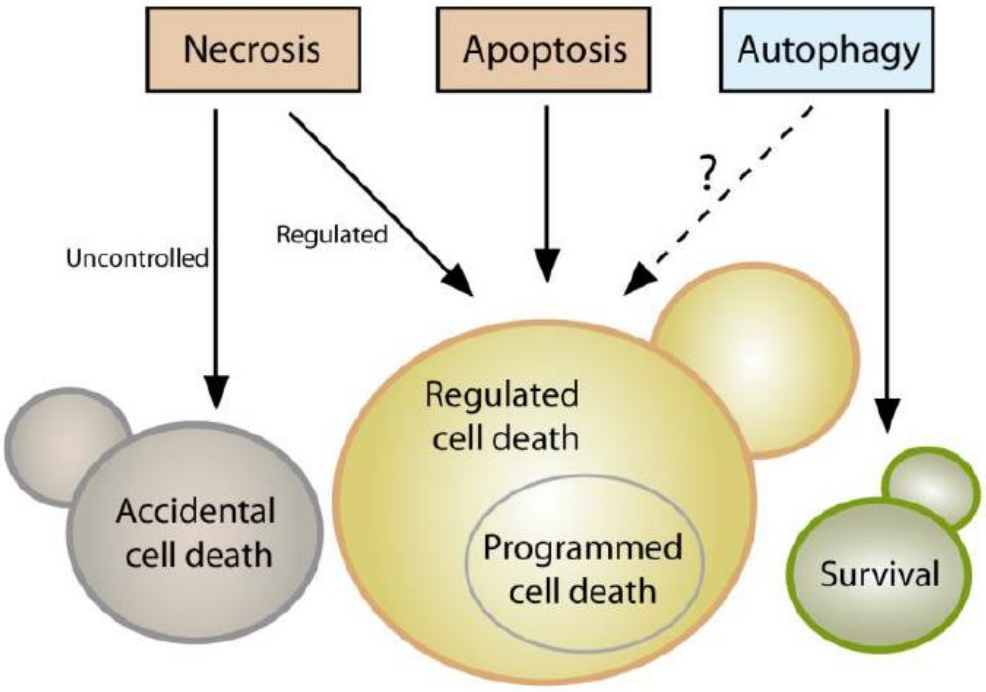

Figure 2. Different types of yeast cell death (Carmona-Gutierrez *et al.*, 2018).

I.2.1.1. Accidental cell death

ACD describes a cellular death following exposure to very harsh microenvironmental conditions, resulting in a rapid, uncontrollable, and unavoidable form of death, allowing no form of genetic or pharmacological inhibition. ACD may result from a series of extreme stimuli, including physical conditions, such as very high temperatures or pressures, severe chemical insults like strong detergents and high concentrations of acids or bases, as well as mechanical challenges, for instance, vigorous shearing or ultrasonic treatment (Carmona-Gutierrez *et al.*, 2018). ACD is frequently used as a synonym of necrosis. However, as is the case in human cells, there is evidence

suggesting that a regulated type of necrosis does also exist in yeast. In fact, it the use of the term accidental necrosis was suggested to distinguish both (Carmona-Gutierrez *et al.*, 2018). Necrosis was first described in detail in 1988 (Walker *et al.*, 1988) and it is morphologically characterized by an increase in cell volume, swelling of organelles and plasma membrane rupture followed by loss of intracellular contents (Zong & Thompson, 2006). Eventually, necrotic cells also show the complete breakdown and disintegration of subcellular structures (Eisenberg *et al.*, 2009). Features commonly used to assess a necrotic cell death in yeast are loss of plasma membrane integrity and nucleo-cytosolic translocation of Nhp6a, homolog of the human protein high mobility box group 1 (HMGB1) (Carmona-Gutierrez *et al.*, 2018).

I.2.1.2. Regulated cell death

RCD is executed by a genetically encoded, dedicated molecular machinery. It describes a cellular death occurring in the context of a failing response to internal or external mild stress and can exhibit a spectrum of morphologies that can be modulated with pharmacologic or genetic means. Consequently, modulating or avoiding stress responses can prevent RCD before the point-of-no-return. After that, it can only be accelerated or delayed. However, more studies are needed to identify the exact boundary between the reversibility of a stress stimulus (Carmona-Gutierrez *et al.*, 2018).

Yeast RCD encompasses both PCD as well as all other instances of cell death that can be differentiated from each other by a series of morphological and biochemical features. PCD designates a specific type of regulated cell death, which occurs in strictly physiological scenarios (Tang *et al.*, 2019). For example, during gametogenesis, when yeast meiotic mother cell death eliminates a superfluous cell remnant during a developmental transition (Eastwood & Meneghini, 2015), and during chronological ageing, when old yeast cells release substances into the medium that stimulate survival of the clone, sparing most of the dwindling resources for the healthy cells (Herker *et al.*, 2004). Cell death executed in the frame of such physiologic programs is considered PCD and all other forms of regulated demise, like cell death induction upon stress, are referred to with the superordinate term of RCD (Carmona-Gutierrez *et al.*, 2018).

I.2.1.2.1. Apoptosis

Apoptosis is the best studied and most common morphological form of RCD in multicellular organisms. Since the discovery that yeast can commit apoptosis in 1997 (Madeo *et al.*, 1997), multiple yeast orthologs of crucial mammalian apoptotic proteins have been identified, as well as the involvement of several organelles and compartments. The regulation of yeast apoptosis requires a complex interplay between small molecules, proteins, and pathways, which perform their functions at different locations in the cell. It is a complex orchestra that has been constantly adding information. Figure 3 summarizes the interconnection of the different players involved in yeast apoptosis (Carmona-Gutierrez *et al.*, 2010).

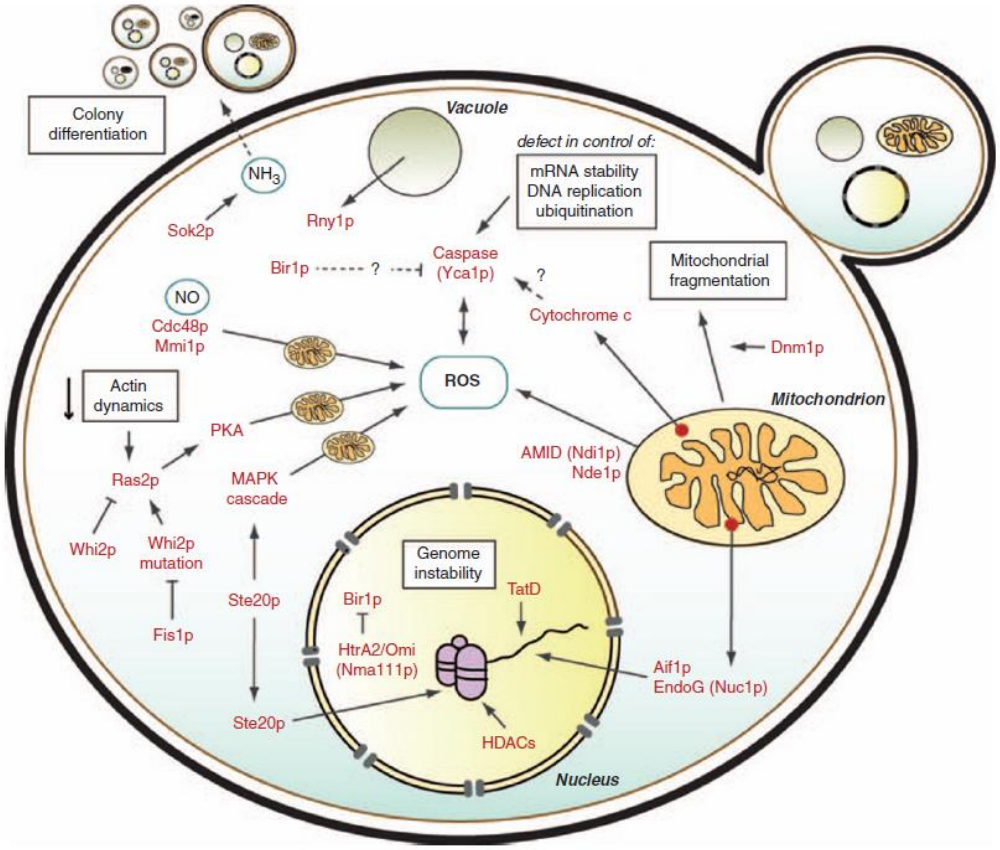

Figure 3. Basic molecular machinery of yeast apoptosis. It includes the yeast caspase Yca1p, the apoptosis inducing factor 1 (Aif1p), HtrA2/Omi (Nma111p), and AMID (Ndi1p), among others. Yeast regulated cell death has also been connected to mitochondrial fragmentation, cytochrome *c* release, caspase activation, cytoskeletal perturbations, ROS production and DNA fragmentation (Carmona-Gutierrez *et al.*, 2010).

Activation of an inside-outside phosphatidylserine translocase is an early and widespread event during apoptosis (Martin *et al.*, 1995). Other hallmarks include chromatin condensation, DNA fragmentation and plasma membrane integrity *(Madeo et al.*, 1999), ROS accumulation and caspase activation (Váchová & Palková, 2005). Apoptotic cell death often follows mitochondrial outer membrane permeabilization, which culminates with the release of pro-apoptotic proteins from the inter-membrane space and irreversible loss of mitochondrial transmembrane potential (Ludovico *et al.*, 2002). Even though maintenance of plasma membrane integrity is an apoptosis hallmark, an apoptotic cell eventually suffers metabolic collapse with plasma membrane integrity loss, culminating in a necrotic phenotype called secondary necrosis (Silva, 2010).

I.2.1.2.2. Regulated necrosis

For some time apoptosis was considered to be the standard cell death form, whereas necrosis was considered to be an accidental cell death that occurred in response to extreme stimuli (Suzanne & Steller, 2013; Taylor *et al.*, 2008). However, necrotic cell death does not only occur after a brutal chemical or physical stimulus but can also occur under normal reasonable concentrations of cell death-inducing substances. Regulated necrosis is defined as a genetically controlled cell death process that eventually results in cellular leakage. It is morphologically characterized by cytoplasmic granulation, as well as organelle and/or cellular swelling and loss of plasma membrane integrity (Carmona-Gutierrez *et al.*, 2010). Under regulated necrosis, it is possible to further distinguish between primary and secondary necrosis. Secondary necrosis is, almost all the time, a consequence of apoptosis (Galluzzi & Kroemer, 2017). That happens because yeast populations do not have a process to eliminate apoptotic cells, like phagocytic activity for example, causing apoptotic cells to eventually collapse, lose plasma membrane integrity and display a necrotic phenotype (Silva, 2010). Secondary necrosis can be distinguished from primary necrosis by the presence of apoptotic markers. Primary necrosis describes the phenotype of cellular necrosis occurring since the beginning, occurring without any preceding apoptotic traits (Silva, 2010). So, primary necrosis may result from ACD, outcome of severe insults and be called accidental necrosis, already discussed, or result from RCD as an event orchestrated by a genetically controlled machinery and be called regulated necrosis (Eisenberg *et al.*, 2010). Thus, in order to differentiate regulated from accidental necrosis, it is necessary to test whether a pharmacological

or genetic intervention is capable of inhibiting necrosis in the scenario that is being studied (Carmona-Gutierrez *et al.*, 2018). In fact, genetic and chemical manipulations have shown that yeast necrosis can be inhibited, indicating that it results from the activation of molecular mechanisms. For example, ageing-associated necrotic death can be inhibited by adding spermidine, a polyamine compound, or by genetic modification of yeast machinery (Eisenberg *et al.*, 2009). The protease Pep4p, homolog of human cathepsin D, was shown to specifically exhibit anti-necrotic characteristics, establishing that the capacity of yeast cells to undergo regulated necrosis is under tight molecular control (Carmona-Gutiérrez *et al.*, 2011). In the developmental coordination of gamete differentiation, necrosis occurs after the spores have reached the final phases of development, suggesting a controlled coordination that allows for gamete differentiation prior to the elimination of the mother cell (Carmona-Gutierrez *et al.*, 2018). However, mechanistic insights into the control of necrosis are still very limited at this point and it remains to be seen whether known inhibitors of regulated necrosis in mammals also interfere with some cell death scenarios in yeast (Conrad *et al.*, 2016). Considering the contribution that yeast provided to the understanding of mammalian apoptosis, and the likely possibility of similar value to the understanding of regulated necrosis, it is relevant and expected there will be a lot of research into this area, associated with different diseases and aging (Eisenberg *et al.*, 2009).

I.2.1.2.3. Other types of yeast regulated cell death

Autophagy defines a predominantly cytoprotective process that orchestrates the digestion of intracellular material in the vacuole. Historically, “autophagic cell death” was one of the three morphologically distinct processes of cell death with necrosis and apoptosis (Kroemer & Levine, 2008). In yeast, the term has been used to describe cellular demise occurring under specific external stress conditions, but more recently the NCCD has agreed to identify such forms of cell death as “autophagy-dependent cell death” (Galluzzi *et al.*, 2018). Autophagy-dependent cell death describes a lethal subroutine in which the molecular machinery of autophagy causally contributes to cellular demise. However, in human cells and yeast, cell death is accelerated and not repressed upon autophagy inhibition (Alvers *et al.*, 2009; Aris *et al.*, 2013; Boya *et al.*, 2005). Although there is some evidence for autophagy activation in the course of cell death, in most cases cells showing biomarkers of autophagy might be dying with, and not by, autophagy (Carmona-Gutierrez *et al.*,

2018). Therefore, autophagy-dependent cell death has been questioned as an actual cell death type (Shen *et al.*, 2011; Shen *et al.*, 2012). Another type of cell death is mitotic catastrophe, upon aberrant mitosis (Galluzzi *et al.*, 2011), that may result from genome instability, microtubule destabilization, DNA damage, or alterations in cell cycle checkpoints (Lombardi & Lasagni, 2016). It is considered an independent molecular avenue that precedes RCD (Vitale *et al.*, 2011).

I.3. Yeast RCD stimuli

Numerous stimuli or even physiological processes such as chronological aging can induce yeast cell death. A variety of agents have been reported to induce an apoptotic phenotype in yeast. These include acetic acid, hydrogen peroxide, ethanol, hypochlorous acid, high salt, UV irradiation, or heat stress (Carmona-Gutierrez & Madeo, 2009; Ludovico *et al.*, 2001; Madeo *et al.*, 1999). Several compounds, which normally constitute nutrients or oligo-elements, can also trigger apoptosis when they are applied at supraphysiological and toxic concentrations. This applies to glucose, sorbitol, copper, manganese, and iron (Carmona-Gutierrez & Madeo, 2009). There are also a variety of agents capable of inducing yeast necrosis. Acetic acid or hydrogen peroxide, which trigger apoptosis when applied at low doses, shift cell death to a rather necrotic phenotype when used at higher concentrations (Ludovico *et al.*, 2001; Madeo *et al.*, 1999). This also applies to copper and manganese (Liang & Zhou, 2007) and the antifungal agent amphotericin B (Phillips *et al.*, 2003). Therefore, a concentration-dependent switch between apoptosis and necrosis might be a common aspect of cell death inducing agents (Lieberthal & Levine, 1996).

I.3.1. Acetic acid

Acetic acid is a weak organic acid best known as a frequent by-product of the alcoholic fermentation carried out by *Saccharomyces cerevisiae* and the main component of volatile acidity in wine. It was shown that exposure of exponentially growing *S. cerevisiae* cells to low acetic acid doses at pH 3.0 induced cell death associated with typical mammalian apoptotic markers, including chromatin condensation, phosphatidylserine exposure and DNA strand breaks (Ludovico *et al.*, 2001). Adding to that, under the same experimental conditions, high acetic acid concentrations induced a necrotic phenotype, as suggested by extensive intracellular

disorganization and compromised plasma membrane integrity (Ludovico *et al.*, 2001). However, as more research was carried on, it became increasingly clear that an apoptotic-like cell death process can occur in yeast cells exposed to acetic acid, since two additional apoptotic markers, namely cytochrome *c* release from the mitochondria to the cytosol and mitochondrial reactive oxygen species accumulation, were identified. It was shown that acetic acid induces the accumulation of mitochondrial ROS, transient hyperpolarization followed by depolarization of mitochondria, decrease in cytochrome *c* oxidase activity and respiration, as well as release of cytochrome *c* (Ludovico *et al.*, 2002a), and causes some mitochondrial ultrastructural changes, such as decrease of cristae number, formation of myelinic bodies, and swelling (Ludovico *et al.*, 2003). The effects, genes involved and consequences of yeast cells exposure to acetic acid were reviewed recently (Chaves *et al.*, 2021).

I.3.2. Hydrogen peroxide

Among the oxidants used to stress cells, the most reported to initiate cell death is hydrogen peroxide (Perrone *et al.*, 2008). Furthermore, ROS accumulation has been found to play an important role in mediating cell death, such as apoptosis, or even – at very high concentrations – necrosis (Madeo *et al.*, 1999). Hydrogen peroxide exposure causes Aif1p to undergo a mitochondrial-nuclear shuttling, similar to its mammalian equivalent AIF, Pep4p, mammalian cathepsin-D homolog, to migrate out of the vacuoles and degrade nucleoporins (Mason *et al.*, 2005), and cells to exhibit features like chromatin condensation, DNA fragmentation and actin depolymerization (Madeo *et al.*, 1999; Vilella *et al.*, 2005). Yeast cells can recover from oxidative stress; however, when the stress is too high, prolonged, or sudden, there is a failure of cell antioxidant defences, which inevitably results in oxidative stress. This potentially leads important cell biomolecules to suffer severe oxidative damage, thus compromising cell viability (Halliwell & Aruoma, 1991). In fact, accumulated ROS have been shown to inflict oxidative damage upon nucleic acids, proteins, and lipids (Farrugia & Balzan, 2012).

I.3.3. Chronological ageing

Chronological ageing is defined as the length of time that a non-dividing yeast cell survives. It is typically measured by growing a culture of yeast cells into stationary phase (Fabrizio & Longo, 2003) and serves as a model for aging of human post-mitotic cells (Breitenbach *et al.*, 2012; Eisenberg *et al.*, 2009). The benefits of old cells undergoing suicide is tied to nutrient availability for fitter cells and, as dead cells release nutrients for the remaining culture (Büttner *et al.*, 2006). Then, death of chronologically aged yeast cells is extremely important so ensure life of the culture and so, it is logical that is not random, but a regulated process promoted by PCD. During chronological aging, cells eventually die exhibiting biochemical and morphological markers of apoptosis as well as necrosis (Allen *et al.*, 2006; Fabrizio *et al.*, 2004; Herker *et al.*, 2004). Interestingly, the fraction of cells dying by primary necrosis actually represents the majority of the dying population (Carmona-Gutiérrez *et al.*, 2011). The nucleo-cytosolic translocation of Nhp6Ap appeared in a large fraction of yeast cells and it is one of the most common markers used to identify necrosis during chronological ageing (Eisenberg *et al.*, 2009). ROS are considered to be the key regulators of ageing (Madeo *et al.*, 1999). It was shown that chronologically aged yeast cells die exhibiting ROS accumulation, caspase activation and oxidative stress increase (Fabrizio & Longo, 2003). Known necrosis-modulatory approaches include the exogenous administration of the naturally occurring polyamine spermidine, or genetic modulation of polyamine biosynthesis, which can specifically reduce primary necrotic cell death in the context of chronological aging (Eisenberg *et al.*, 2009).

As described above, cell balance can be lost for multiple reasons, and cell death is very complex, stressing the relevance of the cell death field and the study of their pathways and underlying mechanisms. Furthermore, a better understanding of such mechanisms has the potential for development or improvement of pharmacological interventions, either to prevent disease-induced tissue death or to remove unwanted tissues. Specifically, the acquired knowledge from the yeast cell death field may help not only the productivity of yeast during large-scale processes in the pharmaceutical and industrial areas, as they largely depend on its viability and ultimately on its tolerance to stress and its demise in stationary cultures, but also help decipher eukaryotic cell death pathways, as it serves as an important model for human disease. Adding to that, novel pharmacological approaches of yeast pathogens may bypass the ever-increasing resistance to classical antimycotics, which is an emerging public health problem. However, there

are still numerous cell death regulators that remain to be discovered, and others whose function and hierarchy in cell death has not yet been determined, as the precise sequence of events and the appearance of the earlier markers in these subroutines are yet not fully outlined.

CHAPTER II – OBJECTIVE

To study the cell death subroutines in yeast, more specifically RCD, it is common to use acetic acid and hydrogen peroxide as stimuli. High doses were shown to lead to a necrotic phenotype, while low doses were shown to induce apoptosis. Apoptotic cells can be distinguished from necrotic cells by staining cells with the fluorescent nucleic acid intercalator propidium iodide (PI), since PI can only enter cells upon cell membrane rupture, so enters in necrotic cells but is excluded from apoptotic cells. Thereby, maintenance of plasma membrane integrity is considered a hallmark of apoptosis. However, since plasma membrane integrity is assessed during the exposure of cells to a stimuli and cell viability assessed only days after plating those cells, the number of cells with plasma membrane integrity and the CFU counts do not always correlate in an apoptotic scenario. Since cell death occurs only upon irreversible plasma membrane breakdown or complete cellular fragmentation, novel methods to better correlate these hallmarks are needed.

The main objective of this work was to develop a simple method able to explore at what point, after an apoptotic stimulus and plating cells, do cells die as result of plasma membrane disruption, i.e., when cells achieve the point-of-no-return and undergo a secondary necrosis process.

To accomplish this aim, the following tasks were designed:

1. Optimization of yeast apoptotic cell death conditions in response to acetic acid and hydrogen peroxide.
2. Monitorization of Nhp6Ap-GFP localization and nuclear integrity in response to the same compounds.

Ideally, the results will contribute to better understand and characterize different types of RCD. They will also support setting out chronological time points for different events covered by these complex and dynamic processes of RCD. This may help not only on better defining the process of secondary necrosis after apoptosis, but also on distinguishing regulated from accidental necrosis.

CHAPTER III – MATERIALS AND METHODS

III.1. Yeast strains and growth conditions

The yeast *S. cerevisiae* strains BY4741 Wild-type, BY4741 pUG35-NHP6A–EGFP and BY4741 HTA2-mCherry pUG35-NHP6A–EGFP were used throughout this work. Cells were maintained in rich medium [YPD; 1% (w/v) yeast extract, 2% (w/v) glucose, 2% (w/v) bactopeptone, 2% (w/v) agar] and grown in synthetic complete medium [SC-Glu; 0.17% (w/v) yeast nitrogen base w/o amino acids and ammonium sulphate, 2% (w/v) glucose, 0.5% (w/v) ammonium sulphate, 0.14% (w/v) Dropout mix lacking histidine, leucine, tryptophan, and uracil, 0.008% (w/v) histidine, 0.04% (w/v) leucine, 0.008% (w/v) tryptophan and 0.008% uracil). Strains transformed with plasmids were grown in the same medium lacking the appropriate amino acids.

III.2. Glucose assay

For the glucose assay, cells were inoculated in growth medium overnight until OD_{600nm} ≈ 0.5. Then, they were centrifuged and resuspended in sterile water for 2h, after which 100 or 200mM of glucose were added. Samples were taken up to 4 hours for staining with PI and to observe Nhp6a-GFP release by fluorescent microscopy.

III.3. Chronological ageing assay

Cells were inoculated in growth medium overnight until OD600nm ≈ 1.0 – 1.5. Then, they were centrifuged and resuspended in the same volume of starvation medium [0.17% (w/v) yeast nitrogen base w/o amino acids and ammonium sulphate, 4% (w/v) Glucose and 0.5% (w/v) (NH4)2 SO4)]. After 24h, the cells were centrifuged and resuspended in sterile water or in NH_4^+ solution (0.5% $(NH4)_2SO_4$, pH 7.0) at the concentration of $3.8x10^7$ cells/mL. Samples were taken for staining with PI and to observe Nhp6a-GFP protein release by flow cytometry and fluorescent microscopy, respectively.

III.4. Acetic acid and hydrogen peroxide assay

Yeast cells were grown overnight in liquid SC-Glu with or without uracil until exponential growth-phase (OD600nm= 0.4-0.6) at 30°C with agitation (200 rpm). For acetic acid treatment, cells were harvested and suspended in fresh SC-Glu medium with or without uracil (pH 3.0) with 100 or 140mM acetic acid. For hydrogen peroxide treatment, 1.5 or 5.0mM hydrogen peroxide was added to the yeast cultures. In both cases, cells were incubated for 180 minutes at 30°C in 50mL Erlenmeyer flasks with an air: liquid ratio of 5:1 in a mechanical shaker at 200 rpm. Samples were taken at different time points, diluted to 10^{-4} in 1:10 serial dilutions in deionized sterilized water, and 40µL drops were spotted on YPD agar plates in replicates of five. Colony forming units (CFU) were counted after a 48h incubation at 30°C. Cell viability was calculated as a percentage of CFU in relation to time zero. Samples were also taken for staining with PI and to observe Nhp6a-GFP protein release by flow cytometry and fluorescent microscopy, respectively.

III.5. Flow cytometry

During acetic acid and hydrogen peroxide treatments, samples were taken to assess loss of plasma membrane integrity by flow cytometry, using a Beckman Coulter Cytoflex System B4-R2-V0 flow cytometer. Cells were collected, suspended in phosphate buffered saline (PBS, 80mM Na_2HPO_4, 20mM NaH_2PO_4 and 100mM NaCl) and stained with 1µg/mL propidium iodide (PI, Sigma) for 10 min, at room temperature, in the dark. Monoparametric detection of PI fluorescence was performed using FL-3 (488/620nm).

III.6. Microscopy

Yeast strains expressing Nhp6a-GFP were collected to evaluate the localization of the protein. In the experiments using epifluorescence microscopy, a Leica Microsystems DM-5000B microscope was used, with appropriate filter settings (red, green and DIC (Differential Interference Contrast)) with a 100x oil immersion objective. Images were obtained with a Leica DFC350 FX Digital Camera and processed with LAS AF Microsystems software.

III.7. Reproducibility and statistical analysis of the results

The results obtained are represented by mean and standard deviation (SD) values of at least three independent experiments. Statistical analyses were carried out using GraphPad Prism Software v5.00 (GraphPad Software, California, USA). P-values lower than 0.05 were assumed to represent a significant difference.

CHAPTER IV – RESULTS

IV.1. Acetic acid and hydrogen peroxide induces an apoptotic cell death followed by a secondary necrosis in yeast cells

Exposure to acetic acid and hydrogen peroxide have been used as a stimulus to study cell death processes in yeast, including apoptosis (Fannjiang *et al.*, 2004; Ludovico *et al.*, 2001; Madeo *et al.*, 2002). More specifically, exposure of yeast cells to low doses of this stimuli leads to a decrease in cell viability without plasma membrane integrity loss, which is indicative of the execution of an apoptotic cell death (Madeo *et al.*, 1999). Because regulated cell death of cells *in vitro* must ultimately culminate in plasma membrane integrity loss, as they reach the point-of-no-return and undergo secondary necrosis, we developed a simple method to determine at what point after an apoptotic stimulus do cells die as result of plasma membrane disruption.

First, different concentrations of acetic acid were tested to determine which induce an apoptotic cell death and replicate results described previously (Ludovico *et al.*, 2001). BY4741 cells were exposed to 100mM of acetic acid in SC Glu medium, pH 3.0 for 3 hours. During that time, cell viability and plasma membrane integrity was determined by standard dilution plate counts and PI staining, respectively (Figure 4 and 5). After acetic acid exposure, cells were washed and resuspended in fresh medium, in an attempt to replicate in liquid media what occurs when cells are platted on new solid media when assessing CFU. After cells were resuspended in fresh medium, plasma membrane integrity was assessed up to 4 hours (Figure 5).

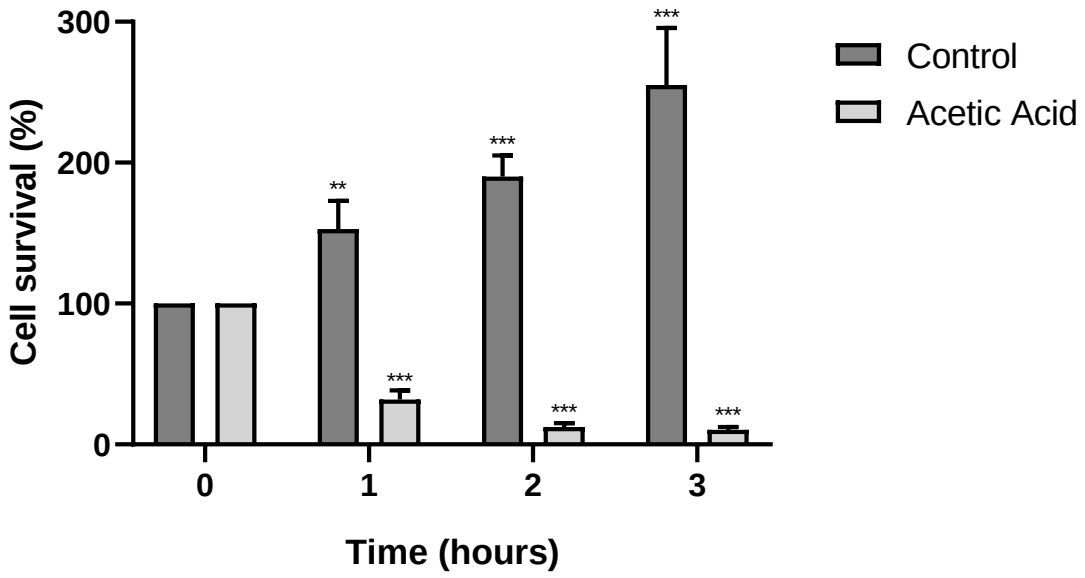

Figure 4. Survival of BY4741 cells after exposure to acetic acid. Cells were exposed to 100mM acetic acid, pH 3.0, for 3 hours. Viability was determined at the indicated times by standard dilution plate counts and expressed as a percentage of CFU in relation to time 0. Values are mean + SD of at least three independent

experiments. Values significantly different from 0: ** P<0.01 and *** P<0.001, Two-way ANOVA and Tukey Test.

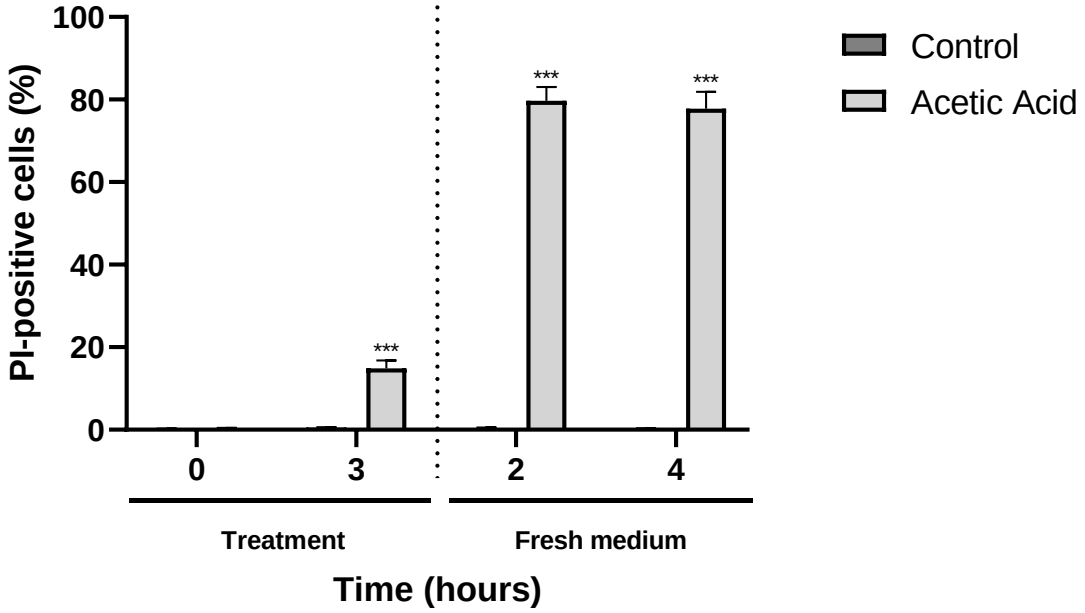

Figure 5. Percentage of BY4741 cells stained with propidium iodide after exposure to acetic acid. Cells were exposed to 100mM acetic acid, pH 3.0, for 3 hours and then washed and resuspended in fresh medium for 4 hours. The dotted line indicates the moment of when cells were washed and resuspended in fresh medium. PI-positive cells were determined at the indicated times by flow cytometry. Values are mean + SD of at least three independent experiments. Values significantly different from 0: *** P<0.001, Two-way ANOVA and Tukey Test.

After 3 hours of exposure to 100mM of acetic acid, it was possible to observe a large decrease in cell viability: there were around 90% of non-viable cells but only approximately 15% of cells exhibiting loss of plasma membrane integrity. After cells were washed and resuspended in fresh medium, the number of cells with losing plasma membrane integrity increased to almost 80% within 2 hours.

Similarly to acetic acid, different concentrations of hydrogen peroxide were tested, to replicate results described previously (Madeo *et al.*, 1999). BY4741 cells were exposed to 1.5 and 5mM of hydrogen peroxide in SC Glu medium, for 3 hours. During that time, cell viability and plasma membrane integrity were determined by standard dilution plate counts and PI staining, respectively (Figure 6 and 7). After hydrogen peroxide exposure, cells were washed and resuspended in fresh medium, and plasma membrane integrity was assessed up to 24 hours (Figure 7).

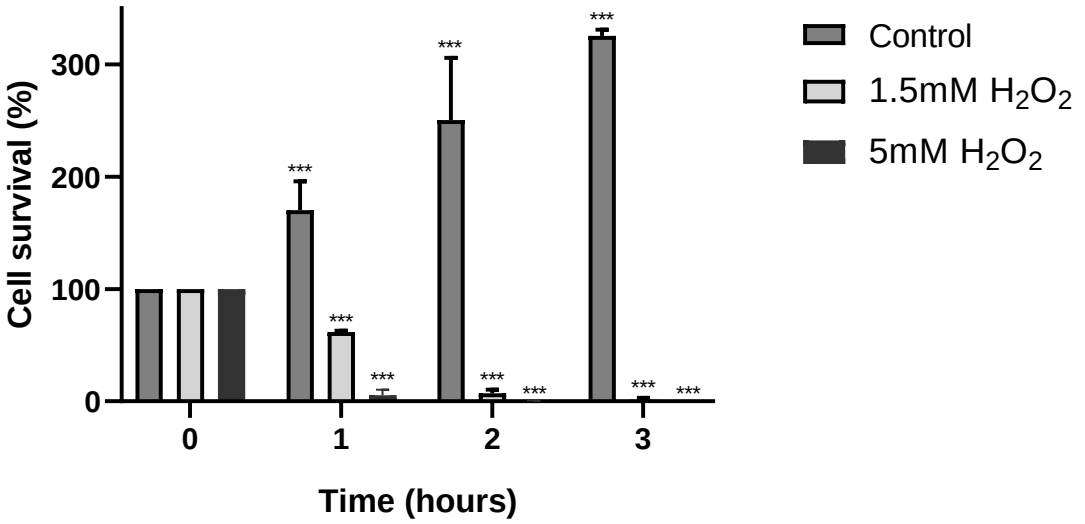

Figure 6. Survival of BY4741 cells after exposure to hydrogen peroxide. Cells were exposed to 1.5 and 5mM of hydrogen peroxide for 3 hours. Viability was determined at the indicated times by standard dilution plate counts and expressed as a percentage of CFU in relation to the time 0. Values are mean + SD of at least three independent experiments. Values significantly different from 0: *** P<0.001, Two-way ANOVA and Tukey Test.

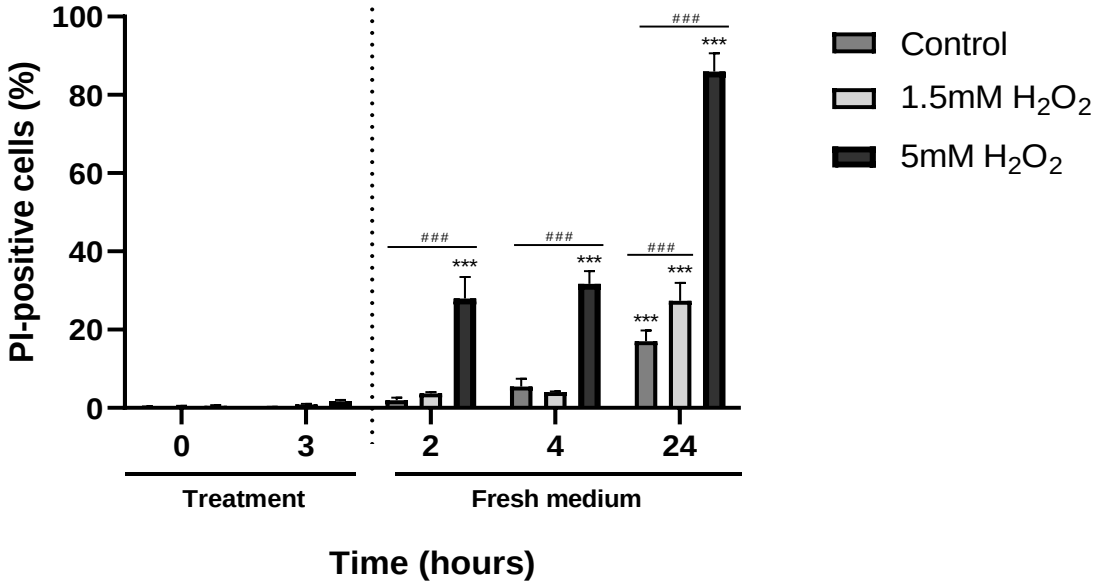

Figure 7. Percentage of BY4741 cells stained with propidium iodide after exposure to hydrogen peroxide. Cells were exposed to 1.5 and 5mM of hydrogen peroxide for 3 hours and then washed and resuspended in fresh medium for 24 hours. The dotted line indicates the moment of when cells were washed and resuspended in fresh medium. PI-positive cells were determined at the indicated times by flow cytometry. Values are mean + SD of at least three independent experiments. Values significantly different from 0: *** P<0.001, values significantly different from 0 and Control: ### P<0.001, Two-way ANOVA and Tukey Test.

After 3 hours of exposure to 1.5 and 5mM of hydrogen peroxide, cell viability was almost null for both concentrations, with no significant increase of PI-positive cells in comparison with control cells. The number of cells with loss of plasma membrane integrity increased to almost 30% after cells were exposed to 1.5mM hydrogen peroxide, washed and resuspended in fresh medium for 24 hours. A lower percentage of PI-positive cells was observed for control cells. The same percentage of PI-positive cells for the cells exposed to 5mM hydrogen peroxide was reached only 2 hours after cells have been resuspended in fresh medium, increasing to almost 85% after 24h.

IV.2. Acetic acid and hydrogen peroxide induce Nhp6Ap-GFP translocation in yeast cells

Nucleo-cytosolic translocation of Nhp6Ap is one of the most common hallmarks used to identify necrosis upon exposure of yeast cells to several stimuli. This includes glucose, ammonium, and ageing (Büttner *et al.*, 2006; Santos *et al.*, 2012; Valiakhmetov *et al.*, 2019). To better understand and characterize the cell death subroutines in yeast cells exposed to acetic acid and hydrogen peroxide, the BY4741 wild-type strain was transformed with pUG35-NHP6A-EGFP vector, to express the Nhp6Ap-GFP protein constitutively to further study its localization during both treatments.

In order to validate the methodology in our experimental conditions, we first assessed the localization of Nhp6Ap-GFP using glucose, ageing and ammonium as stimuli, as all have already been described to cause nucleo-cytosolic translocation of Nhp6Ap. Multiple controls were performed to ensure that the images obtained did not result from autofluorescence or contamination from red fluorescence to green or vice versa (Figure S1). In glucose exposure experiments, cells were grown in SC Glu medium and then transferred to water. After 2 hours, cells were centrifuged and resuspended in 100 and 200mM of glucose and incubated for 4 hours. Cells were analysed by fluorescence microscopy regarding Nhp6Ap-GFP localization and plasma membrane integrity through PI staining (Figure S2). In contrast with previous reports and after several attempts, glucose exposure did not cause nucleo-cytosolic translocation of Nhp6Ap-GFP and, for this reason, other stimuli were tested.

In ageing experiments, cells were grown in SC Glu medium and then transferred to starvation medium. After 24 hours, cells were centrifuged and resuspended in sterile water and

incubated for 120 hours. Cells were analysed by fluorescence microscopy until 5 days regarding Nhp6Ap-GFP localization and plasma membrane integrity (Figure 8).

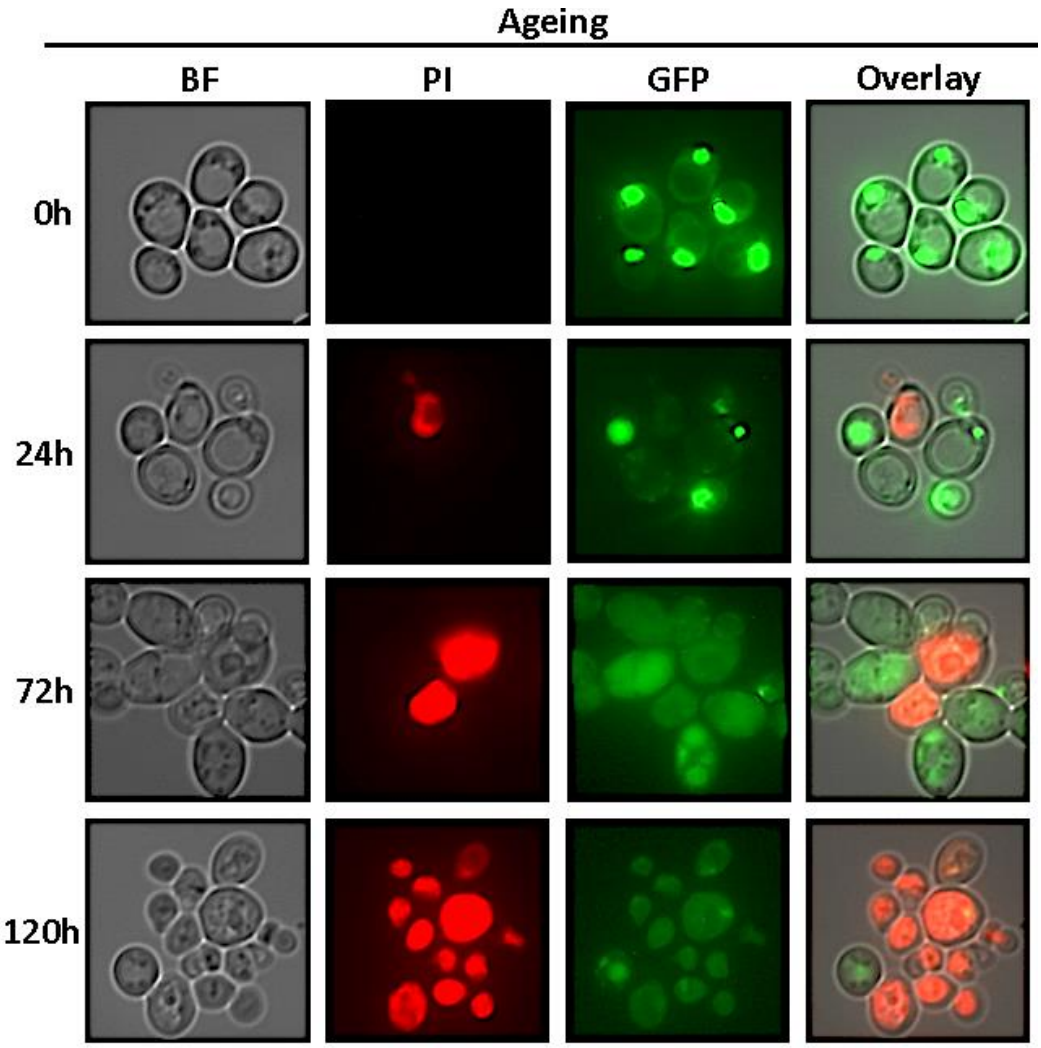

Figure 8. Images of BY4741 cells expressing Nhp6Ap-GFP stained with propidium iodide during chronological ageing. Cells were maintained in regular medium up to 120 hours. Fluorescence microscopy images were captured at the indicated times. BF stands for bright field, PI stands for propidium iodide.

As expected, at the beginning of the ageing process, cell exhibited Nhp6Ap-GFP only localized in the nucleus. After 24h, most of the cells still had the same Nhp6Ap-GFP localization, although the culture was less homogeneous, since a small number of cells also exhibited Nhp6Ap-GFP in the cytosol. After 72h, some cells exhibited PI staining as result of plasma membrane integrity loss and Nhp6Ap-GFP in the cytosol. There were also cells with Nhp6Ap-GFP localization in the cytosol without PI staining, which suggested that Nhp6Ap-GFP translocation occurs before loss of plasma membrane integrity. Some cells also appeared to have a diffused location of

Nhp6Ap-GFP in the cytosol and others more fragmented. After 72h, the majority of the cells exhibited red fluorescence simultaneously with Nhp6Ap-GFP in the cytosol.

Ammonium sulphate experiments were also performed with very similar conditions of ageing experiments. Ammonium-induced cell death was show to shorten the chronological lifespan by inducing an initial small increase of apoptotic cells followed by extensive necrosis (Santos *et al.*, 2012). Cells were grown in SC Glu medium and then transferred to starvation medium. After 24 hours, cells were centrifuged and resuspended in 0.5% ammonium sulphate and incubated for 72 hours. Cells were analysed by fluorescence microscopy until 72 hours regarding Nhp6Ap-GFP localization and plasma membrane integrity (Figure S3). Under our conditions, ammonium exposure did not speed up the process, when compared with the ageing assay, regarding nucleo-cytosolic translocation of Nhp6Ap-GFP.

After validation of the methodology and to better characterize the death process occurring in cells during acetic acid treatment, the BY4741 pUG35-NHP6A-EGFP strain was exposed to acetic acid and characterized regarding Nhp6Ap-GFP localization. Since we started working with a transformed strain and we were not able to obtain the same phenotypes observed previously using 100mM of acetic acid, the dose of acetic acid was increased to 140mM to obtain the same results of cell viability and plasma membrane integrity (Figures 9 and 10).

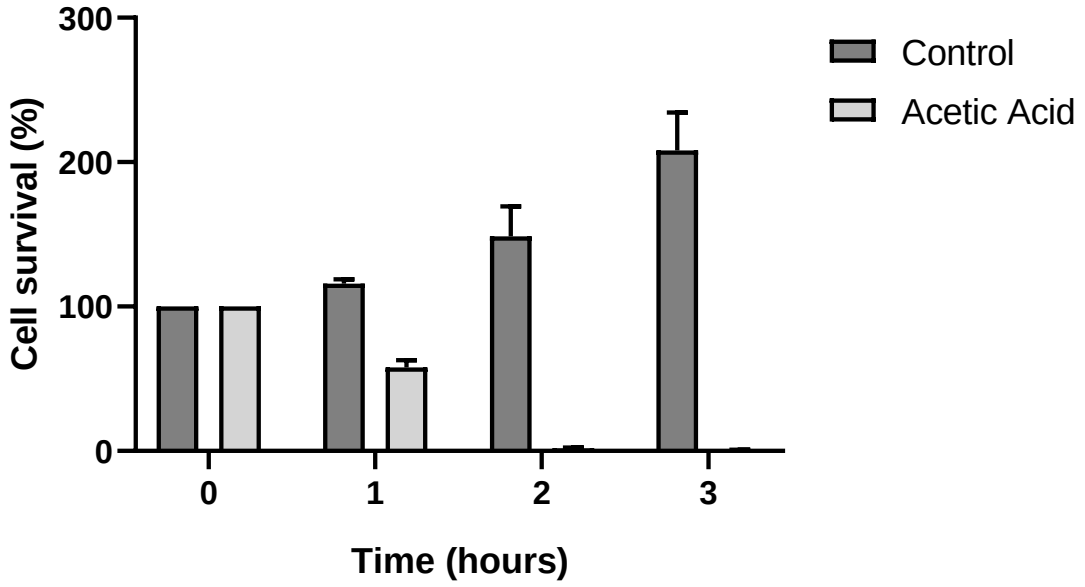

Figure 9. Survival of BY4741 cells expressing Nhp6Ap-GFP after exposure to acetic acid. Cells were exposed to 140mM acetic acid, pH 3.0, for 3 hours. Viability was determined at the indicated times by standard dilution plate counts and expressed as a percentage of CFU in relation to in relation to time 0, n=2.

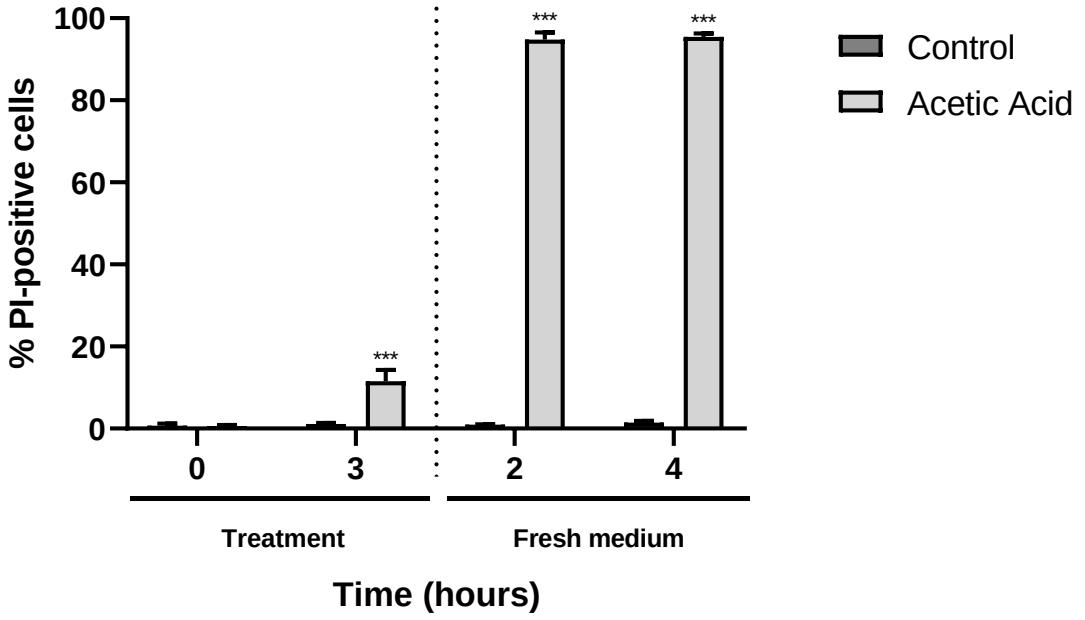

Figure 10. Percentage of BY4741 cells expressing Nhp6Ap-GFP stained with propidium iodide after exposure to acetic acid. Cells were exposed to 140mM acetic acid, pH 3.0, for 3 hours and then washed and resuspended in fresh medium for 4 hours. The dotted line indicates the moment of when cells were washed and resuspended in fresh medium. PI-positive cells were determined at the indicated times by flow cytometry. Values are mean + SD of at least three independent experiments. Values significantly different from 0: *** P<0.001, Two-way ANOVA and Turkey Test.

Cells were also analysed by fluorescence microscopy regarding Nhp6Ap-GFP localization and PI staining along of the 3h of acetic acid treatment, and until 4h after cells were washed and resuspended in fresh medium (Figure 11).

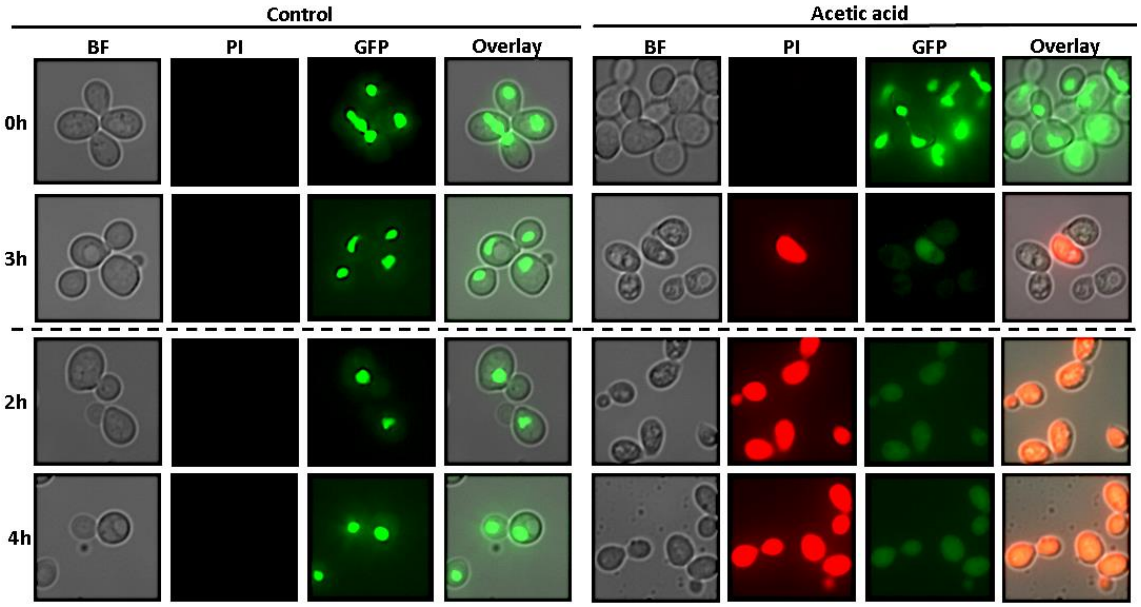

Figure 11. Images of BY4741 cells expressing Nhp6Ap-GFP stained with propidium iodide after exposure to acetic acid. On the left, cells were on pH 3.0 for 3 hours and then washed and resuspended in fresh medium for 4 hours and are used as control. On the right, cells were exposed to 140mM acetic acid, pH 3.0, for 3 hours and then washed and resuspended in fresh medium for 4 hours. Fluorescence microscopy images were captured at the indicated times. The dotted line indicates the moment of when cells were washed and resuspended in fresh medium. BF stands for bright field, PI stands for propidium iodide.

It was possible to observe that control cells exhibited Nhp6Ap-GFP in the nucleus during all the experiment. Cells treated with acetic acid started exhibiting Nhp6Ap-GFP in the nucleus, as the control cells, but after 3h of exposure to acetic acid a few cells began exhibiting loss of plasma membrane integrity, being stained with PI. The same PI-positive cells also exhibited Nhp6Ap-GFP in the cytosol. Cells resuspended in fresh medium after being exposed to acetic acid exhibited the same phenotype: all the cells which exhibited loss of plasma membrane integrity also exhibited Nhp6Ap-GFP in the cytosol, but an increasing number of cells losing plasma membrane integrity was observed 4h after resuspension in fresh medium, as was expected due to the previous experiment.

A study of our group identified, at a genome-wide scale, several functional categories involved in the resistance and sensitivity of yeast cells to death induced by acetic acid. Among these, cell proliferation, differentiation and increase in cell size were shown to be associated with

higher susceptibility to acetic acid-induced cell death (Sousa *et al.*, 2013). This raised the hypothesis that washing the cells and resuspend them in fresh medium after acetic acid treatment and promoting such events may decrease the survival of cells. Therefore, yeast cells were exposed to 140mM of acetic acid for a longer time without being washed. Cell viability was assessed by CFU (not shown) and plasma membrane integrity by internalization of PI using flow cytometry (Figure 12).

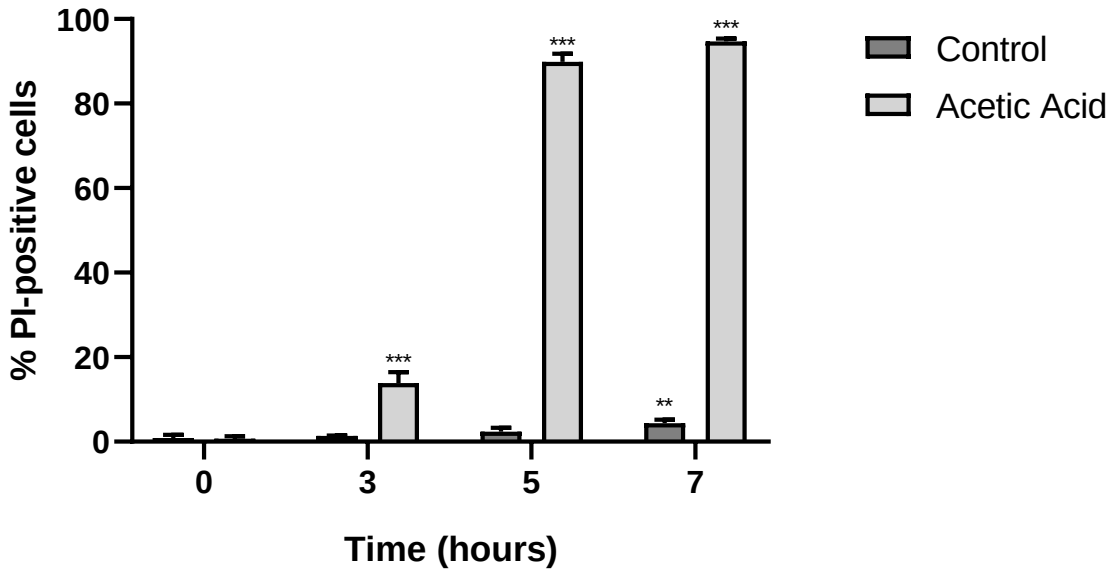

Figure 12. Percentage of BY4741 cells expressing Nhp6Ap-GFP stained with propidium iodide after exposure to acetic acid. Cells were exposed to 140mM acetic acid, pH 3.0, for 7 hours. PI-positive cells were determined at the indicated times by flow cytometry. Values are mean + SD of at least three independent experiments. Values significantly different from 0: ** P<0.01 and *** P<0.001, Two-way ANOVA and Turkey Test.

Cell viability was consistent with previous experiments of acetic acid treatment (not shown). After 3 hours of treatment, only approximately to 15% of cells exhibited loss of plasma membrane integrity, as shown before. After 5h the number of cells which had lost plasma membrane integrity increased to around 90%. During the same experiment, Nhp6Ap-GFP protein localization was also evaluated along the acetic acid treatment by fluorescence microscopy (Figure 13).

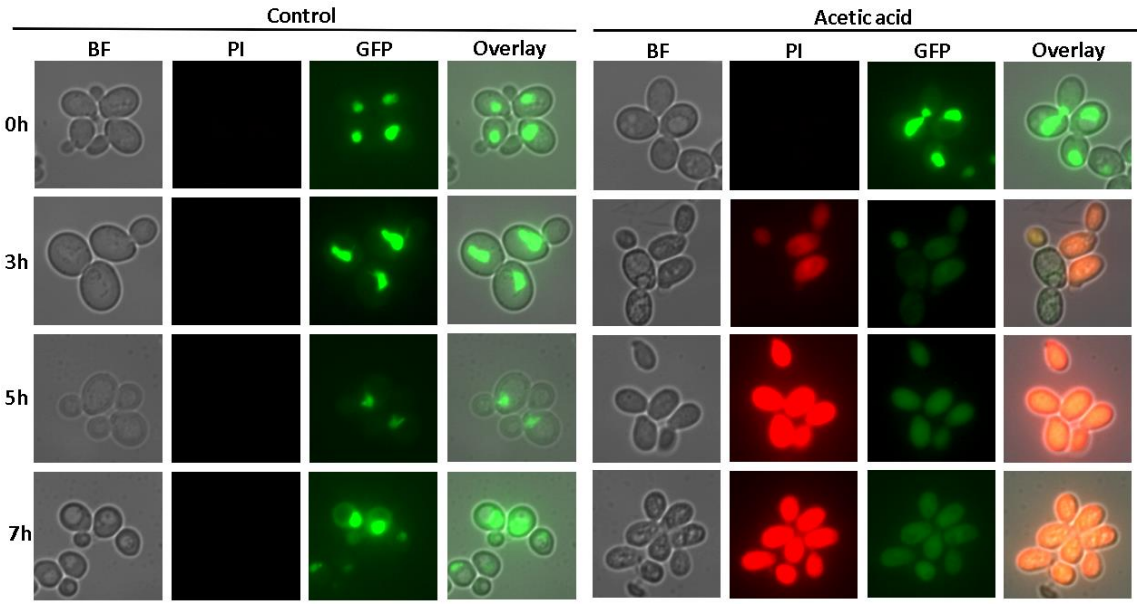

Figure 13. Images of BY4741 cells expressing Nhp6Ap-GFP stained with propidium iodide after exposure to acetic acid. On the left, cells were on pH 3.0 for 7 hours and are used as control. On the right, cells were exposed to 140mM acetic acid, pH 3.0, for 7 hours. Fluorescence microscopy images were captured at the indicated times. BF stands for bright field, PI stands for propidium iodide.

The results obtained were consistent with previous experiments. Control cells exhibited Nhp6Ap-GFP in the nucleus during all the experiment. Acetic acid-treated cells exhibited the same Nhp6Ap-GFP alterations as previously observed: after 3h of exposure to acetic acid some cells already exhibit loss of plasma membrane integrity, being stained red with PI, and those cells also exhibited Nhp6Ap-GFP in the cytosol. After 5 and 7 hours of exposure to acetic acid, all the cells exhibited loss of plasma membrane integrity and Nhp6Ap-GFP in the cytosol.

To better characterize the death process occurring in cells during hydrogen peroxide treatment, cells exposed to hydrogen peroxide were characterized regarding Nhp6Ap-GFP localization until 3 hours, and then cells were washed and resuspended in fresh medium for up to 24 hours (Figure 14). As shown previously, hydrogen peroxide treatment decreased cell viability without loss of plasma membrane integrity. Only after 24 hours in fresh medium did cells exposed to 1.5mM of hydrogen peroxide start exhibiting PI-positive staining as a result of loss of plasma membrane integrity. For cells exposed to 5mM of hydrogen peroxide, the loss of plasma membrane integrity was more rapid and about 90% of cells exhibited PI-positive staining after 24h in fresh medium.

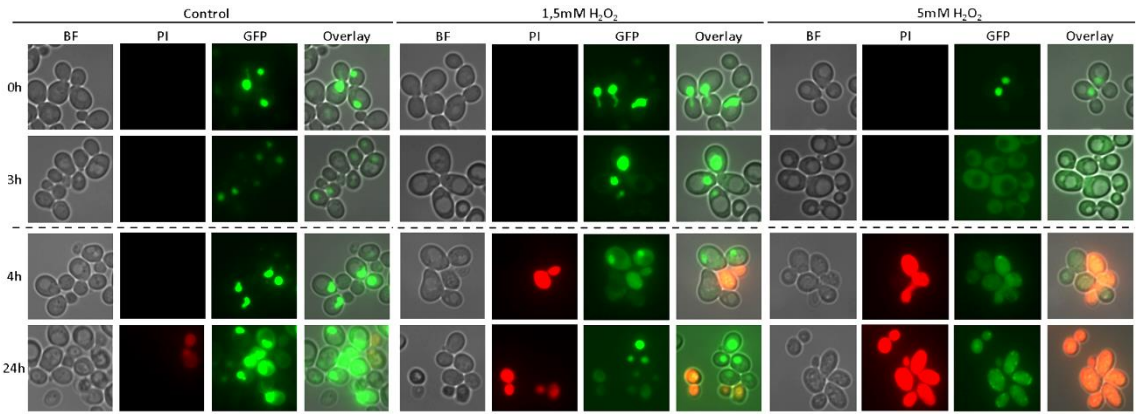

Figure 14. Images of BY4741 cells expressing Nhp6Ap-GFP stained with propidium iodide after exposure to hydrogen peroxide. On the left, cells had no stress for 3 hours and then washed and resuspended in fresh medium for 24 hours and are used as control. On the middle, cells were exposed to 1.5mM hydrogen peroxide for 3 hours and then washed and resuspended in fresh medium for 24 hours. On the right, cells were exposed to 5mM hydrogen peroxide for 3 hours and then washed and resuspended in fresh medium for 24 hours. The dotted line indicates the moment of when cells were washed and resuspended in fresh medium. Fluorescence microscopy images were captured at the indicated times. BF stands for bright field, PI stands for propidium iodide.

It was possible to observe that the control cells exhibited Nhp6Ap-GFP in the nucleus during all the experiment, but after 24 hours, some cells started exhibiting loss of plasma membrane integrity, as observed previously through flow cytometry. Cells exposed to 1.5mM of hydrogen peroxide exhibited Nhp6Ap-GFP in the nucleus, as the control cells, during all the treatment. Only after 4h in fresh medium did some cells start exhibiting loss of plasma membrane integrity, being stained red with PI. Those cells were the same that also exhibited Nhp6Ap-GFP in the cytosol. The same phenotype was observed after 24h in fresh medium. Cells exposed to 5mM of hydrogen peroxide started exhibiting Nhp6Ap-GFP in the cytosol after 3 hours of treatment, however cells maintained the plasma membrane integrity, since they did not exhibit PI staining. During the 24 hours in fresh medium, Nhp6Ap-GFP localization was maintained in the cytosol. After 4 hours, some cells started exhibiting loss of plasma membrane integrity. The number of PI-positive cells increased until 24 hours in fresh medium, which was consistent with the previous experiment with hydrogen peroxide.

IV.3. Acetic acid induces nuclear fragmentation in yeast cells

In the previous experiments, some cells seemed to exhibit clusters of Nhp6Ap-GFP. This raised questions regarding the integrity of the nucleus and its effect on the localization of Nhp6Ap-

GFP during the cell death process. To clarify this issue, a BY4741 yeast strain harbouring the pUG35-Nhp6Ap-EGFP vector and the Hta2-mCherry in the genome, encoding a core histone protein required for chromatin assembly and chromosome function, was constructed. The ageing assay was repeated with this new strain, with the objective of analysing the integrity of the nucleus during the experiment by following the Hta2-mCherry protein using fluorescence microscopy simultaneously with Nhp6Ap-GFP (Figure 15).

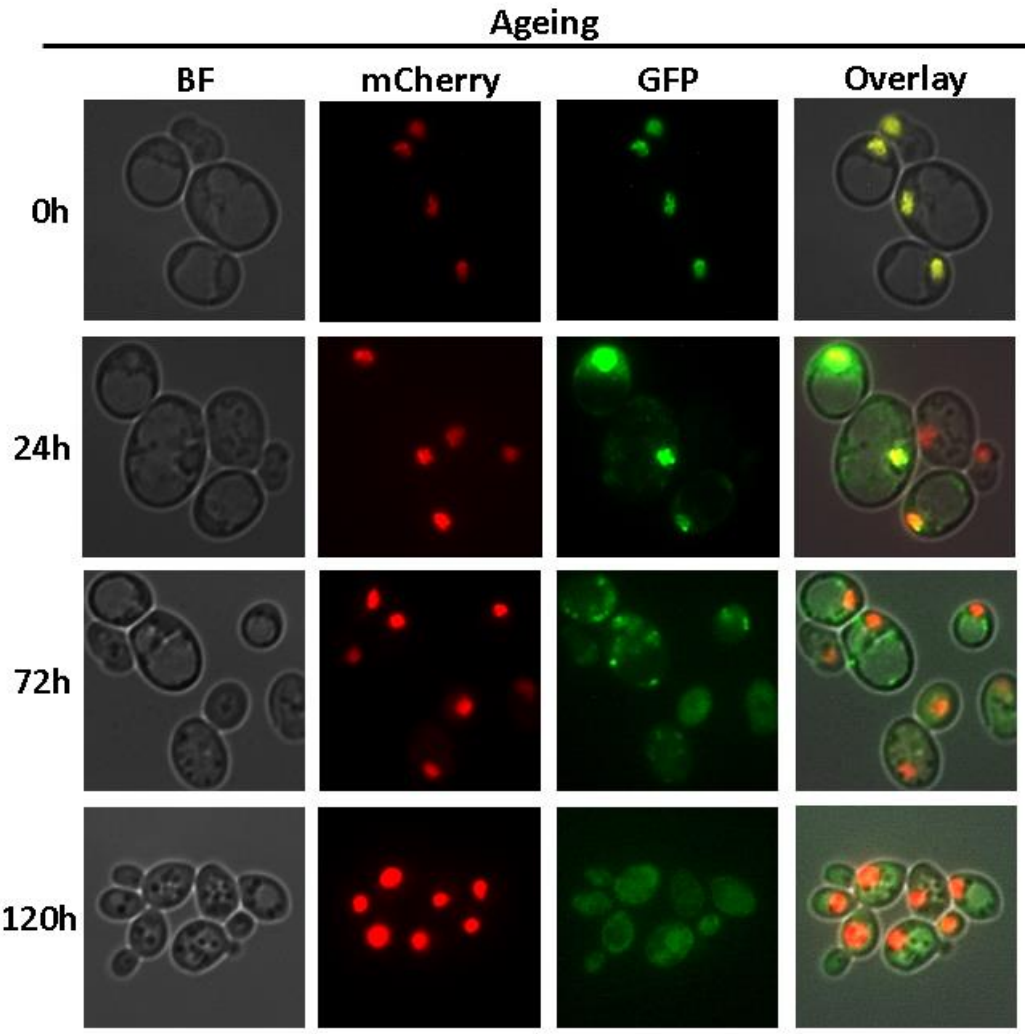

Figure 15. Images of BY4741 cells expressing Nhp6Ap-GFP and Hta2-mCherry during chronological ageing. Cells were kept in regular medium up to 120 hours. Fluorescence microscopy images were captured at the indicated times. BF stands for bright field.

Cells were observed by fluorescence microscopy until 120h. The localization of Nhp6Ap-GFP was similar to the previous experiment: only after 72h was it possible to observe an increasing number of cells exhibiting clusters of Nhp6Ap-GFP. However, Hta2-mCherry maintained the same localization during all the experiment, never indicating a compromised or fragmented nucleus.

Since cells maintained the integrity of the nucleus during all the experiment, we concluded that the Nhp6Ap-GFP localization observed was not a consequence of nucleus fragmentation.

Nuclear integrity during acetic acid treatment was also evaluated by following Hta2-mCherry protein localization using the BY4741 strain harbouring the pUG35-Nhp6Ap-EGFP vector and with Hta2-mCherry in the genome along the 3 hours of exposure to 100mM of acetic acid (Figure 16). Since we started working with a different transformed strain and we were not able to obtain the same phenotypes observed previously using 140mM of acetic acid, the dose of acetic acid was decreased to 100mM to obtain the same results of cell viability and plasma membrane integrity (not shown).

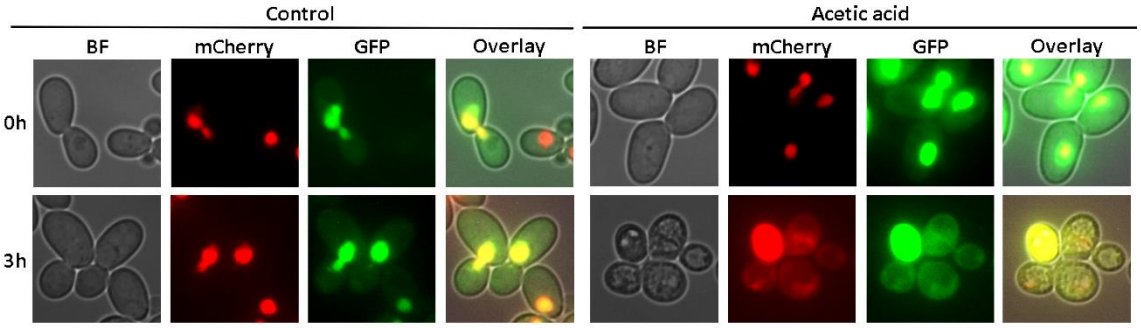

Figure 16. Images of BY4741 cells expressing Nhp6Ap-GFP and Hta2-mCherry after exposure to acetic acid. On the left, cells were on pH 3.0 for 3 hours and are used as control. On the right, cells were exposed to 100mM acetic acid, pH 3.0, for 3 hours. Fluorescence microscopy images were captured at the indicated times. BF stands for bright field.

At the beginning of the experiment, both fluorescent proteins overlapped in the nucleus. After 3 hours, the control cells still exhibited both fluorescent proteins overlapping in the nucleus. In contrast, after 3 hours, the cells exposed to acetic acid exhibited Nhp6Ap-GFP in the cytosol. Those cells also exhibited red fluorescence in the cytosol, indicating the release of Hta2-mCherry from nucleus and/or its fragmentation, although it did not perfectly colocalize with the green fluorescence. This suggest that although acetic acid can induce nuclear fragmentation, the Nhp6Ap-GFP translocation does not seem to be exclusively the result of nuclear fragmentation.

CHAPTER V – CONCLUDING REMARKS AND FUTURE PERSPECTIVES

As already discussed, yeast cells die through ACD with a necrotic morphotype, or through RCD from a variety of pathways such as regulated necrosis and apoptosis. Regulated necrosis comprises primary and secondary necrosis. Primary necrosis characterizes a cellular necrosis occurring since the beginning, without apoptotic traits (Silva, 2010). Cells undergoing secondary necrosis exhibit apoptotic markers, most likely a consequence of apoptosis (Galluzzi & Kroemer, 2017). Plasma membrane integrity loss and nucleo-cytosolic translocation of Nhp6A are, usually, the hallmarks tested to characterize a necrosis process (Carmona-Gutierrez *et al.*, 2018). On the other hand, one of the apoptosis hallmarks is the maintenance of plasma membrane integrity, however an apoptotic yeast cell ultimately undergoes metabolic collapse losing plasma membrane integrity, starting secondary necrosis (Silva, 2010).

Acetic acid and hydrogen peroxide are commonly used to induce apoptosis in yeast and are associated with multiple apoptotic markers (Ludovico *et al.*, 2001; Madeo *et al.*, 1999). We were able to replicate the kinetics of cell death already described for both acetic acid and hydrogen peroxide. In both treatments, cells don't lose membrane integrity but lose their viability, which suggests that both stimuli induced an apoptotic cell death at the concentrations used. Cell death occurs only when the point-of-no-return is surpassed, which follows upon irreversible plasma membrane breakdown or complete cellular fragmentation (Galluzzi *et al.*, 2014). For the first time, this study showed when yeast cells exposed to different apoptotic stimuli lose the integrity of the plasma membrane. To accomplished that, we developed a simple method capable of following cell survival as the processes of cell death unfolds. After exposure to both stimuli, cells were washed and resuspended in fresh medium, in an attempt to replicate in liquid media what occurs when cells are platted on new solid media when assessing CFU. Through this methodology, we observed that the number of cells losing plasma membrane integrity increased, which confirms that even though cells during treatment still preserved plasma membrane integrity, they continue the cell death process and lose plasma membrane integrity and their ability to form colonies. It was also possible to observe that, for the concentrations used, the point-of-no-return for cells after exposure to hydrogen peroxide occurs much later than in acetic acid treatments (4h vs 24h) and for lower concentrations that still induce regulated cell death, the kinetics and the time until the point-of-no-return is even longer. Overall, this method allowed a better correlation between the number of cells with plasma membrane integrity and the CFU counts, which was not the case in most studies published, where plasma membrane integrity was assessed during the exposure to stimuli and cell viability after plating cells in solid media.

Nucleo-cytosolic translocation of Nhp6Ap is one of the most frequent markers used to identify necrosis. For that reason, it was used to identify necrosis during acetic acid and hydrogen peroxide treatments. First, the methodology was validated during chronological ageing, a physiological scenario that induces PCD with necrotic characteristics of old yeast cells to ensure life of the culture (Eisenberg *et al.*, 2009). It was also possible to observe Nhp6Ap-GFP nucleo-cytosolic translocation as a consequence of chronological ageing occurs before loss of plasma membrane integrity. Furthermore, when analysing nuclear integrity using Hta2-mCherry, it was possible to conclude that cells maintained nuclear integrity. Thus, Nhp6Ap-GFP nucleo-cytosolic translocation was not a result of nucleus fragmentation. Following Nhp6Ap-GFP localization in cells during and after acetic acid exposure not only allowed to determine when the point-of-no-return is reached, but also showed, for the first time, that acetic acid induces Nhp6Ap-GFP translocation, simultaneously with loss of plasma membrane integrity. All the cells that exhibited loss plasma membrane integrity also exhibited Nhp6Ap-GFP localization in the cytosol, most likely due to a fast transition from apoptosis to secondary necrosis. Adding to that, we also demonstrated that washing and resuspending cells in fresh medium does not promote cell proliferation, differentiation and increase in cell size to a significant enough degree to induce a different cell death subroutine, and consequently an increase of PI-positive cells nor a change in Nhp6Ap-GFP localization, even though these processes are described as associated with higher susceptibility to acetic acid-induced cell death (Sousa *et al.*, 2013). However, when following Hta2-mCherry localization, cells exposed to acetic acid also exhibited red fluorescence in the cytosol, indicating the release of Hta2-mCherry from nucleus and/or its fragmentation. Although it did not perfectly colocalize with the green fluorescence, it remained unclear if Nhp6Ap-GFP localization and translocation after acetic acid exposure was exclusively due to nucleus fragmentation or a direct consequence of acetic acid exposure. Known hallmarks of acetic acid-induced cell death include chromatin condensation and DNA strand breaks (Ludovico *et al.*, 2001). In the future, a more extensive characterization of the nuclear integrity during this experiment would be useful to elucidate this aspect, for example following a protein from the nuclear envelope using fluorescence microscopy, allowing simultaneously observation with the other proteins already studied in this work. Regarding hydrogen peroxide exposure, we also showed that Nhp6Ap-GFP nucleo-cytosolic translocation does not always occur simultaneously with loss of plasma membrane integrity. Most likely due to a slower transition to secondary necrosis, and in contrast with acetic acid, cells exposed to hydrogen peroxide exhibited Nhp6Ap-GFP localization in the cytosol without exhibiting loss of plasma

membrane integrity. These results demonstrated that the chronological order of the events is: first, nucleo-cytosolic translocation of Nhp6Ap and only then loss of plasma membrane integrity. We speculate that this separation of the events is observable because the point-of-no-return for cells after exposure to hydrogen peroxide occurs much later than during acetic acid treatments, although this chronological separation of the events was not observed with lower concentrations. The integrity of nucleus was not assessed in hydrogen peroxide treatments. For the future, it would be interesting to repeat this experiment using a strain with Hta2-mCherry, as was done for acetic acid, to compare and evaluate nuclear integrity following this protein.

Exposing yeast cells to higher concentrations of acetic acid and hydrogen peroxide that undoubtedly are able to induce a necrotic cell death would certainly add value to this work. This would enable to compare the moment of loss of plasma membrane integrity and changes in the Nhp6Ap-GFP and Hta2-mCherry localization. The protease Pep4p, homolog of human cathepsin D, was shown to specifically exhibit anti-necrotic characteristics (Carmona-Gutiérrez *et al.*, 2011). It would also be interesting to analyse if overexpression of this protein can change or inhibit some of the events that were analysed throughout this work, or even alter their chronological order. After the treatment with an insult and resuspension in a fresh medium, it would be interesting to find proteins or compounds that could avoid reaching the point-of-no-return.

In conclusion, we successfully developed a method capable of demonstrating the moment of cell integrity loss caused by exposure to acetic acid and hydrogen peroxide in yeast. These results will contribute to a better characterization of yeast cell death induced by these stimuli and set some chronological times points for different events helping to better define the process of secondary necrosis after apoptosis.

CHAPTER VI – REFERENCES

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CHAPTER VII – SUPPLEMENTAL MATERIAL

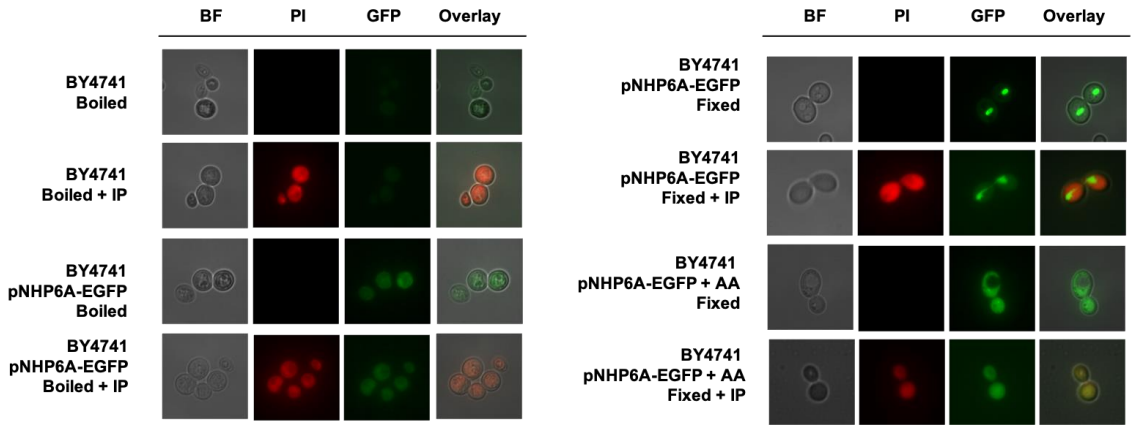

Figure S1. Images of BY4741 cells and BY4741 cells expressing Nhp6Ap-GFP with or without propidium iodide staining. Fluorescence microscopy images were captured for the different conditions indicated. BF stands for bright field, PI stands for propidium iodide and AA stands for 140mM acetic acid. Boiled cells were incubated for 5 minutes at 100°C using a thermoblock. For the cell fixation, cells were centrifuged, the supernatant was removed, cells were resuspended in paraformaldehyde and incubated for 15min. Then cells were centrifuged, the supernatant was removed, and cells were washed and resuspended in KPO_4/sorbitol.

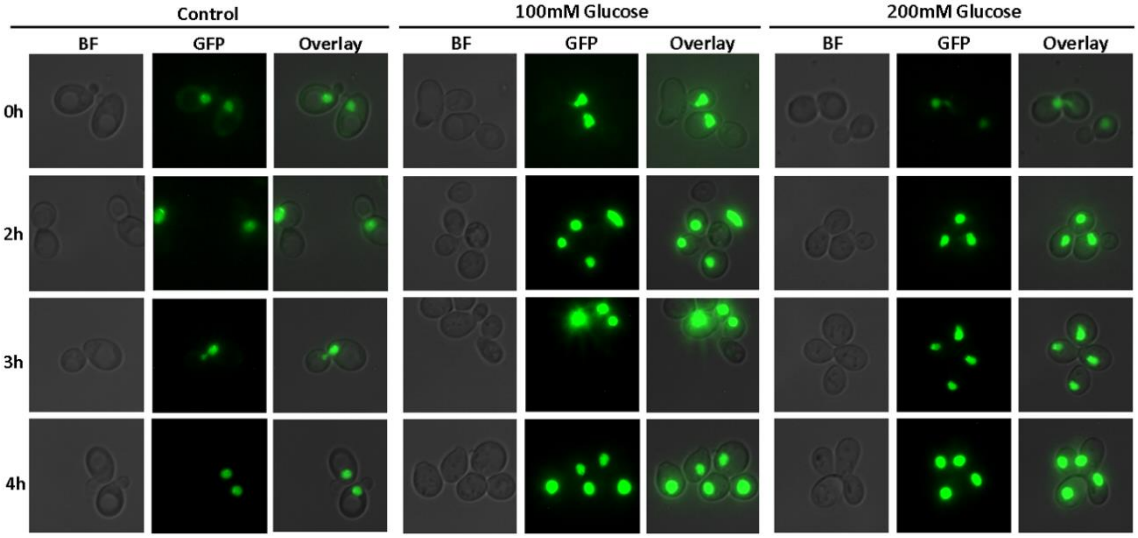

Figure S2. Images of BY4741 cells expressing Nhp6Ap-GFP after exposure to glucose. After 2 hours in water with no carbon source, cells were maintained in water (left panel), 100mM glucose or 200mM glucose were added (centre and right panels) and cells were incubated for an additional 4 hours. Fluorescence microscopy images were captured at the indicated times. BF stands for bright field.

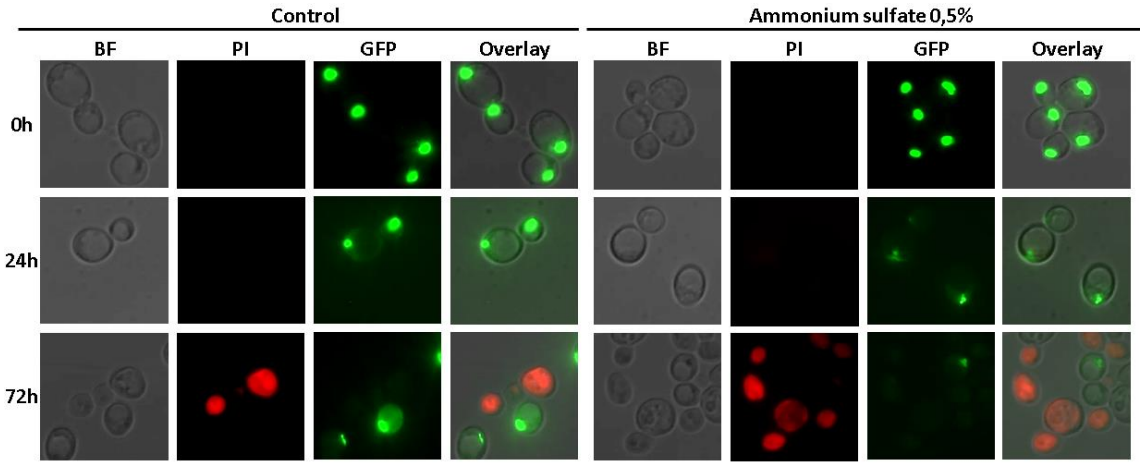

Figure S3. Images of BY4741 cells expressing Nhp6Ap-GFP stained with propidium iodide after exposure to ammonium sulphate. On the left, cells are in water with no carbon source for 72 hours after 1 day in starvation medium and are used as control. On the right, cells were exposed to 0.5% ammonium sulphate for 72 hours after 1 day in starvation medium. Fluorescence microscopy images were captured at the indicated times. BF stands for bright field.