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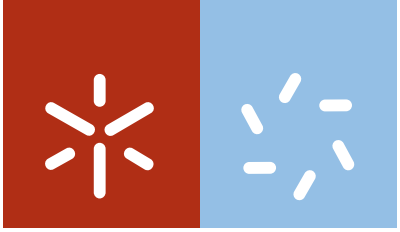

Universidade do Minho
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Helena Paula Fernandes Pereira

The role of Pep4p, the vacuolar yeast protease ortholog of human cathepsin D, in mitochondria-dependent apoptosis

Doctoral Thesis
PhD Programm on Molecular and Environmental Biology

Elaborated under the supervision of
Professora Doutora Manuela Côrte-Real
and
Doutora Susana Chaves

January, 2015

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The role of Pep4p, the vacuolar yeast protease ortholog of human cathepsin D, in mitochondria-dependent apoptosis

Abstract

Lysosomal cathepsins play a crucial role in cell homeostasis by participating in the degradation of heterophagic and autophagic material. Additionally, following their release into the cytosol, these proteases are involved in pro-apoptotic and anti-apoptotic processes, particularly the aspartic cathepsin D (CatD). Indeed, CatD released into the cytosol triggers a mitochondrial apoptotic cascade. However, CatD can have anti-apoptotic effects in some cellular types and specific contexts. Therefore, targeting this apoptosis regulator in therapies for apoptosis deficiency-associated diseases, such as cancer, requires detailed elucidation of its mechanisms of action. Understanding the molecular mechanisms connecting lysosomal to mitochondrial membrane permeabilization is thus particularly relevant.

More recently, vacuolar membrane permeabilization and consequent release of vacuolar proteins into the cytosol was also observed in yeast. It was demonstrated, that Pep4p (yeast CatD), a pepsin-like aspartic protease found in the yeast vacuole and ortholog to human CatD, is released from the vacuole during hydrogen peroxide- or actin stabilization-induced apoptosis. It also translocates into the cytosol during acetic acid-induced apoptosis, and is required for autophagy-independent degradation of mitochondria and for increased cell survival in response to this acid. Furthermore, acetate in colorectal carcinoma (CRC) cells seems to behave as acetic acid in yeast, triggering lysosomal membrane permeabilization (LMP), CatD release and mitochondria-dependent apoptosis. Recently, we found that CatD is involved in autophagy-independent degradation of damaged mitochondria, which renders CRC cells more resistant to apoptosis induced by acetate. These observations, combined with the hints provided by the yeast cell model, support the idea that LMP associated with the release of CatD protects CRC cells from mitochondrial dysfunction during acetate-induced apoptosis through its involvement in degradation of damaged mitochondria. Thus, it has become apparent that the approaches with yeast have already provided and can further offer new perspectives for an enhanced understanding of the role of CatD in mammalian apoptosis, as well of the molecular basis

of the crosstalk between the lysosome and mitochondria. Thereafter, we set out to exploit acetic acid-induced apoptosis in *Saccharomyces cerevisiae* to study the yeast vacuolar protease Pep4p, both concerning its role in mitochondrial degradation and its involvement in the course of apoptosis.

In this thesis, it is shown that the protective role of Pep4p in acetic acid-induced apoptosis is independent of the yeast voltage dependent channel Por1p (which has no role on mitochondrial degradation) but dependent on AAC proteins, the yeast adenine nucleotide translocator. Moreover, it has shown that both the Pep4p anti-apoptotic function and its role in mitochondrial degradation depend on Pep4p proteolytic activity. In this study, we also demonstrated that the pro-survival role of Pep4p in acetic acid-induced apoptosis is dependent on mitochondrial respiratory function, and that deficiency in mitochondrial respiration suppresses its role in mitochondrial degradation.

Altogether, these results contributed to unveil a novel pro-survival function of CatD in autophagy-independent mitochondrial degradation, which can lead to enhanced cell survival in CRC cells undergoing acetate-induced apoptosis. Moreover, these studies reinforce the use of yeast as a valuable model to elucidate the role of CatD in mammalian apoptosis, as well as the molecular mechanisms involved in the crosstalk between the lysosome and mitochondria.

O papel da Pep4p, a protease vacuolar da levedura ortóloga da catepsina D humana, na apoptose dependente da mitocôndria

Resumo

As catepsinas lisossomais têm um papel crucial na homeostasia celular, participando na degradação de material hetero- e autofágico. Adicionalmente, estas proteases estão envolvidas em processos pró- e anti-apoptóticos após a sua libertação para o citosol, particularmente a catepsina aspártica D (CatD). Com efeito, uma vez no citosol, a CatD desencadeia a cascata apoptótica mitocondrial. Contudo, esta protease pode ter um papel anti-apóptótico. A utilização deste regulador apoptótico como alvo molecular na terapia de doenças associadas a deficiências no processo apoptótico requer portanto uma elucidação detalhada dos seus mecanismos de acção. Por este motivo, a compreensão dos mecanismos moleculares que conectam a permeabilização da membrana lisossomal (PML) à permeabilização da membrana mitocondrial é particularmente relevante.

Mais recentemente, observou-se que a permeabilização da membrana vacuolar e consequente libertação de proteases vacuolares para o citosol também ocorre na levedura. Foi demonstrado que a Pep4p, a protease aspártica encontrada no vacúolo da levedura e ortóloga da CatD humana, é libertada do vacúolo para o citosol durante a apoptose induzida pelo peróxido de hidrogénio ou estabilização da actina. Esta protease também é translocada para o citosol durante a apoptose induzida pelo ácido acético desempenhando um papel crucial na degradação mitocondrial independente da autofagia e na sobrevivência celular em resposta a este ácido. Adicionalmente, o acetato em linhas celulares derivadas do carcinoma colorectal (CRC) comporta-se de modo análogo ao ácido acético na levedura, induzindo PML, libertação da CatD e apoptose dependente da mitocôndria. Nós demonstrámos recentemente que a CatD está envolvida na degradação mitocondrial independente da autofagia, o que torna as células do CRC mais resistentes à apoptose induzida pelo acetato. Estas observações, mais as indicações obtidas através do modelo de levedura, reforçam a ideia de que a PML associada à libertação da CatD protege as células do CRC de uma disfunção mitocondrial durante a apoptose induzida pelo acetato, através do seu envolvimento na degradação de mitocôndrias danificadas. Tornou-se então aparente que as abordagens na levedura forneceram informação importante e podem vir a oferecer

perspectivas adicionais, contribuindo assim para uma melhor compreensão do papel da CatD na apoptose em mamíferos, bem como das bases moleculares do "crosstalk" entre o lisossoma e a mitocôndria. Por conseguinte, decidimos explorar o modelo da apoptose induzida pelo ácido acético na *Saccharomyces cerevisiae* para estudar a protease vacuolar da levedura Pep4p, relativamente ao seu papel na degradação mitocondrial e consequentemente seu envolvimento na apoptose.

Nesta tese, mostra-se que o papel protector da Pep4p na apoptose induzida pelo ácido acetico é independente do canal de aniões dependente da voltagem de levedura Por1p (que por sua vez não desempenha um papel na degradação mitocondrial), mas é dependente das proteínas AAC, o antiportador mitocondrial de ATP/ADP da levedura. Também foi demonstrado que a função anti-apoptótica da Pep4p, bem como o seu papel na degradação mitocondrial dependem da sua actividade proteolítica. Neste estudo, foi também demonstrado que o papel protector da Pep4p na apoptose induzida pelo ácido acético é dependente da função respiratória mitocondrial, e também que a deficiência na respiração mitocondrial suprime o papel da Pep4p na degradação mitocondrial. Em conjunto, os resultados aqui descritos contribuíram para revelar uma nova função da CatD na degradação mitocondrial independente da autofagia, que pode conduzir a um aumento da sobrevivência nas células do CRC durante a apoptose induzida pelo acetato. Além disso, estes estudos reforçam o uso da levedura como modelo para elucidar o papel da CatD na apoptose de mamíferos, bem como os mecanismos moleculares envolvidos no "crosstalk" entre o lisossoma e a mitocôndria.

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Abbreviations

RCD	regulated cell death
MOMP	mitochondrial outer membrane permeabilization
AIF	apoptosis inducing factor
PTP	permeability transition pore
ROS	reactive oxygen species
LMP	lysosomal membrane permeabilization
CatD	cathepsin D
VMP	vacuolar membrane permeabilization
CRC	colorectal carcinoma
Cat	Cathepsin
ER	endoplasmic reticulum
tBid	truncated Bid
Pep4p	proteinase A
Prb1p	proteinase B
CPY	carboxypeptidase Y
CPS	carboxypeptidase S
Ape1	aminopeptidase I
Ape3	aminopeptidase Y
Dap2	dipeptidylaminopeptidase B
Pff1	Metalloprotease
EGFP	enhanced green fluorescent protein
AAC	ADP/ATP carrier
ANT	adenine nucleotide translocator
VDAC	voltage-dependent anion channel
WT-Pep4p	wild type Pep4p
DPM-Pep4p	double point mutant Pep4p
PI	propidium iodide
DHE	Dihydroethidium
3-AT	3-aminotriazole

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Chapter 1

General introduction

This chapter comprises parts from the following publication:

Pereira H, Oliveira CSF, Castro L, Preto A, Chaves SR, Côrte-Real M (2015). A novel function of human cathepsin D unveiled by the yeast vacuolar protease Pep4p: potential impact on colorectal cancer prevention/therapy. Submitted manuscript.

1.1. Introduction

In multicellular organisms, regulated cell death (RCD) is an active process that consists in the removal of mutated, infected or simply dispensable cells. It is crucial for normal tissue homeostasis, development, and regulation of the immune system. RCD insufficiency can result in cancer, autoimmune diseases and spreading of viral infections, while neurodegenerative disorders, AIDS and ischaemic diseases are caused or enhanced by RCD. Importantly, RCD can occur as part of physiologic programs but can also be activated when adaptive responses to perturbations of the extracellular or intracellular microenvironment fail (reviewed in [1,2]).

Apoptosis is considered the primary form of RCD and is defined by a set of cytological alterations like cell shrinkage, chromatin condensation, membrane blebbing, nuclear fragmentation, chromosomal DNA fragmentation, and formation of apoptotic bodies (reviewed in [3]). Apoptosis is also often characterized as a caspase-dependent form of RCD and may be elicited through several molecular pathways. These pathways have been best characterized in higher eukaryotes, and the most prominent are the extrinsic and intrinsic pathways. Mitochondria are obligate participants in intrinsic apoptotic signalling, and play important roles also in extrinsic, receptor-mediated apoptosis [4].

In mammalian cells, mitochondrial outer membrane permeabilization (MOMP) is widely considered as the critical point in the cascade of events leading to cell death via intrinsic apoptosis, but is also involved in the amplification of the death signal in the extrinsic pathway (Figure 1.1). MOMP leads to the dissipation of the mitochondrial transmembrane potential ($\Delta\psi_m$), which immediately results in the arrest of mitochondrial ATP synthesis and the release of pro-apoptotic factors, like cytochrome *c*, second mitochondria-derived activator of caspases/direct IAP-binding protein (Smac/DIABLO), apoptosis inducing factor (AIF) and endonuclease G (EndoG). Once in the cytosol, these proteins activate caspase-dependent and -independent mechanisms that altogether mediate the execution of cell death. Among apoptotic factors, cytochrome *c* plays a crucial role in intrinsic apoptosis, by inducing the apoptotic peptidase-activating factor 1 (APAF1) and ATP/dATP to assemble the apoptosome, which in turn activates caspase 9 and caspase 3. SMAC/DIABLO participates in both intrinsic and extrinsic pathways, by suppressing the inhibitory activity of inhibitors of apoptosis (IAPS), allowing the activation of caspase 3 by

caspase 9 and caspase 8 (reviewed in [5,6]). Evidence from mammalians cells has been attributing the permeabilization of the mitochondrial membrane to opening of the permeability transition pore (PTP); the formation of pores in the mitochondrial outer membrane, either by Bcl-2 pro-apoptotic family members or by ceramide molecules; and through interactions between the different processes and components [7]. Furthermore, it was established that the first regulatory step for mitochondrial apoptosis is mediated by Bcl-2 family proteins through the regulation of MOMP by physical and functional interactions between pro- and anti-apoptotic functions of these proteins (reviewed in [5]).

More recently, apoptotic markers were also observed in yeast in response to several stimuli, indicating the presence of the basic mechanisms of RCD already in unicellular eukaryotes [8–11]. Moreover, because there is a surprisingly high degree of conservation between yeast and higher eukaryotes, this organism has been used as a relevant experimental model to discover the regulation and molecular mechanisms of eukaryotic RCD. In addition, the simple and easy biochemical and genetic manipulations, as well as the extensive molecular tools available are important advantages of yeast as a model system.

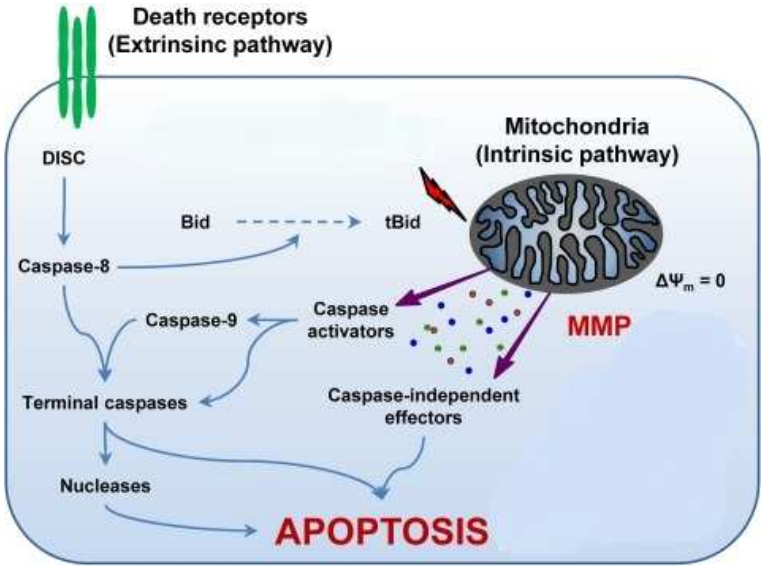

Figure 1.1. Mitochondria participation in cell death signaling. Representation of the two major apoptotic pathways in mammalian cells: the involvement of mitochondrial membrane permeabilization (MMP) in both the extrinsic death receptor pathway and the intrinsic mitochondrial pathway. Scheme adapted from Galluzi L, *et al.* 2008.

In last two decades, important contributions were made to the advancement of the yeast apoptosis field, most notably through the early recognition of a mitochondria-mediated apoptotic pathway showing similarities to the mammalian intrinsic pathway (reviewed in [12]). Several studies have shown that acetic acid triggers a mitochondria-dependent apoptotic pathway in yeast associated with chromatin condensation, formation of DNA strand breaks, accumulation of mitochondrial reactive oxygen species (ROS) and MOMP with subsequent release of cytochrome *c* and yeast apoptosis inducing factor 1 (Aif1p) [10,13,14]. Additionally, the involvement of yeast orthologs of some of the mammalian permeability transition pore components in mitochondria permeabilization and cytochrome *c* release, in the absence of heterologous expression of Bcl-2 family members, was also addressed [15]. It was also found that acetic acid induces yeast caspase-dependent and -independent apoptotic pathways [16], and that caspase activation was partly dependent on cytochrome *c* in cells undergoing hyperosmotic stress-induced apoptosis [17].

Besides mitochondria, the lysosome has also been implicated in the regulation of RCD in mammalian cells. Indeed, in addition to mitochondrial destabilization, lysosomal permeabilization has also been shown to initiate a cell death pathway, under specific circumstances. Furthermore, it was established that the extent of lysosomal damage determines cell fate; limited lysosomal release results in cell death by apoptosis, while massive lysosomal breakdown leads to necrosis [18]. Several studies in mammalian systems demonstrated that selective lysosomal membrane permeabilization (LMP) and subsequent release of cathepsins or other hydrolases induces apoptosis through a mitochondria-dependent pathway [18–20]. Among the cathepsins released from the lysosome into the cytosol, cathepsin D (CatD) has been shown to play an important role in RCD by triggering a mitochondrial apoptotic cascade [21].

Several studies have also revealed that the yeast lysosome-like vacuole is also involved in yeast cell death. In fact, vacuolar membrane permeabilization (VMP) and consequent release of vacuolar proteins into the cytosol was also observed in these unicellular organisms. It was demonstrated that Pep4p (yeast CatD), a pepsin-like aspartic protease found in the yeast vacuole and ortholog to human CatD, translocates from the vacuole to the cytosol in response to different apoptosis stimuli, as observed in mammalian cells [22–24]. Additionally, it was reported that Rnp1p, an RNase T2 family member, is

released from the vacuole into the cytosol during oxidative stress, directly promoting cell death [25].

Although it has become apparent that the lysosome/vacuole is intrinsically connected with mitochondria in modulation of RCD through its permeabilization and the consequent release of proteins to the cytosol, the molecular mechanisms triggered by LMP/VMP, as well as the signaling to mitochondria remain to be clarified. In the next sections, the role of LMP/VMP in cell death of mammalian/yeast cells, as well as the involvement of cathepsins/vacuolar proteases in this process, in particular the role of CatD/Pep4p, are reported. Additionally, the role of CatD in colorectal carcinoma (CRC), including insights from the yeast model is also discussed.

1.2. Lysosomal permeabilization: the role of cathepsins in cellular physiology and pathology

Cathepsins are members of a large protease family, which can be subdivided according to their structure and active-site amino acid into cysteine (cathepsins B, C, F, H, K, L, O, S, V, W, and X), serine (cathepsins A and G), and aspartic cathepsins (cathepsins D and E) (reviewed in [26]). Cathepsins (Cat) B, L, H, C e D are ubiquitously expressed in human tissues, while expression of CatA, G, K, S, V, X and W depends on the type of cell and tissue [27–29]. In general, cathepsins are found in acidic cellular organelles (lysosomes and endosomes), where they efficiently cleave a wide variety of substrates; some cathepsins, such as CatL, B and D, are most abundant in the lysosomes [28,30]. Similarly to caspases, cathepsins are activated by proteolytic cleavage of the synthetized inactive zymogens. They are composed of an N-terminal signal peptide, a propeptide, and a catalytic domain. The signal peptide directs the nascent chain into the endoplasmic reticulum (ER). After its cleavage in the lumen, the procathepsin is N-glycosylated and directed to the acidic endosomal/lysosomal compartment, where the proregion is cleaved, resulting in the active cathepsin catalytic domain [31]. It is now also recognized that the propeptide is a multifunctional region. Indeed, it assists in the folding of the protein after cleavage of the signal peptide, acts as an inhibitor preventing the premature activation of the catalytic domain, and is responsible for the transport of the procathepsin to both endosomes and lysosomes [32].

In the last decades, numerous physiological functions of cathepsins have been uncovered [28]. Initially, it was thought the function of cathepsins was confined to the lysosomal compartment, where they were responsible for the turnover of proteins delivered to the lysosome by endocytosis or autophagocytosis. However, it was later found that cathepsins can be secreted from the cell and degrade substrates like collagen, fibronectin, proteoglycans and laminin. As discussed below, this extracellular proteolytic activity facilitates cell migration and invasion by cancer cells [33]. Cathepsins are also involved in Ag processing, bone and tissue remodeling, wound healing, prohormone and proenzyme activation [27,29,34–40] and, more recently, have been implicated in the immune response [34]. Other studies also showed that cathepsins are translocated from the lysosomal lumen to the cytosol of mammalian cells through LMP in response to a variety of apoptotic signals such as TNF receptor ligation (the best-studied inducer of LMP [30]), sphingosine [41,42], staurosporine [21], reactive oxygen species [19], DNA damaging agents (e.g. cisplatin, etoposide [37]), resveratrol [43], p53 [19], lysosomotropic agents (eg the antibiotics ciprofloxacin, norfloxacin and hydroxychloroquine), and kinase inhibitors (e.g. imatinib and staurosporine [44]).

The use of cathepsin inhibitors like pesptatin A and Ca-074-ME, inhibitors of CatD and CatB, respectively, were instrumental to demonstrate that these non-caspase proteases are also involved in apoptosis execution [33]. Their use also led to the discovery that cathepsins, like caspases, may be activated in a cascade-like manner during apoptosis, as is the case in the activation of CatB through direct cleavage by CatD. However, little is known about potential interactions between cathepsin- and caspase-mediated pathways, although it seems likely that these pathways communicate [33]. Indeed, in several paradigms of apoptosis, lysosomes may function as death signal integrators [30], and partial or moderate LMP and the ensuing release of lysosomal hydrolases into the cytosol can activate either the intrinsic caspase-dependent apoptosis pathway or the caspase-independent alternative cell death program [30,44,45]. However, LMP can initiate other pathways depending on the cell type, death stimuli and dose (Figure 1.2), as well as on the extent of lysosomal leakage (especially of cathepsin release) [46,47]. In the case of necrosis, LMP is an early event associated with a massive release of lysosomal contents into the cytosol [30,45,47]. In necroptosis, LMP is a late process, which coincides with the disintegration phase through proteolysis [46,47].

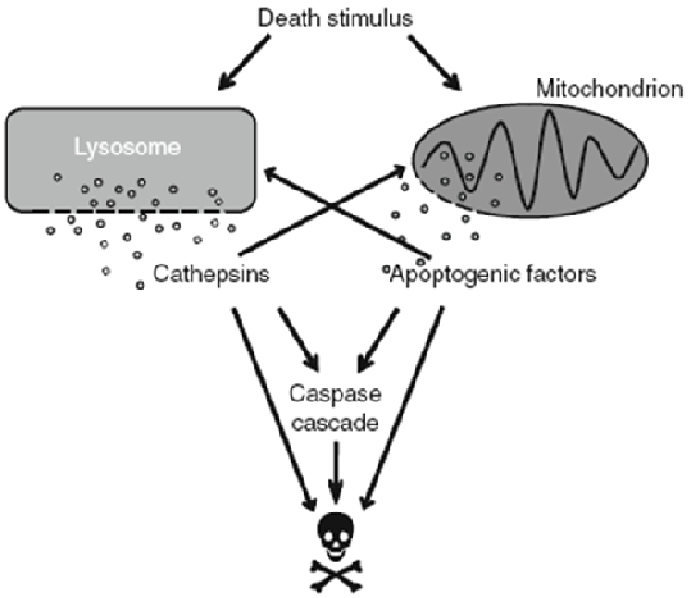

Figure 1.2. Involvement of the lysosome in cell death. Lysosomal membrane permeabilization (LMP), and consequent release of cathepsins from the lysosome into the cytosol is an important step in cell death pathways induced by several death stimuli. LMP can activate caspase-dependent and -independent pathways. Although the mitochondrial pathway is a common downstream event of LMP, cathepsins may also cause cell death without the involvement of mitochondria. Scheme from Johansson AC, *et al*. 2010.

Despite its obvious importance, the mechanism involved in LMP remains to be elucidated. However, it is now apparent that lysosomal membrane composition, including sphingolipid and cholesterol levels, plays a key role in the maintenance of lysosomal integrity [42]. On one hand, a high content of sphingolipids and cholesterol renders the lysosome membrane thicker and more ordered and rigid [42,46]. In contrast, damage to lysosomal membrane components or changes in the membrane structure and fluidity can result in lysosomal destabilization [19]. For instance, accumulation of sphingosine-like ceramide in lysosomes can induce LMP [41], which can occur when sphingomyelin present in the lysosomal membrane is converted to ceramide and further to sphingosine by acid sphingomyelinase (ASMase) and ceramidase, respectively [41]. Accordingly, it is known that generation of sphingosine is increased in TNF-α-induced apoptosis, which is accompanied by LMP. In the case of LMP triggered by oxidative stress, there is increased hydrogen peroxide diffusion into the lysosome. The acidic milieu of the lysosome lumen and the presence of low-molecular-weight iron, derived from degraded iron- containing proteins, are conducive to the reduction of iron and the generation of hydroxyl radicals.

These hydroxyl radicals induce peroxidation of membrane lipids and thereby cause leakage of lysosomal constituents into the cytosol [19].

Besides their physiological roles, cathepsins have been associated with several pathologies, such as cardiovascular diseases, osteoporosis, rheumatoid arthritis, artherosclerosis and cancer (where they are involved in cell differentiation, tumor progression, angiogenesis and metastasis, though cancer cells appear more sensitive to lysosomal destabilization and induction of LMP has thus emerged as an effective way to kill resistant cancer cells) [37,38,48,49]. Elucidating the mechanisms underlying the involvement of cathepsins in the pathogenesis of these diseases, and how they can be modulated to develop new prevention and therapeutic strategies, has therefore taken center stage. In particular, the role of CatD in apoptosis and consequently its relevance in both physiological and pathological cellular contexts is discussed below.

1.3. Opposing functions of cathepsin D in apoptosis

CatD is a lysosomal aspartic protease found in most mammalian cells [50]. Human CatD is synthesized as a precursor (52 kDa) and processed into an intermediate (~48 kDa) and then active double-form (34 kDa and 14 kDa) [50–52]. Like other cathepsins, CatD is engaged in many physiological processes such as protein degradation [26], necrosis, autophagy [52–54], and apoptosis [37,43,55], and is associated with different pathological scenarios such as cancer progression and metastasis [49,55,56], Alzheimer's disease [37], artherosclerosis [37] and inflammatory disorders [34].

In recent years, many studies have shown that CatD can have opposite roles in apoptosis. Indeed, depending on the environment, CatD can induce or inhibit apoptosis, acting through different mechanisms [50]. Some authors reported that CatD can directly induce apoptosis triggered by several stimuli, such as staurosporine [21], etoposide [37], resveratrol [43] and 5-fluorouracil [37], and that the pro-apoptotic function of CatD can be mediated both by the intrinsic or the extrinsic pathway [50]. The extrinsic pathway begins at the surface of the cell when specific ligands interact with surface death receptors, which leads to activation of caspases 8, 10 and 3[50]. Activation of caspase 3 as a terminal effector of apoptosis can be enhanced by additional activation of caspase 9 through the intrinsic pathway [50]. The role of CatD in the intrinsic pathway is linked to the lysosomal

release of mature 34 kDa CatD into the cytosol [45,55], followed by MOMP [45]; as a result of MOMP, pro-apoptotic molecules such as cytochrome *c* and AIF are released to the cytosol [45,57]. Both pathways are linked when caspase 8 induces proteolysis of Bid to truncated Bid (tBid) [50]. Mitochondrial membrane binding by tBid initiates an ordered series of events culminating in Bax-mediated MOMP [58]. Indeed, it was shown that CatD in human fibroblasts mediates cytochrome *c* release and caspase activation in staurosporine-induced apoptosis [21], and that CatD cleaves Bid and promotes apoptosis in human neutrophils via oxidative stress-induced lysosomal membrane permeabilization [59]. The involvement of CatD in caspase-independent apoptosis by activating Bax independently of Bid cleavage was also demonstrated [60]. In that study, it was reported that CatD mediates the selective release of AIF through activation of the pro-apoptotic protein Bax in a Bid-independent manner in T lymphocytes entering the early commitment phase to apoptosis [60]. Furthermore, other studies strongly suggest that cytosolic CatD may have an additional role involving protein-protein interactions. It was shown that overexpression of both catalytically active and -inactive CatD by cancer cells enhances apoptosis-dependent chemo-sensitivity [61], and that stress-induced apoptosis is not affected in fibroblasts synthesizing a catalytically inactive CatD [62]. Additionally, it was demonstrated that microinjection of the inactive pro-catD into the cytosol of both human fibroblasts and HeLa cells induces apoptosis [63].

On the other hand, other studies describe an anti-apoptotic role for CatD in cancer cells. Most of these show that CatD is up-regulated in many types of cancer (breast cancer, ovarian cancer, endometrial cancer, cancer of the head and neck, bladder cancer, malignant glioma, melanoma), and that the high levels of CatD expression are correlated with increased risk of relapse and poor prognosis [45,49,51,55]. In several cases, this is due to hypersecretion of the pro-enzyme to the tumor micro-environment, where it is proteolytically active due to the acidic pH [55]. In fact, it was reported that Pro-CatD outside the cells induces proliferation, angiogenesis, invasion and metastasis [37]. Intracellularly, CatD has been shown to protect human neuroblastoma cells from doxorubicin-induced cell death [64]. In that study, it was shown that CatD downregulation sensitizes neuroblastoma cells to doxorubicin-induced apoptosis, while the opposite effect was observed for CatD overexpression [64]. In this case, CatD attenuates apoptosis via the PI3K-Akt pathway and promotes drug resistance independently of p53 signaling [64]. Additionally, it was demonstrated that inhibition of CatD with pepstatin A induces

caspase-dependent apoptosis in neuroblastoma cell lines [65] and that overexpression of intracellular CatD in mouse xenografs using rat-derived cell lines inhibits apoptosis [55]. Another study in glioblastoma cells proposed that CatD stimulates induction of autophagy, inhibiting apoptotic cell death under genotoxic conditions [53]. As discussed below in more detail, it was also shown that inhibition of CatD sensitizes CRC-derived cells to acetate-induced apoptosis. These results demonstrate an important role of CatD in anti-apoptotic signaling in cancer cells and suggest a mechanism for the development of chemotherapy resistance in many types of tumors. However, the exact role of CatD in apoptosis, particularly what determines whether this protease plays an anti-apoptotic or pro-apoptotic function, and how to exploit them in cancer therapy, remains poorly understood.

1.4. Vacuolar proteases as players in yeast cell survival and death

The yeast vacuole is functionally similar to the lysosome of higher eukaryotes, in particular regarding proteolysis. It harbors seven characterized proteases, namely three aminopeptidades, three serine proteases and one aspartyl protease. Among these, two are endopeptidases, proteinases A (Pep4p) and B (Prb1p), and five are exopeptidases, carboxypeptidases Y (CPY) and S (CPS), aminopeptidases I (Ape1) and Y (Ape3), and dipeptidylaminopeptidase B (Dap2). More recently, Hecht *et al.* reported an eighth vacuolar protease, a transmembrane metalloprotease (Pff1) [66]. Although evidence of Pff1 vacuolar localization was shown, its proteolytic activity has yet to be demonstrated. Yeast vacuolar proteases, like their lysosomal counterparts, are mainly synthesized as inactive zymogens, traveling via the ER and Golgi to the acidic vacuoles, where they are activated through the proteolytic removal of the inhibitory propeptide (reviewed in [67]).

Endopeptidases are responsible for the majority of bulk protein degradation, including of plasma membrane proteins, as well as for the activation of the vacuolar proteolytic cascade. Using null mutants, it was demonstrated that Pep4p is a key enzyme in the vacuolar protease activation cascade, since it is involved in proteolytic activation of Prb1p, CPY and Ape1 [68,69]. Prb1p, in turn, participates in the activation of Pep4p, CPY, CPS, Ape1 and Ape3. Both carboxypeptidases and Ape1 are involved in peptide and glutathione degradation, respectively, but are not required for zymogen activation [69,70].

Substrates of the vacuolar proteases are mostly imported via endocytosis (extracellular and cell surface proteins) or autophagy (cytoplasmic material and organelles). Using yeast strains lacking Pep4p and Prb1p, it was shown that active autophagy occurs under nutrient deprivation conditions, as well as that both proteases are implicated in the dissolution of autophagic bodies [71]. In addition, vacuolar proteases have been shown to play a role in sporulation. While absence of Prb1p activity alone results in partial reduction of sporulation, absence of Prb1p activity in a mutant lacking both CPY and CPS leads to almost complete loss of sporulation ability [72]. Vacuolar proteolysis therefore appears to be mainly a stress-responsive process, particularly under nutrient stress conditions and sporulation. However, additional roles for vacuolar peptidases have emerged in recent years. Indeed, although vacuolar proteases are mainly located in the vacuole, migration of Pep4p to the cytosol was observed in response to different cell death stimuli, including H_2O_2 [22], actin stabilization [23] and acetic acid [24]. Both vacuolar membrane permeabilization and the role of Pep4p in yeast programmed cell death are discussed below.

1.5. Vacuolar membrane permeabilization and regulated cell death in yeast

During the last years, it has become apparent that core components of the RCD machinery are conserved in yeast. Moreover, yeast can undergo RCD that exhibits typical markers of apoptosis, autophagy and necrosis (reviewed in [2,73–75]). Thus, this eukaryotic organism has been used as a relevant model to study the molecular mechanisms of RCD pathways. The role of the lysosome-like vacuole in the regulation of RCD has also been investigated in yeast, where it has been demonstrated that the vacuole has a function similar to lysosomes in the regulation of this process [76,77].

Like in mammalian LMP, VMP and consequent release of vacuolar proteins into the cytosol can also occur in yeast. The first report relating the vacuole with yeast cell death showed that the vacuolar protease Pep4p, a pepsin-like aspartic protease ortholog to human CatD, translocates from the vacuole to the cytosol and is involved in the degradation of nucleoporins following H_2O_2-induced apoptosis [22]. In H_2O_2-treated cells, the release of a Pep4p-EGFP (Enhanced Green Fluorescent Protein) fusion from the vacuole is not associated with major rupture of the vacuolar membrane, as cells maintain a

vacuolar lumen morphologically distinct from the cytosol. This is indicative of a selective VMP, as observed in the lysosomes of apoptotic mammalian cells. Later, it was shown under this same VMP- and death- inducing conditions that the RNase T2 family member Rny1p is also released from the vacuole into the cytosol, directly promoting cell death independently of its catalytic activity [25]. In that study, Rny1p-GFP was localized in the vacuole of control cells, but the levels of fluorescence decreased after exposure to H_2O_2, which indicates that Rny1p is released from the vacuole. Meanwhile, the vacuolar luminal dye CMAC (7-amino-4-chloromethylcoumarin) was retained in the vacuoles, suggesting that vacuolar membrane integrity is preserved during oxidative stress.

VMP and release of Pep4p-EGFP into the cytosol was also observed in *END3*-deficient cells, exhibiting apoptotic cell death induced by actin cytoskeleton stabilization [23]. In that study, Pep4p-EGFP was visualized exclusively in the vacuole lumen in wild type cells by fluorescence microscopy, while in *END3*-deficient cells appeared distributed throughout the entire cell. In another study, it was shown that deletion of class C vacuolar protein sorting genes results in drastically enhanced sensitivity of yeast to treatment with acetic acid and leads to a necrotic death, while death is mainly apoptotic in the wild type strain [78], indicating that a functional vacuole is required for an RCD process through apoptosis. More recently, it was found that Pep4p-EGFP also translocates to the cytosol during acetic acid-induced apoptosis involving selective VMP typical of apoptotic death [24], showing by transmission electron microscopy that acetic acid-treated cells preserve both vacuolar and plasma membrane integrities. In addition, VMP promoted by vacuolar H^+-ATPase was also observed in tunicamycin-induced necrosis [79], where fluorescence microscopy of yeast cells stained with the fluorescent dye FM4-64, which stains the vacuolar membrane, showed that tunicamycin induces vacuolar fragmentation in a manner dependent on V-ATPase.

Finally, the involvement of VMP in programmed nuclear destruction (PND) during yeast gametogenesis was also reported [80]. Using cells co-expressing Pep4p-mCherry (red fluorescent protein) and a GFP-tagged vacuolar membrane protein (Vma1-GFP), the authors showed that Pep4p translocates from the vacuole into the ascal compartment of early postmeiotic cells during sporulation, with preservation of vacuolar integrity. Altogether, these data provide evidence that the vacuole is intrinsically connected with modulation of RCD in yeast. Therefore, both vacuole and mitochondria destabilization appear to be events in the RCD cascade.

1.6. Yeast cathepsin D plays opposing roles in cell fate

As mentioned above, the involvement of the yeast CatD (Pep4p) in RCD was first demonstrated in hydrogen peroxide [22] and actin cytoskeleton stabilization-induced apoptosis [23]. In the first study, an increase in nuclear permeability associated with an increase in ROS accumulation during H_2O_2-induced cell death was observed. Later, after loss of cell viability, Pep4p is released into the cytosol and degrades nucleoporins [22]. However, Pep4p deficient cells are not more resistant to H_2O_2-induced cell death, probably because Pep4p migrates out of vacuoles and degrades nucleoporins after the cells are effectively unviable. In the second study, the authors showed that stabilization of the actin cytoskeleton caused by lack of the End3 protein leads to loss of mitochondrial membrane potential, accumulation of ROS, increase in VMP and consequent migration of Pep4p to the cytosol, as well as apoptotic cell death [23]. However, no role was ascribed for this protease in actin-stabilized dying cells. It was also reported that Pep4p is involved in protein degradation and removal of oxidized proteins during H_2O_2-induced cell death, and that it has a pro-survival role during chronological aging, since a Pep4p-deficient mutant displayed a shortened lifespan associated with higher levels of carbonylated proteins [81].

More recently, it was found that Pep4p also translocates to the cytosol and has a pro-survival role during acetic acid-induced apoptosis. *PEP4* disrupted cells displayed higher susceptibility to acetic acid, while the opposite phenotype was observed for *PEP4* overexpression [24]. In addition, the role of Pep4p in the autophagic process was also reported. In fact, it was observed that deletion of Pep4p results in an accumulation of authophagic bodies in the vacuole under nutrient-deficient conditions [71,82]. Surprisingly, it was shown that, once in the cytosol, Pep4p also plays an important role in mitochondrial degradation through an autophagic-independent process [24]. Depletion and overexpression of Pep4p delayed and enhanced mitochondrial degradation in response to acetic acid, respectively.

Degradation of mitochondria during mammalian apoptosis is a widespread phenomenon, in which lysosomes play an important role [83,84]. Though removal of mitochondria is mainly mediated by mitophagy/autophagy, it still occurs normally in cells where autophagy is abrogated, implying the existence of alternative pathways. Since autophagy is not active in yeast cells undergoing acetic acid-induced apoptosis, VMP

associated with the release of Pep4p may act as an alternative mitochondrial degradation process. Although the molecular role of Pep4p in mitochondrial degradation is unknown, it is apparent that it also involves non-vacuolar proteins. In fact, it was showed that absence of ADP/ATP carrier (AAC) proteins is also associated with a decrease in mitochondrial degradation during apoptosis induced by acetic acid. Moreover, Pep4p is still released from the vacuole in *AAC*-deleted cells [24]. These results indicate that degradation of mitochondria depends on the mitochondrial AAC proteins at a step downstream of Pep4p release.

Another study found that Pep4p has a dual pro-survival role composed of both anti-apoptotic and anti-necrotic functions, conferred by its proteolytic activity and its proteolitically inactive propeptide, respectively [85]. The authors showed that deletion of *PEP4* results in both apoptotic and necrotic cell death during chronological aging, while prolonged overexpression promotes survival by reducing necrosis. In addition, they demonstrated that the anti-necrotic function of Pep4p that mediates lifespan extension involves histone hypoacetylation and depends on polyamine biosynthesis, through increased intracellular levels of putrescine, spermidine and its precursor S-adenosyl-methionine. In contrast, in a functional genomics screen of the yeast Euroscarf knock-out mutant collection for differential sensitivity to acetic acid, it was found that absence of *PEP4* resulted in increased resistance to acetic acid-induced RCD [86], which was confirmed by viability assays using a newly constructed *pep4*Δ mutant in the BY4741 background. These results, in contrast with those previously described in W303 cells [24], suggest that Pep4p may play a dual function in acetic acid-induced RCD depending on the different mitochondrial mass of the strain [87,88]. Since the involvement of Pep4p in mitochondrial degradation protects yeast cells during acetic acid-induced RCD [24] a lower mitochondrial mass may explain the pro-apoptotic role of Pep4p in BY4741 cells.

As mentioned above, human CatD is also involved in both apoptosis and necrosis, and can also have both anti-survival and pro-survival roles depending on cellular type and context. Whether human CatD also plays a role in mitochondrial degradation, as well as whether its proteolytic activity is required for its role in cell fate has been recently addressed by us in CRC cell lines, as discussed below.

1.7. Role of cathepsin D in colorectal cancer – insights from the yeast model

As discussed in the sections above, CatD plays several important roles in cancer. Indeed, this protease can have both pro- and anti-survival functions depending on its proteolytical activity, tumor cell type, stress stimulus and context [21,39,59–64,89]. Moreover, it has been demonstrated that CatD is often overexpressed and hypersecreted by human cancer cells, including some CRC cells [37,55,90,91]. For instance, elevated secretion of pro-CatD along with a tumor-generated acidic extracellular environment was observed in the AA/CI/SB10/M human colorectal adenocarcinoma cell line [92]. In that study, the authors demonstrated that the medium of adenocarcinoma cells displays more secreted CatD than the non-tumorigenic cell line. In addition, both the increase in the amount of CatD secreted and the acidic tumor microenvironment coincided with the presence of mature CatB in the medium of the more malignant cell lines. In the case of colorectal carcinoma, the role of CatD remains controversial. In fact, divergent patterns of CatD expression in CRC clinical cases were reported [93–99]. Highly increased CatD expression in main tumor body (MTB) cells in late stage CRC, showing significant correlation with subsequent distant metastasis and shorter cancer-specific survival, was recently reported [56]. In addition, CatD expression was found to be an independent prognostic marker for poorer colorectal cancer-specific survival, suggesting that this protease can be used in the future as an indicator to identify patients with both early-stage cancer and a potentially poor prognosis [100]. Increased activity of CatD in CRC has also been shown. A study by Waszkiewicz N. *et al.* demonstrated an increased degradation and remodeling of glycoconjugates in colon adenocarcinoma tissue associated with an increase in the specific activity of both lysosomal exoglycosidases and CatD [101].

CatD has also been attributed a pro-death role in an *in vitro* study with resveratrol, which triggers mitochondria-dependent apoptosis in CRC [43]. Using DLD1 and HT29 cell lines, the authors showed that resveratrol induces overexpression of CatD, lysosomal membrane permeabilization and consequent translocation of CatD into the cytosol, as well as MOMP and caspase activation. Moreover, both Pepstatin A and knockdown of CatD expression by RNA interference prevents resveratrol toxicity, impeding Bax oligomerization, mitochondrial membrane permeabilization, cytochrome *c* release and caspase 3 activation. These data indicated that both lysosomal and mitochondrial

membrane permeabilization are implicated in resveratrol-induced apoptosis in CRC cells, and showed that human CatD is involved in this process, acting upstream of caspase activation.

More recently, it was shown that acetate, previously implicated in mitochondria-dependent apoptosis in CRC, also leads to LMP and consequent migration of CatD to the cytosol, as well as apoptotic cell death in CRC cell lines [102]. Indeed, acetate induced DNA fragmentation, caspase activation, cell surface exposure of phosphatidylserine and the appearance of a sub-G1 population in the CRC-derived cell lines HCT-15 and RKO. However, CatD release protected CRC cells from acetate- induced apoptosis instead of triggering apoptosis as in response to resveratrol treatment, since inhibition of this protease with Pepstatin A increased apoptosis in the human CRC cell line HCT-15 [102]. These results indicate that CatD has a protective role rather than a pro-apoptotic function in this process, as was shown for its ortholog Pep4p in yeast acetic acid-induced apoptosis [24]. Furthermore, acetate in CRC cells seems to behave as acetic acid in yeast, triggering LMP, CatD release and mitochondria-dependent apoptosis. In fact, it has been demonstrated that acetate produced by human intestinal propionibacteria induces nuclei shrinkage, chromatin condensation, nuclei fragmentation into apoptotic bodies and activation of pro-caspase 3 in human CRC-derived cell lines [103]. In addition, acetate-induced apoptotic cell death involves mitochondrial outer membrane permeabilization, ROS accumulation and the mitochondrial adenine nucleotide translocator (ANT) [104].Thus, as observed in yeast cells, it seems apparent that both lysosomes and mitochondria are involved in acetate-induced apoptosis of CRC cells.

In yeast, the mitochondrial contribution to RCD in response to acetic acid has been intensively investigated (reviewed in [12,105]), and alterations in mitochondria similar to those observed in CRC cells in response to acetate were identified, including mitochondrial swelling and decrease of mitochondrial membrane potential ($\Delta\Psi m$) [106], mitochondrial fragmentation/degradation [107], production of ROS and MOMP, with consequent release of pro-apoptotic factors like cytochrome *c*, Aif1p and Nuc1p (yeast ortholog of EndoG) [5,13,14]. In addition, the yeast orthologs of the mammalian VDAC (voltage-dependent anion channel) and ANT were shown to play a role in MOMP and cytochrome *c* release during acetic acid-induced apoptosis in yeast [15]. Deletion of *POR1* (yeast VDAC) enhances apoptosis triggered by acetic acid, whereas absence of AAC proteins, orthologs of the ANT, protects cells exposed to acetic acid and impairs MOMP and cytochrome *c*

release. It was also shown that the pro-death role of AAC does not depend on the translocase activity, suggesting that acetic acid might induce AAC to form an unregulated channel leading to an increase in mitochondrial volume and to MOMP, either directly or through upstream signaling events [108]. Notably, acetate was proposed to target mitochondrial ANT since it specifically permeabilises liposomes containing ANT [104].

Similarly to the role of Pep4p in mitochondrial degradation and cell survival through an autophagic-independent process we also found that acetate blocks starvation-induced autophagy in human CRC cells with a decrease in the levels of autophagy-related proteins such as Beclin-1 (involved in autophagosome-lysosome fusion regulation), Atg5 (early autophagosomal marker, essential for autophagosome formation) and LC3-II (product of the authophagic flux). Thus, as we discuss below for yeast, impairment of autophagosome and lysosome fusion during acetate induced-apoptosis in CRC cells, associated with the release of CatD into the cytosol, may provide the conditions for degradation of dysfunctional mitochondria through a process alternative to autophagy with a similar protective role in cell survival. Indeed, we also show that inhibition of CatD by both RNA interference and pepstatinA enhances apoptosis associated with higher mitochondrial dysfunction and increases mitochondrial mass, mimicking the effect of Pep4p deficiency in yeast. However, while deleting *PEP4* in yeast only results in a delay in mitochondrial degradation in response to acetic acid, inhibiting CatD in RKO cells leads to an increase in mitochondrial mass after exposure to acetate. This could be due to more prominent consequences of autophagy inhibition in these cells, which present high levels of basal autophagy, than in yeast, or to specific degradation of mitochondria by the released CatD (since the E64d CatB specific inhibitor does not increase cell death), whereas other yeast vacuolar proteases eventually released could ensure mitochondrial degradation, though in a less rapid manner.

These results, combined with the hints provided by the yeast cell model, support the idea that LMP associated with the release of CatD protects CRC cells from mitochondrial dysfunction during acetate-induced apoptosis through its involvement in degradation of damaged mitochondria. Thus, it has become apparent that the approaches with yeast have already provided and can further offer new perspectives for an enhanced understanding of the role of CatD in mammalian apoptosis, as well of the molecular basis of the crosstalk between the lysosome and mitochondria. Elucidating the molecular mechanisms underlying the involvement of CatD in mitochondrial degradation will be crucial to

develop novel strategies to specifically inhibit this protease in apoptosis deficiency-associated diseases, such as cancer.

1.8. Main goals and thesis overview

As discussed previously in this chapter, the studies with yeast have provided valuable insights on the role of CatD in mammalian apoptosis, as well on the regulation and interplay between mitochondria and the lysosome in RCD. The mail goal of this thesis was to exploit acetic acid-induced apoptosis in *Sacharomyces cerevisiae* to study the yeast vacuolar protease Pep4p, both concerning its role in mitochondrial degradation and its involvement in the course of apoptosis. We expected that the approaches planned with yeast, harbouring a lysosome-like vacuole, would more efficiently provide new clues on the crosstalk between lysosomal components and mitochondria than the more complex mammalian cells.

Chapter 2 includes the materials and methods used in the present work. All experimental results are presented in Chapter 3: in Subchapter 3.1 the interplay between yeast CatD and mitochondrial proteins involved in apoptosis regulation was studied. In addition, experimental evidence is included showing that the role of yeast CatD in acetic acid-induced apoptosis and mitochondrial degradation depends on its catalytic activity and is complemented by human CatD. Evidence demonstrating that the protective role of yeast CatD in acetic acid-induced apoptosis depends on mitochondrial respiration are included in Subchapter 3.2. In Subchapter 3.3 we describe and discuss the development of a split-ubiquitin yeast membrane two-hybrid assay to find new protein substrates/partners of Pep4p. Finally, Chapter 4 is dedicated to the discussion, including concluding remarks and future perspectives, highlighting the main contributions of the present work to the understanding of the role of Pep4p in mitochondrial alterations occurring during RCD and the use of the yeast model as a powerful tool for these studies.

Chapter 2

Materials and methods

2.1. Strains and plasmids

Saccharomyces cerevisiae strains used are listed in Table 1. *pep4*Δ and *aac1/2/3*Δ*pep4*Δ mutants were constructed in W303-1A (MATa, *ura3-1, trp1-1, leu2-3, 112, his3-11,15 ade2-1, can1-100)* and JL1-3Δ2Δ3, respectively, by homologous recombination using a *PEP4*::*kanMX4* disruption cassette amplified from the respective Euroscarf deletion strain by PCR. The *kanMX4* cassette in a *POR1*::*kanMX4* strain (Euroscarf) was replaced with *URA3* by transformation with a *kanMX4*::*URA3* cassette. After selection of uracil-resistant, G418-sensitive colonies, a fragment containing *POR1*::*URA3* was amplified by colony PCR and transformed into W303-1A and W303-1A *pep4*Δ strains to obtain *por1*Δ and *pep4*Δ*por1*Δ mutants, respectively. The *pep4*Δ mutant was also constructed in W303-1A (MAT*a*, *ade2*, *his3*, *leu2*, *trp1*, *ura3*, *can1*), by homologous recombination as described above. Correct integration of the cassettes was confirmed by PCR. To construct $CatD^{FLAG}$, the insert was amplified by PCR from the plasmid pJP1520-*CTSD* (containing human cathepsin D cDNA) and integrated by homologous recombination into the pESC-His vector. Correct integration was verified by sequencing.

For depletion of the mtDNA (Rho^0), W303-1A and W303-1A *pep4*Δ strains were grown in YPD medium containing 10 µg/ml ethidium bromide for 24 hours. Respiratory deficiency was confirmed by complete lack of growth on glycerol.

Strains were transformed with pYX232-mtGFP or pGAL-CLbGFP vectors for mitochondrial fragmentation/degradation studies. The *aac1/2/3*Δ strain was transformed with the empty vector (pDP34) and the pDP34-*PEP4* vector for wild type Pep4p (WT-Pep4p) overexpression. The *aac1/2/3*Δ*pep4*Δ mutant was transformed with the empty vector (pRS314) and the pRSop1 vector expressing a R96H mutated form of Aac2p (op1). Wild type strains were transformed with the empty vector (pESC) and the *pep4*Δ strains were transformed with the empty vector (pESC), pESC-*PEP4*, pESC-*DPM* or pESC-*CTSD* plasmids for expression of WT-Pep4p, double point mutant (DPM-Pep4p) or human CatD, respectively.

All transformations were performed by the lithium acetate method.

Table 1. List of *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Reference/ Source
W303-1A	MATa, *ura3-1, trp1-1, leu2-3, 112, his3-11,15 ade2-1, can1-100*	R. Rothstein
*pep4*Δ	W303-1A *pep4* :: *kanMX4*	This study
*por1*Δ	W303-1A *por1* :: *URA3*	This study
*pep4*Δ *por1*Δ	W303-1A *pep4* :: *KanMX4 por1*:: *URA3*	This study
W303-1A pYX232-mtGFP	W303-1A harboring pYX232-mtGFP	This study
*pep4*Δ pYX232-mtGFP	W303-1A *pep4*Δ harboring pYX232-mtGFP	This study
*por1*Δ pYX232-mtGFP	W303-1A *por1*Δ harboring pYX232-mtGFP	This study
*pep4*Δ *por1*Δ pYX232-mtGFP	W303-1A *pep4*Δ *por1*Δ harboring pYX232-mtGFP	This study
JL-3Δ2Δ3 (*aac1/2/3*Δ)	MATα, *leu2-3*, *112*, *his3-11, 15*, ade2-1, *trp1-1*, *ura3-1*, *can1-100*, *aac1* :: *LEU2*, Δ*aac2* :: *HIS3*, Δ*aac3*	[109]
*aac1/2/3*Δ *pep4*Δ	*aac1/2/3*Δ *pep4* :: *kanMX4*	This study
*Aac1/2/3*Δ pDP34	*aac1/2/3*Δ harboring pDP34	This study
*Aac1/2/3*Δ pDP34-*PEP4*	*aac1/2/3*Δ harboring pDP34-*PEP4*	This study
*aac1/2/3*Δ *pep4*Δ pRS314	*aac1/2/3*Δ *pep4*Δ harboring pRS314	This study
*aac1/2/3*Δ *pep4*Δ pRS314 op1	*aac1/2/3*Δ *pep4*Δ harboring pRS314 op1	This study
W303 pESC	W303-1A harboring pESC	This study
*pep4*Δ pESC	W303-1A *pep4*Δ harboring pESC	This study
*pep4*Δ pESC-*PEP4*	W303-1A *pep4*Δ harboring pESC-*PEP4*	This study
*pep4*Δ pESC-*DPM*	W303-1A *pep4*Δ harboring pESC-*DPM*	This study
W303 Rho^0	W303-1A lacking mitochondrial DNA	This study
*pep4*Δ Rho^0	W303-1A *pep4*Δ lacking mitochondrial DNA	This study
W303 Rho^0 pYX232-mtGFP	W303-1A Rho^0 harboring pYX232-mtGFP	This study
*pep4*Δ Rho^0 pYX232-mtGFP	W303-1A *pep4*Δ Rho^0 harboring pYX232-mtGFP	This study
BY4741	MATa, *his3*Δ*1, leu2*Δ*0*, *met15*Δ*0*, *ura3*Δ*0*	Euroscarf
*pep4*Δ	BY4741 *pep4* :: *kanMX4*	Euroscarf
BY4741 pGAL-CLbGFP	BY4741 harboring pGAL-CLbGFP	This study
*pep4*Δ pGAL-CLbGFP	BY4741 *pep4*Δ harboring pGAL-CLbGFP	This study
BY4741 pESC	BY4741 harboring pESC	This study
*pep4*Δ pESC	BY4741 *pep4*Δ harboring pESC	This study
*pep4*Δ pESC-*PEP4*	BY4741 *pep4*Δ harboring pESC-*PEP4*	This study
*pep4*Δ pESC-*DPM*	BY4741 *pep4*Δ harboring pESC	This study
BY4741 pESC + pGAL-CLbGFP	BY4741 harboring both pESC and pGAL-CLbGFP	This study
*pep4*Δ pESC + pGAL-CLbGFP	BY4741 *pep4*Δ harboring both pESC and pGAL-CLbGFP	This study
*pep4*Δ pESC-*PEP4* + pGAL-CLbGFP	BY4741 *pep4*Δ harboring both pESC-*PEP4* and pGAL-CLbGFP	This study
*pep4*Δ pESC-*DPM* + pGAL-CLbGFP	BY4741 *pep4*Δ harboring both pESC-*DPM* and pGAL-CLbGFP	This study
W303-1A	MATa, *ade2*, *his3*, *leu2*, *trp1*, *ura3*, *can1*	
*pep4*Δ	W303-1A *pep4* :: *kanMX4*	This study
W303 pESC	W303-1A harboring pESC	This study
*pep4*Δ pESC	W303-1A *pep4*Δ harboring pESC	This study
*pep4*Δ pESC-*PEP4*	W303-1A *pep4*Δ harboring pESC-*PEP4*	This study
*pep4*Δ pESC-*DPM*	W303-1A *pep4*Δ harboring pESC-*DPM*	This study

Table 1. List of *Saccharomyces cerevisiae* strains used in this study (continuation).

Strain	Genotype	Reference/ Source
*pep4*Δ pESC-*CTSD*	W303-1A *pep4*Δ harboring pESC-*CTSD*	This study
W303 pESC + pYX232-mtGFP	W303-1A harboring both pESC and pYX232-mtGFP	This study
*pep4*Δ pESC + pYX232-mtGFP	W303-1A *pep4*Δ harboring both pESC and pYX232-mtGFP	This study
*pep4*Δ pESC-*PEP4* + pYX232-mtGFP	W303-1A *pep4*Δ harboring both pESC-*PEP4* and pYX232-mtGFP	This study
*pep4*Δ pESC-*DPM* + pYX232-mtGFP	W303-1A *pep4*Δ harboring both pESC-*DPM* and pYX232-mtGFP	This study
*pep4*Δ pESC-*CTSD* + pYX232-mtGFP	W303-1A *pep4*Δ harboring both pESC-*CTSD* and pYX232-mtGFP	This study

2.2. Growth conditions and treatments

Strains were grown in synthetic complete medium [SC; 0.67% (w/v) yeast nitrogen base without aminoacids, 2% (w/v) glucose, 0.14% (w/v) drop-out mixture lacking histidine, leucine, tryptophan and uracil, 0.008% (w/v) histidine, 0.04% (w/v) leucine, 0.008% (w/v) tryptophan and 0.008% (w/v) uracil] to early exponential phase (OD_{600nm} = 0.5–0.6) at 30ºC in an orbital shaker at 200 rpm, with a ratio of flask volume/medium of 5:1. Strains transformed with plasmids were grown in the same medium but without the appropriate amino acids. For strains carrying a pGAL-CLbGFP and/or pESC plasmids, 2% galactose (SCG) was used for induction of expression. For acetic acid treatment, strains were cultured under the conditions described above, harvested and suspended in SC or SCG at pH 3.0 (set with HCl) containing 120 mM of acetic acid and incubated for up to 200 min at 30ºC, in an orbital shaker at 200 rpm. Inhibition of oxidative phosphorylation was performed by adding 5 µg/ml oligomycin (Merck) to the growth and treatment medium. Control cells were grown and treated with ethanol (0.25%, v/v), used as solvent for oligomycin. To inhibit glycolysis, strains were pre-incubated with 100 nM 2-deoxyglucose (Sigma–Aldrich) for 10 minutes. Cell viability was measured as a percentage of colony forming units (c.f.u.) on YPD agar plates.

2.3. Propidium iodide staining

Plasma membrane integrity was assessed by flow cytometry using propidium iodide (PI) (Sigma–Aldrich) staining. Acetic acid-treated cells were harvested by centrifugation, resuspended in 500 µl PBS (80 mM Na_2HPO_4, 20 mM NaH_2PO_4 and 100 mM NaCl) and incubated with 5 µg/ml PI for 20 min in the dark. Cells with red fluorescence [FL-3 channel (488/620 nm)] were considered to contain plasma membrane disruption.

2.4. DHE staining

ROS production was quantified by flow cytometry using dihydroethidium (DHE) (Molecular Probes, Eugene, USA) staining. Acetic acid-treated cells (120 mM) were harvested by centrifugation, resuspended in 500 µl PBS and incubated with 5 µg/ml DHE for 30 min in the dark. Cells with red fluorescence [FL-3 channel (488/620 nm)] were considered to accumulate superoxide anion.

2.5. Pep4p activity assay

Cells were grown as described above, and harvested at the exponential phase (OD_{600nm} = 0.7–0.9) without acetic acid treatment. Yeast extracts were prepared in 0.1 M Tris, pH 7.6, by vigorous shaking of the cell suspension in the presence of glass beads, for 5 min. Short pulses of 1 min were used, with 1 min intervals on ice. Pep4p activity was determined using 0.250 mg total protein, by measuring the release of tyrosine-containing acid-soluble peptides from acid-denatured haemoglobin [expressed as µg Tyr min^{-1} (mg protein)$^{-1}$ [110].

2.6. Mitochondrial fragmentation

Mitochondrial network fragmentation of cells transformed with a plasmid expressing mitochondrial GFP (pYX232-mtGFP) was analyzed using a fluorescence microscope. One hundred cells per sample were counted as “thread” or “grain”.

2.7. Mitochondrial degradation

Mitochondrial degradation was determined by flow cytometry using cells transformed with a plasmid expressing mitochondrial GFP (pYX232-mtGFP or pGAL-CLbGFP). The percentage of cells that still exhibit mtGFP fluorescence after exposure to acetic acid (120 mM) was determined in biparametric histograms [ratio (FL-1 area (log)/FS (log)) × GFP fluorescence (FL-1 peak)] to eliminate variations in fluorescence due to cell size and to discriminate between the cells with intense spots of mitochondrial-GFP and diffuse GFP resultant from mitochondrial degradation.

2.8. Western blot analysis

Protein samples for Western blot were prepared by suspending approximately $2x10^6$ cells in 0.5 ml of water and adding 50 µl of a mixture of 3.5% β-mercaptoethanol in 2 M NaOH. After a15 min incubation on ice, proteins were precipitated with 50 µl of 3 M Trichloracetic acid for 15 min on ice. After a rapid centrifugation, the pellet was resuspended in Laemmli buffer for SDS-PAGE. The primary antibodies used were mouse monoclonal anti-yeast FLAG (1:5000, Sigma) and mouse monoclonal anti-yeast phosphoglycerate kinase (PGK1) (1:5000, Molecular Probes). Secondary antibody against mouse IgG, coupled to horseradish peroxidase (Jackson Laboratories), was used at 1:10000 and revealed by chemiluminescence (ECL, Amersham).

2.9. Chronological aging assay

Yeast cells were growth in SC medium for two days, until stationary phase, at 30ºC in an orbital shaker at 200 rpm, with a ratio of flask volume/medium of 5:1. The first age-points (day 0) were taken and cultures were incubated under the same conditions for up to 15 days. Cultures were performed in triplicate. Cell viability was measured as a percentage of colony forming units (c.f.u.) on YPD agar plates.

2.10. Flow cytometry assays

Flow cytometry assays were performed in an Epics® XL™ (Beckman Coulter) flow cytometer equipped with an argon-ion laser emitting a 488-nm beam at 15 mW. Thirty thousand cells per sample were analyzed. Data were analyzed with WinMDI 2.8 software.

2.11. Split-ubiquitin membrane yeast two-hybrid assays

2.11.1. Bait construction and expression verification

In order to detect an interaction between Pep4p and Por1p, a cDNA sequence encoding Por1p was cloned into the pBT3-N bait vector (Figure 2.1A), fused to the C-terminal half of ubiquitin (Cub) followed by a transcription factor (LexA-VP16). To identify proteins that interact with Pep4p, a cDNA sequence encoding a cytosolic Pep4p was cloned into the pCMBV4 bait vector (Figure 2.1B) in frame with the Cub-LexA-VP16 reporter moiety and the membrane protein Ost4p, which anchors the fusion in the endoplasmic reticulum membrane. These constructs were then separately transformed into the reporter yeast strain DSY-1. Correct expression and lack of self-activation of both baits was assayed using the controls provided by the manufacturer (pAlg5-NubI as a positive control prey and pAlg5-NubG as a negative control prey) (Dualsystems Biotech). 3-Aminotriazole (3-AT), a competitive inhibitor of the *HIS3* gene product, was added to the selection medium to increase the threshold of selection.

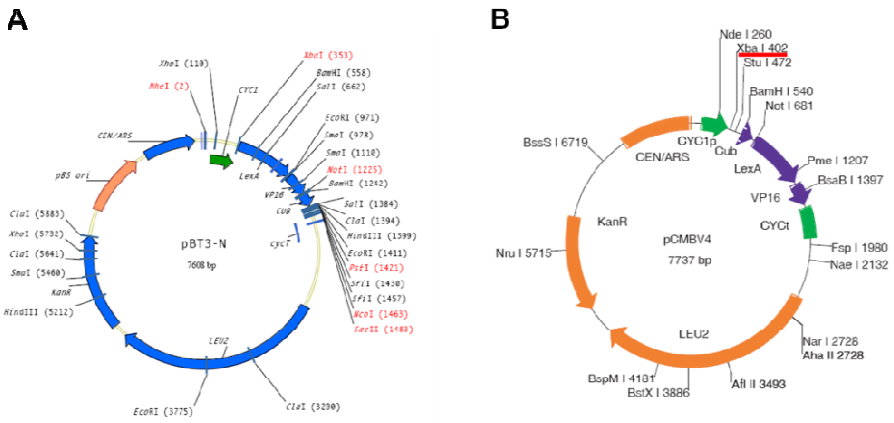

Figure 2.1. Bait plasmids used in split-ubiquitin membrane yeast two-hybrid assays. (A) pBT3-N bait vector, which contains a *LEU2* gene for auxotrophic selection in yeast and a LexA-VP16-Cub cassette, followed by a multiple cloning site and expresses the protein of interest as a LexA-VP16-Cub-Bait fusion (fusion at N-terminus of protein of interest). (B) pCMBV4 bait vector, which contains a *LEU2* gene for auxotrophic selection in yeast and a multiple cloning site, followed by a LexA-VP16-Cub cassette and expresses the protein of interest as a Bait-LexA-VP16-Cub fusion. Figure adapted from Dualsystems Biotech [www.dualsystems.com].

2.11.2. Prey vector construction and transformation

A cDNA sequence encoding a cytosolic Pep4p was cloned into the pDL2 prey vector, fused to the N-terminal half of ubiquitin (NubG) (Figure 2.2). This construct was then transformed into the bait (pBT3-Por1) bearing strain. Lack of self-activation of the prey was assayed using the control bait pMBV-Alg5. If Por1p and Pep4p interact, Cub and NubG complement to form split ubiquitin, which attracts cleavage by ubiquitin proteases. As a result, the transcription factor is released and translocates to the nucleus, where it can activate transcription of reporter genes (*HIS3* and *lacZ*). This results in blue cells in the presence of X-Gal and growth of the cells on agar plates lacking histidine.

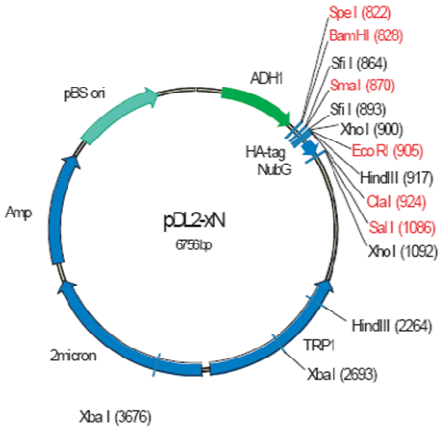

Figure 2.2. Prey plasmid used in split-ubiquitin membrane yeast two-hybrid assay. pDL2-xN prey vector, which contains a *TRP1* gene for auxotrophic selection in yeast and expresses cDNA as an N-terminal fusion to the NubG cassette. Figure adapted from Dualsystems Biotech [www.dualsystems.com].

2.11.3. Filter assay for detection of β-galactosidase activity

Yeast colonies were first transferred to a Whatman filter paper, lysed by a freeze/thaw cycle using liquid nitrogen and then overlayed with an agarose mixture containing the β-galactosidase substrate X-Gal [1x PBS pH 7.4, 0.5% (w/v) agarose, 0.1 mg/ml X-Gal]. Yeast expressing β-galactosidase convert X-Gal into a blue-colored compound, thus the yeast will "turn blue".

2.11.4. Library transformation and selection of positive clones

A yeast cDNA library NXO19 was amplified and then transformed into the bait (pCMBV4-Pep4) bearing strain. Positive interactions resulting in activation of reporter genes *HIS3* and *LacZ* was assessed by growth selection on medium lacking the amino acids tryptophan, leucine and histidine (SD -trp -leu -his medium) and a color readout using a substrate for the enzyme ß-galactosidase, respectively. Colonies that tested positive in both assays were picked up and restreaked on selective plates. These plates were again subjected to a ß-galactosidase test. Only colonies that were positive in both tests were

selected for the next step. Library plasmids were then isolated from all positive clones and retransformed into *E. coli*. Finally, plasmid DNA was prepared.

2.11.5. Bait dependency test

The isolated library plasmids were retransformed into the yeast reporter strain, together with (1) the original bait plasmid pCMBV4-Pep4 and (2) the negative control bait pMBV-Alg5 and the resulting strains were tested again for the two independent reporter readouts. Library plasmids that test positive with the original bait but not with the control bait are considered true positives.

2.12. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, California, USA) and *P*-values <0.05 were considered statistically significant in all experiments.

Chapter 3

Results

Chapter 3 Results

Subchapter 3.1

The protective role of yeast Cathepsin D in acetic acid-induced mitochondrial degradation and apoptosis depends on its catalytic activity and on ANT (Aac2p)

Subchapter 3.2

The role of yeast Cathepsin D in acetic acid-induced apoptosis depends on mitochondrial respiration

Subchapter 3.3

Identification of protein substrates/partners of Pep4p by physical interaction studies

Subchapter 3.1

The protective role of yeast Cathepsin D in acetic acid-induced mitochondrial degradation and apoptosis depends on its catalytic activity and on ANT (Aac2p)

This subchapter comprises parts from the following publications:

Pereira H, Azevedo F, Rego A, Sousa MJ, Chaves SR, Côrte-Real M (2013). The protective role of yeast Cathepsin D in acetic acid-induced apoptosis depends on ANT (Aac2p) but not on the voltage-dependent channel (Por1p). *FEBS Lett* 587(2):200-205.

Oliveira CSF, Pereira H, Alves S, Castro L, Baltazar F, Chaves SR, Preto A, Côrte-Real M (2015). Cathepsin D protects colorectal cancer cells from acetate-induced apoptosis through autophagy-independent degradation of damaged mitochondria. Submitted manuscript.

The protective role of yeast Cathepsin D in acetic acid-induced mitochondrial degradation and apoptosis depends on its catalytic activity and on ANT (Aac2p)

In this subchapter, we sought to investigate the interplay between the vacuole and the mitochondria during acetic acid-induced cell death. For this purpose, we assessed whether the previously described increased sensitivity of *PEP4*-deleted W303-1A mutants to acetic acid depends on two mitochondrial proteins previously implicated in this process, Por1p and AAC proteins. We found that Pep4p and Por1p function independently, as absence of both *PEP4* and *POR1* resulted in increased sensitivity to acetic acid than either individual mutation. Moreover, we demonstrate that Pep4p is not involved in mitochondrial fragmentation in untreated cells, and that Por1p does not play a role in acetic acid-induced mitochondrial degradation. In contrast, deletion or overexpression of Pep4p had no effect on the viability of an *AAC*-deficient strain, indicating that Pep4p function depends on the presence of AAC proteins. In addition, we show that the role of Pep4p in acetic acid induced-cell death and -mitochondrial degradation depend on its proteolytic activity and can be complemented by human CatD. These results demonstrate a differential interplay between yeast vacuolar CatD and mitochondrial proteins involved in apoptosis regulation, as well as shed more light on the role of yeast CatD in mitochondrial degradation and its consequence for cell survival.

3.1.1. The protective role of Pep4p in acetic acid-induced cell death is independent of the Por1 protein

In order to elucidate the role of Pep4p in the mitochondria-dependent death pathway, we tested whether the previously described increased sensitivity of *pep4*Δ mutant cells to acetic acid and delayed mitochondrial degradation depend on the yeast voltage-dependent channel (Por1p), a mitochondrial protein also involved in acetic acid-induced cell death [15]. We therefore constructed *pep4*Δ, *por1*Δ and *pep4*Δ*por1*Δ mutants in the W303-1A background, and assessed acetic acid-induced cell death and ROS production in

these strains. Consistent with previous results [15,24], deletion of *PEP4* or *POR1* increased the sensitivity of yeast cells to acetic acid when compared with wild type cells (Figure 3.1A). This decrease in cell survival was associated with an increase in loss of plasma membrane integrity (Figure 3.1B) and ROS production (Figure 3.1C) in both mutants. In addition, we found that deleting both *PEP4* and *POR1* resulted in higher susceptibility to acetic acid than deleting either gene individually (Figure 3.1A), and that this phenotype was also associated with an increase in loss of plasma membrane integrity (Figure 3.1B) and ROS production (Figure 3.1C). These results indicate that the pro-survival roles of Pep4p and Por1p during acetic acid-induced cell death are independent.

It had been previously demonstrated that absence of Por1p affects mitochondrial morphology in non-treated cells [111]. We therefore also monitored if Pep4p is implicated in mitochondrial morphology alterations by assessing the percentage of cells with mitochondrial fragmentation of untreated wild type, *pep4*Δ, *por1*Δ and *pep4*Δ*por1*Δ strains expressing mtGFP by fluorescence microscopy. Accordingly, the *por1*Δ strain displayed higher mitochondrial network fragmentation than the wild type strain, as previously observed [111], while the *pep4*Δ strain did not (Figure 3.2), suggesting that Pep4p does not affect mitochondrial network fragmentation in untreated cells. In agreement, the *pep4*Δ*por1*Δ strain had a higher number of cells exhibiting mitochondrial fragmentation than the wild type and *pep4*Δ strains but similar to the *por1*Δ strain (Figure 3.2).

We next investigated whether Por1p is also involved in mitochondrial degradation by assessing the loss in mitochondrial mass of wild type, *pep4*Δ, *por1*Δ and *pep4*Δ*por1*Δ strains expressing mtGFP in response to acetic acid by flow cytometry, as previously described [24]. After 120 min of acetic acid treatment, the *pep4*Δ strain had a higher number of cells exhibiting mtGFP fluorescence than the wild type strain, while the *por1*Δ strain did not (Figure 3.3). This indicates deletion of *PEP4* results in a delay in acetic acid-induced mitochondrial degradation, in accordance with an earlier report, while Por1p does not interfere with mitochondrial degradation [24]. Consistently, after 120 min of treatment, the *pep4*Δ*por1*Δ strain presented a percentage of cells exhibiting mtGFP fluorescence that was higher than that obtained for wild type and *por1*Δ strains and similar to the *pep4*Δ strain (Figure 3.3).

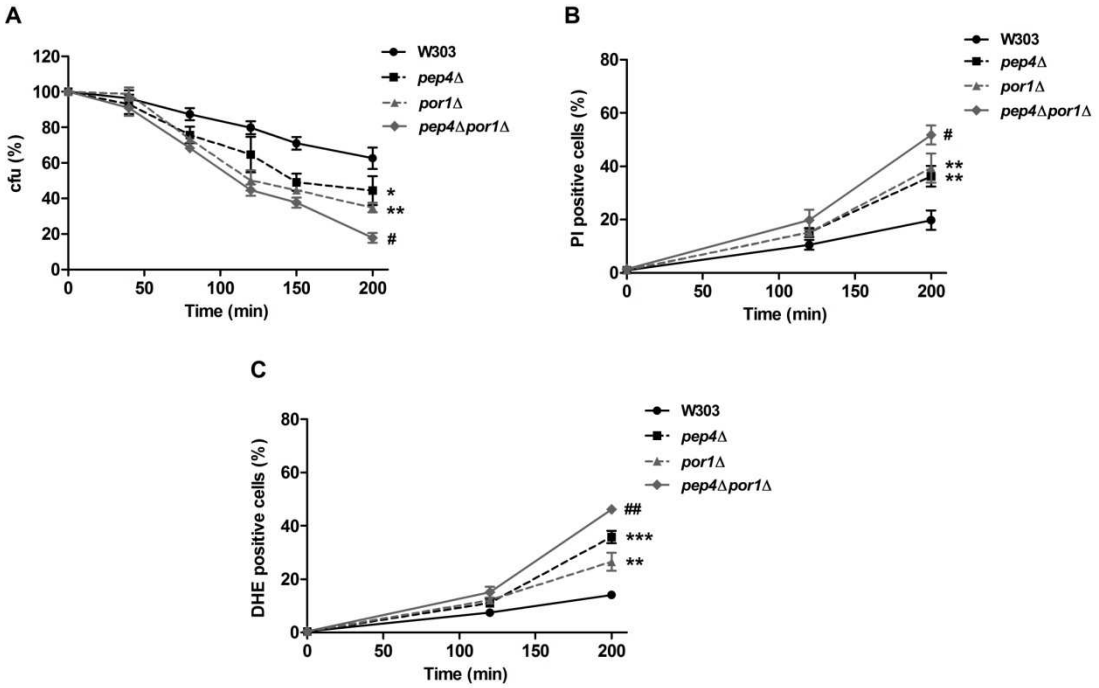

Figure 3.1. *pep4*Δ*por1*Δ cells have increased susceptibility to acetic acid. W303, *pep4*Δ, *por1*Δ and *pep4*Δ*por1*Δ strains were incubated with 120 mM acetic acid for up to 200 min. (A) Cell survival was determined by standard dilution plate counts and expressed as a percentage of c.f.u. in relation to time 0. (B and C) Loss of membrane integrity (B) and ROS production (C) were determined by flow cytometry using PI (B) and DHE staining (C). Data represent means ± S.D. (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to W303; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to both *pep4*Δ and *por1*Δ.

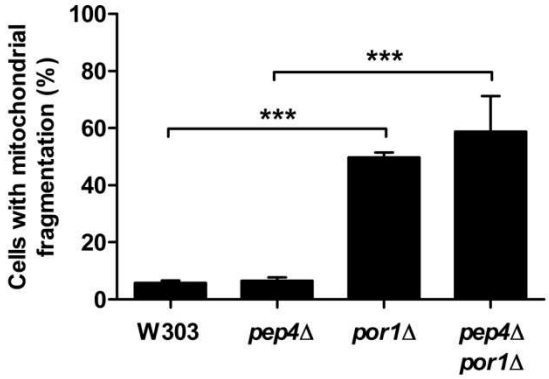

Figure 3.2. Pep4p is not involved in mitochondrial fragmentation in untreated cells. Mitochondrial fragmentation was assessed in untreated W303, *pep4*Δ, *por1*Δ and *pep4*Δ*por1*Δ strains expressing mitochondrial GFP (pYX232-mtGFP), by measuring the percentage of cells displaying mitochondrial network fragmentation (100% corresponds to the total number of cells). Values are means ± S.D. (n = 3). $^{***}P < 0.001$.

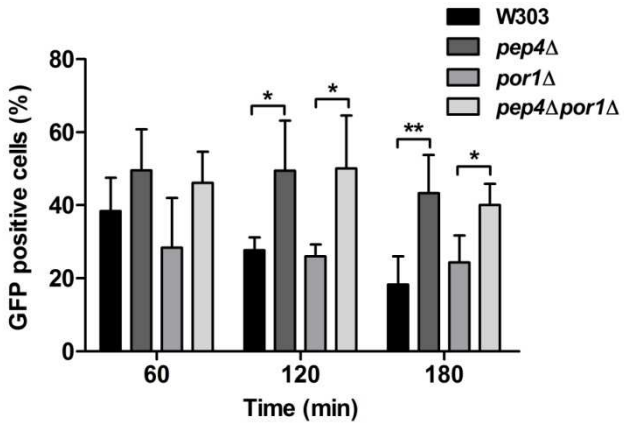

Figure 3.3. Por1p does not interfere with mitochondrial degradation. Mitochondrial degradation was assessed in W303, *pep4*Δ, *por1*Δ and *pep4*Δ*por1*Δ strains expressing mitochondrial GFP (pYX232-mtGFP), by measuring the percentage of cells which displayed loss of mtGFP fluorescence during exposure to 120 mM acetic acid, for up to 180 min (100% corresponds to the number of GFP positive cells at time 0). Values are means ± S.D. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$.

3.1.2. The increased sensitivity of PEP4-disrupted cells to acetic acid depends on AAC proteins

It was previously shown that absence of AAC proteins protects cells exposed to acetic acid and impairs MOMP and cytochrome *c* release [15]. *AAC*-deficient cells, which are not defective in Pep4p release, also show a decrease in mitochondrial degradation in response to acetic acid [24], suggesting that AAC proteins affect mitochondrial degradation at a step downstream of Pep4p release. We therefore also investigated if the observed increased sensitivity of *pep4*Δ cells to acetic acid depended on these proteins, and as a consequence on MOMP and cytochrome *c* release. To this effect, we disrupted *PEP4* in an *aac1/2/3*Δ strain and assessed acetic acid-induced cell death and ROS production in both *aac1/2/3*Δ and *aac1/2/3*Δ*pep4*Δ mutants. Cell survival of the *aac1/2/3*Δ*pep4*Δ strain in response to acetic acid was similar to that of the *aac1/2/3*Δ strain (Figure 3.4A). The increase in the loss of membrane integrity and ROS production in these two strains was indistinguishable (Figure 3.4B and C), further indicating that the increased sensitivity of *pep4*Δ cells to acetic acid depends on the presence of AAC proteins.

We also assessed the effect of overexpressing Pep4p on acetic acid-induced cell death of the *aac1/2/3*Δ strain. We observed that overexpression of Pep4p does not alter cell survival and loss of plasma membrane integrity in the *aac1/2/3*Δ background, as these parameters were not significantly different in the *aac1/2/3*Δ pDP34-*PEP4* and *aac1/2/3*Δ pDP34 strains (Figure 3.4D and E). This is in contrast with our previous results showing an increase in the cell survival and a decrease in loss of plasma membrane integrity of W303 cells expressing pDP34-*PEP4* exposed to acetic acid, compared with W303 cells expressing pDP34 [24].

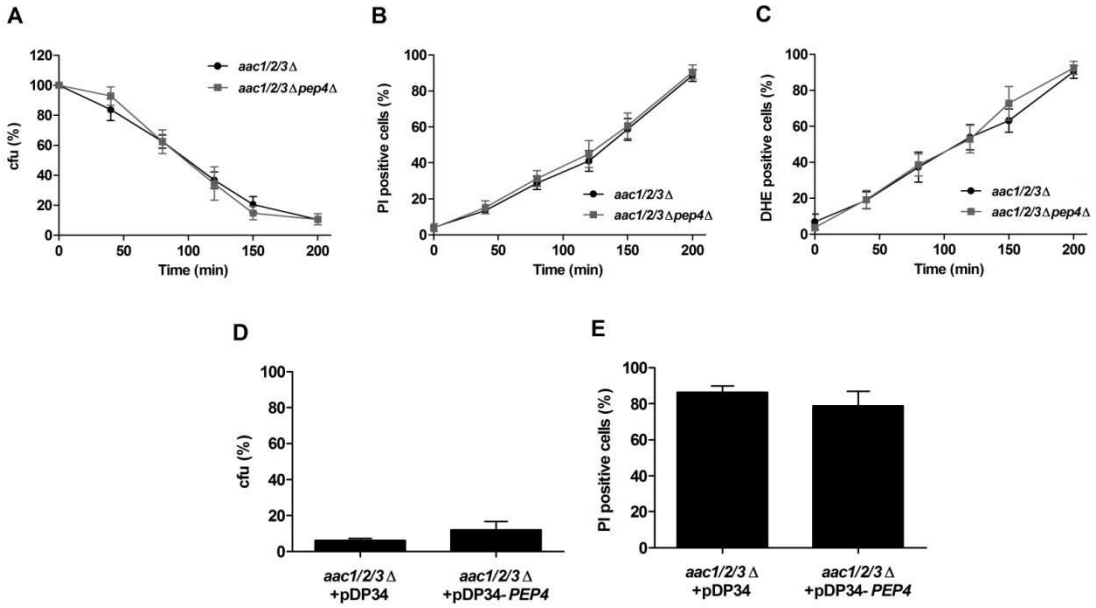

Figure 3.4. Deletion or overexpression of *PEP4* does not alter the resistance of *aac1/2/3*Δ cells to acetic acid. (A–C) *aac1/2/3*Δ and *aac1/2/3*Δ*pep4*Δ strains were incubated with 120 mM acetic acid for up to 200 min. (A) Cell survival was determined by standard dilution plate counts and expressed as a percentage of c.f.u. in relation to time 0. (B and C) Loss of membrane integrity (B) and ROS production (C) were determined by flow cytometry using PI (B) and DHE staining (C). Data represent means ± S.D. (n = 3). (D and E) W303 and *aac1/2/3*Δ strains transformed with the empty vector (pDP34) or pDP34-*PEP4* (expressing WT-Pep4p) were incubated with 120 mM acetic acid for up to 200 min. (D) Cell survival was determined by standard dilution plate counts and expressed as a percentage of c.f.u. in relation to time 0. (E) Loss of membrane integrity was determined by flow cytometry using PI staining. Data represent means ± S.D. (*n* = 3). These results were obtained in collaboration with Flávio Azevedo.

We also transformed *aac1/2/3*Δ*pep4*Δ cells with a mutated form of Aac2p (op1) deficient in translocation activity, which was shown to revert the resistance phenotype of *aac1/2/3*Δ cells to acetic acid [15]. As expected, expression of op1 increased the

susceptibility of *aac1/2/3*Δ*pep4*Δ cells to acetic acid (Figure 3.5A), associated with an increase in the loss of plasma membrane integrity and ROS production (Figure3.5B and C). Taken together, these results further strengthen the idea that AAC proteins act downstream of Pep4p release in the apoptotic cascade and confirm that the protective role of Pep4p in acetic acid-induced cell death is critical only when AAC proteins are present, presumably because MOMP and cytochrome *c* release are not impaired.

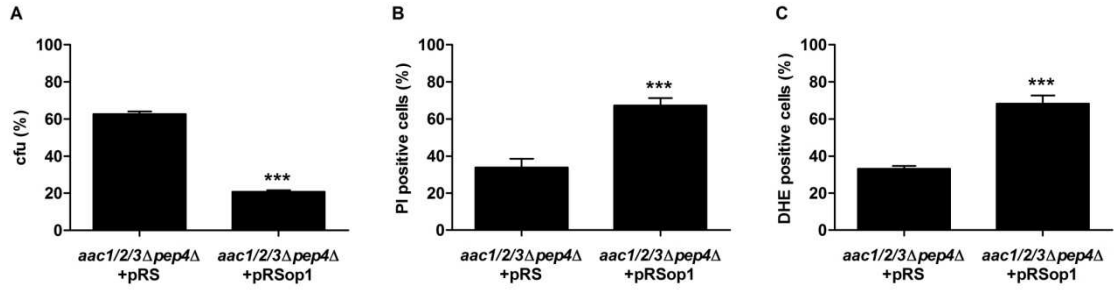

Figure 3.5. Expression of a mutated form of Aac2p (op1) reverts the resistance phenotype of *aac1/2/3*Δ*pep4*Δ cells. *aac1/2/3*Δ*pep4*Δ strains transformed with the empty vector (pRS314) or pRSop1 were incubated with 120 mM acetic acid for 200 min. (A) Cell survival was determined by standard dilution plate counts and expressed as a percentage of c.f.u. in relation to time 0. (B and C) Loss of membrane integrity (B) and ROS production (C) were determined by flow cytometry using PI (B) and DHE staining (C). Data represent means ± S.D. (n = 3). $^{***}P < 0.001$. These results were obtained in collaboration with Flávio Azevedo.

3.1.3. The anti-apoptotic role of Pep4p depends on its proteolytic activity

We next investigated whether the protective role of Pep4p in acetic acid-induced cell death depends on its proteolytic activity. To this effect, we assessed both Pep4p activity and cell survival in *pep4*Δ cells expressing wild type Pep4p (pESC-*PEP4*), a double-point mutant form of Pep4p (pESC-*DPM*) deficient in proteolytic activity, and empty vector control (pESC) and compared it with that of wild type W303 cells expressing the empty vector. As expected, both *pep4*Δ pESC and *pep4*Δ pESC-*DPM* strains did not exhibit Pep4p activity (Figure 3.6). In addition, as shown in Figure 3.7, expression of wild type Pep4p (WT-Pep4p) reverted the sensitivity phenotype of *pep4*Δ cells to acetic acid, while the expression of double point mutant Pep4p (DPM-Pep4p) did not. These results indicate that the pro-survival role of Pep4p during acetic acid-induced cell death is dependent on its proteolytic activity.

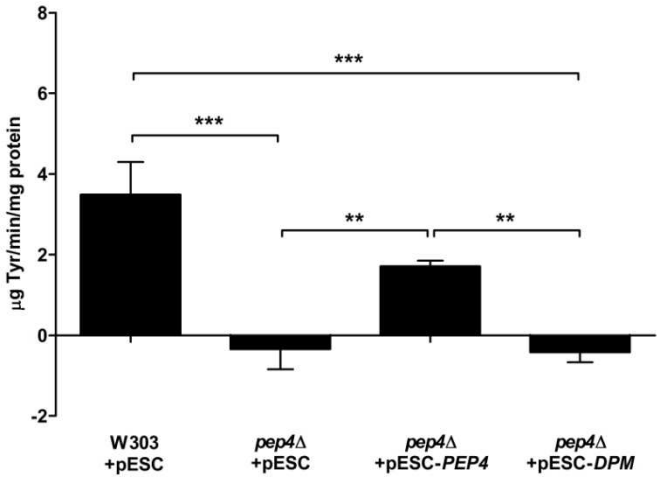

Figure 3.6. *PEP4*-depleted cells expressing a proteolytic inactive Pep4p mutant do not exhibit Pep4p activity. The W303 strain was transformed with the empty vector (pESC) and *pep4*Δ strains were transformed with the empty vector (pESC), pESC-*PEP4* (expressing WT-Pep4p) or pESC-*DPM* (expressing DPM-Pep4p). Pep4p activity was detected in cell extracts, by measuring the release of tyrosine-containing acid-soluble peptides from acid-denatured haemoglobin. Data represent means ± S.D. ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$.

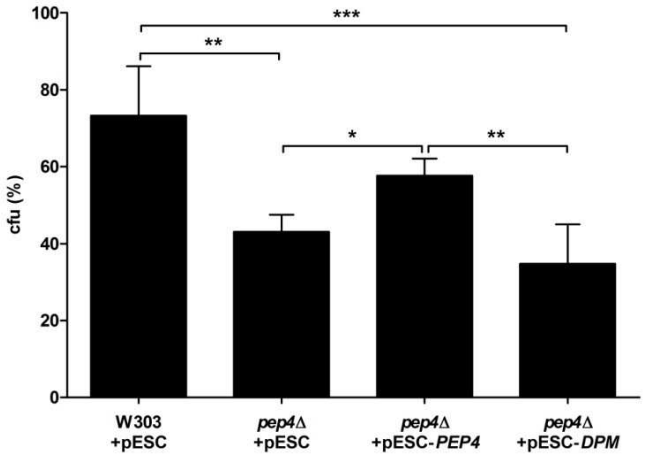

Figure 3.7. Expression of a catalytically inactive Pep4p mutant does not revert the sensitivity phenotype of *pep4*Δ cells to acetic acid. The W303 strain transformed with the empty vector (pESC) and *pep4*Δ strains transformed with the empty vector (pESC), pESC-*PEP4* (expressing WT-Pep4p) or pESC-*DPM* (expressing DPM-Pep4p) were incubated with 120 mM acetic acid for up to 200 min. Cell survival was determined by standard dilution plate counts and expressed as a percentage of c.f.u. in relation to time 0. Data represent means ± S.D. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.1.4. The role of Pep4p in acetic acid-induced mitochondrial degradation depends on its catalytic activity and is complemented by Cathepsin D

In a recent study in CRC cells lines, we showed that the role of CatD in acetate-induced mitochondrial degradation depends on its proteolytic activity. To further support this observation and determine whether this mechanism is conserved, we assessed if the same is true for the yeast CatD (Pep4p), and if human and yeast CatD are functionally equivalent. For this purpose, we constructed strains deficient in Pep4p expressing the empty vector control (pESC), and equivalent levels of FLAG-tagged wild type Pep4p (pESC-*PEP4*), a double-point mutant form deficient in proteolytic activity (pESC-*DPM*) and human CatD (pESC-*CTSD*) (Figure 3.8A) and compared their sensitivity to acetic acid with that of wild type W303 cells expressing the empty vector. As observed above, expression of wild type Pep4p, but not of the catalytically inactive mutant, reverted the sensitivity phenotype of the Pep4p deficient mutant to acetic acid-induced apoptosis. Now, we further show that expression of CatD also compensates for the loss of Pep4p, indicating the two proteins play a similar role in this process (Figure 3.8B). All strains were then transformed with a plasmid expressing mitochondrial GFP, and mitochondrial degradation in response to acetic acid was assessed by estimating the percentage of cells with preserved green fluorescence, as described previously [24]. We show that while expression of wild type Pep4p and CatD reverted the delay in mitochondrial degradation observed in Pep4p-deficient cells exposed to acetic acid, expression of double point mutant Pep4p (DPM-Pep4p) did not (Figure 3.8C). Though the precise mechanism underlying the role of Pep4p in cell survival as well as in mitochondrial degradation is still elusive, we have now determined that both depend on its proteolytic activity and are complemented by CatD.

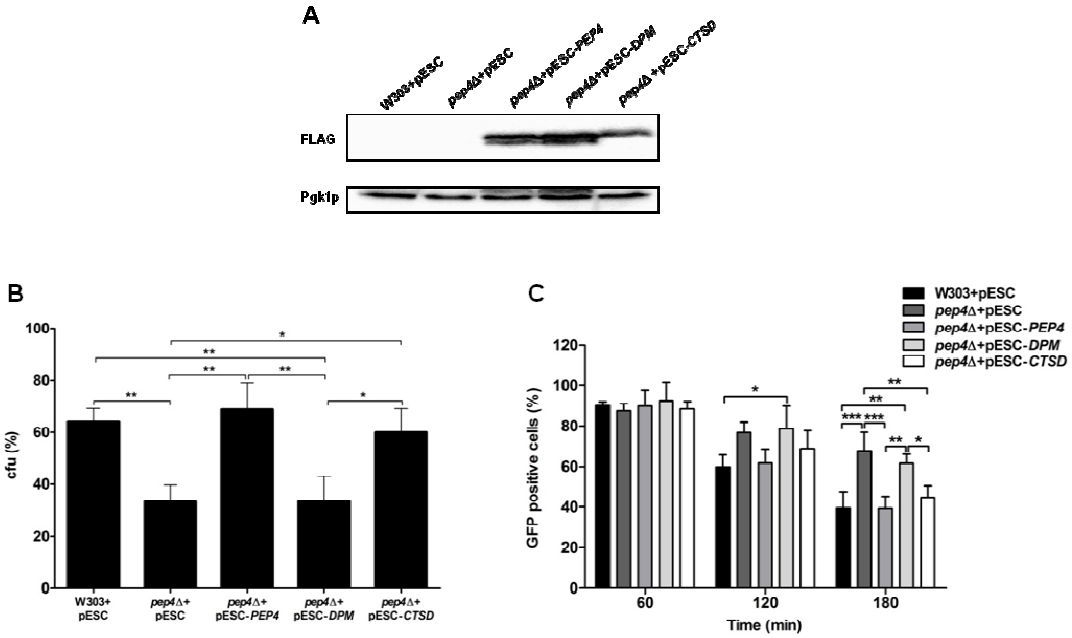

Figure 3.8. Cell survival and mitochondrial degradation in *S. cerevisiae* cells during acetic acid treatment. The W303 strain transformed with the empty vector (pESC) and *pep4*Δ strains transformed with the empty vector (pESC), pESC-*PEP4* (expressing WT-Pep4p), pESC-*DPM* (expressing DPM-Pep4p) or pESC-*CTSD* (expressing human CatD) were incubated with 120 mM acetic acid for up to 180 min. (A) Immunoblot analysis of whole cell extracts of untreated *pep4*Δ cells expressing FLAG-tagged WT-Pep4p, FLAG-tagged DPM-Pep4p, FLAG-tagged CatD and the corresponding empty vector after 20 h of growth. Pgk1p was used as a loading control. (B) Cell survival at time 180 min. was determined by standard dilution plate counts and expressed as a percentage of c.f.u. in relation to time 0. Data represents means ± S.D. (*n=3*). (C) Mitochondrial degradation was assessed by measuring the percentage of cells which displayed loss of mtGFP fluorescence (100% corresponds to the number of GFP positive cells at time 0). Data represents means ± S.D. (*n = 4*). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Western blot was performed by Lisandra Castro.

The role of yeast Cathepsin D in acetic acid-induced apoptosis depends on mitochondrial respiration

The role of yeast Cathepsin D in acetic acid-induced apoptosis depends on mitochondrial respiration

In subchapter 3.1, our results demonstrated a differential interplay between Pep4p and mitochondrial proteins involved in apoptosis regulation. In this subchapter, we intended to elucidate the importance of mitochondrial events in the protective role of Pep4p in acetic acid-induced apoptosis, in order to gain further insight into the involvement of Pep4p in the mitochondria-dependent death pathway. For this purpose, we investigated if the protective role of Pep4p in acetic acid-induced apoptosis, as well as its role in mitochondrial degradation depends of an active mitochondrial function. We assessed cell survival and mitochondrial degradation in respiratory-deficient W303 and *pep4*Δ cells during acetic acid-induced cell death. We show that the pro-survival role of Pep4p in acetic acid-induced apoptosis is dependent on an active mitochondrial function, since absence of mitochondrial respiration resulted in higher resistance to acetic acid in the PEP4-deleted mutant than in the wild type strain. We also show that deficient mitochondrial respiration suppresses the role of Pep4p in mitochondrial degradation. In addition, we found that cell death of *PEP4*-deleted cells in response to acetic acid is dependent on aerobic glycolysis. In contrast, BY4741 *pep4*Δ cells, which are more resistant to acetic acid than wild type isogenic cells, display a delay in mitochondrial degradation in response to acetic acid that is dependent on Pep4p catalytic activity, as previously observed in W303 cells.

This study contributes to increase the understanding on the role of yeast CatD in mitochondrial degradation, its dependence on mitochondrial function and may contribute to an enhanced understanding of the role of CatD in mammalian apoptosis.

3.2.1. The protective role of Pep4p in acetic acid-induced cell death depends on mitochondrial respiratory activity

It was previously shown that absence of Pep4p sensitizes W303-1A cells to acetic acid [24]. In contrast, a later study demonstrated that Pep4p has a pro-apoptotic role in BY4741 cells during acetic acid-induced cell death [86]. To confirm the pro-death role of Pep4p in BY4741 cells, we assessed acetic acid-induced cell death in both wild type BY4741 and *pep4*Δ mutant strains. As shown in Figure 3.9A, deletion of *PEP4* in BY4741 cells resulted in higher resistance to acetic acid when compared with wild type cells, confirming the opposite phenotype of Pep4p observed in this background. This effect was likely not due to unspecific resistance of this strain to apoptotic stimuli, since we verified that disruption of *PEP4* in this BY4741 strain background still resulted in decreased chronological lifespan of yeast cells in comparison with wild type cells (Figure 3.9B), as previously described [85].

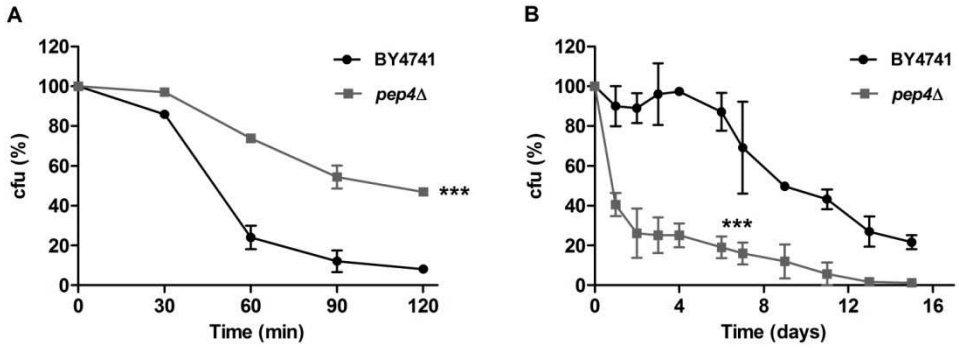

Figure 3.9. Deletion of *PEP4* increases the resistance to acetic acid and decreases chronological lifespan. Cell survival was assessed in BY4741 and *pep4*Δ strains during exposure to 120 mM acetic acid (A) and during chronological aging (B). Cell survival was determined by standard dilution plate counts and expressed as a percentage of c.f.u. in relation to time 0. Data represent means ± S.D. ($n = 3$). $^{***}P < 0.001$.

Since the BY4741 strain has a reduced respiratory capacity, as well as a lower mitochondrial mass when compared with the W303-1A strain [87,88], the resistance of BY4741 *pep4*Δ cells to acetic acid treatment suggested that mitochondrial respiratory activity might be required for the protective role of Pep4p in acetic acid-induced cell death. To investigate this hypothesis, we assessed cell viability in W303 wild type and *pep4*Δ deficient respiratory cells (rho^0 strains) in response to acetic acid. Mitochondrial DNA-null cells are unable to respire and grow only by glycolysis on fermentable carbon sources. Indeed, deletion of *PEP4* in W303 rho^0 cells increased the resistance to acetic acid-induced cell death, in contrast what was observed in rho^+ cells (Figure 3.10A). This suggests that inhibition and consequent deficiency in mitochondrial respiration protects *pep4*Δ cells during acetic acid-induced cell death. It has been demonstrated previously that W303-1A Rho^0 cells are more resistant to acetic acid-induced cell death than wild type cells [13]. In addition, another study demonstrated that respiration enhances apoptosis and ROS production in yeast cells [112]. Accordingly, as shown in Figure 3.10A, both W303 rho^0 and *pep4*Δ rho^0 strains also displayed a higher resistance to acetic acid than the respective rho^+ strains. To exclude pleiotropic effects of mitochondrial DNA depletion that do not account for respiratory deficiency, W303-1A and *pep4*Δ strains were grown in the presence of oligomycin and subsequently treated with acetic acid in the presence of this compound. Oligomycin is an inhibitor of the F0 part of H^+-ATP-synthase, which is involved in proton translocation required for oxidative phosphorylation. Cells grown in the presence of oligomycin therefore have a decreased capacity to respire, becoming more dependent on glycolysis. In agreement with the data obtained with the rho^0 strains, in the presence of oligomycin *pep4*Δ cells displayed higher resistance to acetic acid-induced cell death than wild type cells (Figure 3.10B). In addition, olygomycin had no effect on cell death of the wild type strain, as previously described [13]. Altogether, these results suggest that the pro-survival role of Pep4p in acetic acid-induced apoptosis is dependent on an active mitochondrial function. In fact, when mitochondrial respiration is inhibited, Pep4p has a function in the execution of cell death rather than in cell protection.

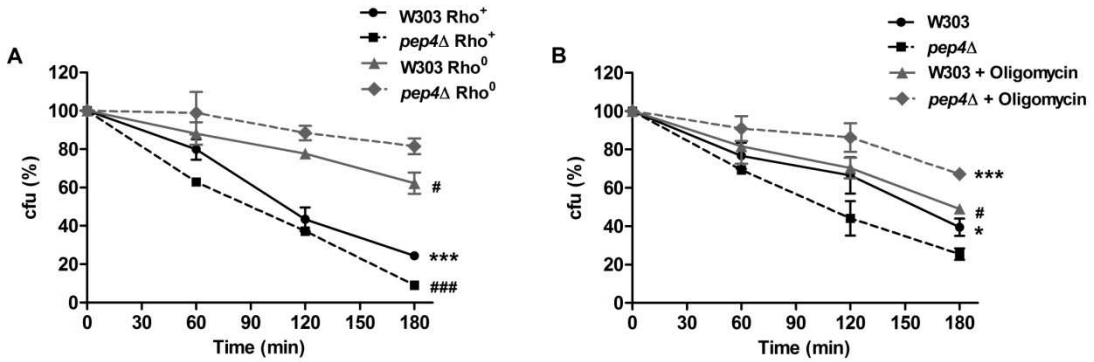

Figure 3.10. Absence of mitochondrial respiration affects the protective role of Pep4p in acetic acid-induced cell death. Cell survival was assessed in the W303-1A background during exposure to 120 mM acetic acid (A) W303, *pep4*Δ, W303 Rho^0 and *pep4*Δ Rho^0 strains. Cell survival was determined by standard dilution plate counts and expressed as a percentage of c.f.u in relation to time 0. Data represent means ± SD (n=3). ***$P < 0.001$ compared to both *pep4*Δ Rho$^+$ and W303 Rho^0, $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ compared to *pep4*Δ Rho^0. (B) W303 and *pep4*Δ strains were grown and treated in the presence of oligomycin or in the presence of ethanol (0.25%, v/v) used as solvent for oligomycin. Cell survival was determined by standard dilution plate counts and expressed as a percentage of c.f.u in relation to time 0. Data represent means ± SD (n=3). *$P < 0.5$, ***$P < 0.001$ compared to *pep4*Δ; $^{\#}P < 0.05$ compared to *pep4*Δ + Oligomycin.

3.2.2. Deficient mitochondrial respiration suppresses the role of Pep4p in acetic acid-induced mitochondrial degradation

It was previously shown that, once in the cytosol, Pep4p plays an important role in mitochondrial degradation during acetic acid-induced cell death [24]. In addition, it was also found that mitochondrial AAC proteins are required for efficient mitochondrial degradation during this process, in a step downstream of Pep4p release [24]. We therefore investigated whether mitochondrial respiration also affects the role of Pep4p in mitochondrial degradation. For this purpose, we used flow cytometry to assess the loss in mitochondrial mass in response to acetic acid-induced apoptosis in W303-1A rho^0 cells expressing mtGFP, as previously described [24]. As shown in Figure 3.11, after 120 min of acetic acid treatment the number of *pep4*Δ rho^0 cells exhibiting mtGFP fluorescence in response to acetic acid was similar to that of W303 rho^0 cells, indicating that deletion of *PEP4* in a W303 respiratory-deficient strain (rho^0) has no effect in mitochondrial degradation during acetic acid treatment. In contrast, as previously demonstrated [24] , we observed a delay in mitochondrial degradation in the *pep4*Δ strain when compared to the

W303 strain (Figure 3.11). These results suggest that Pep4p is not required for the degradation of respiratory-deficient mitochondria.

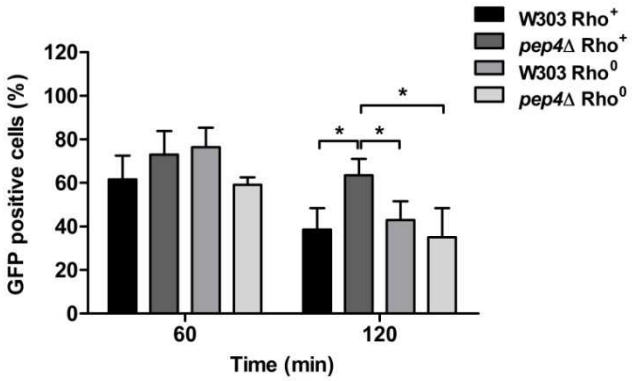

Figure 3.11. Deletion of mtDNA in W303-1A cells bypasses the need for Pep4p in mitochondrial degradation. Mitochondrial degradation was assessed in W303 Rho$^+$, W303 *pep4*Δ Rho$^+$, W303 Rho^0 and W303 *pep4*Δ Rho^0 expressing mitochondrial GFP (mtGFP), by measuring the loss of mtGFP fluorescence during exposure to 120 mM acetic acid (100% corresponds to the number of GFP positive cells at time 0). Values are means ± S.D. (n = 3). $^*P < 0.05$.

3.2.3. Inhibition of aerobic glycolysis protects PEP4-deleted cells from acetic acid-induced cell death

In yeast, aerobic glycolysis is the main metabolic pathway when fermentable carbon sources are available. To evaluate whether the pro-survival role of Pep4p in acetic acid-induced cell death is affected by inhibition of yeast fermentation, both W303-1A and *pep4*Δ strains were pre-incubated for 10 min with 2-deoxyglucose, a inhibitor of glycolytic ATP production. As shown in Figure 3.12, in the presence of 2-deoxyglucose, cell survival of the *pep4*Δ strain in response to acetic acid was similar to that of the wild type strain, though *pep4*Δ cells displayed higher sensitivity in the absence of 2-deoxyglucose. In addition, acetic acid-induced cell death of wild type cells was not affected by this inhibitor (Figure 3.12). These results indicate aerobic glycolysis plays a role in acetic acid-induced cell death of *pep4*Δ but not wild type cells.

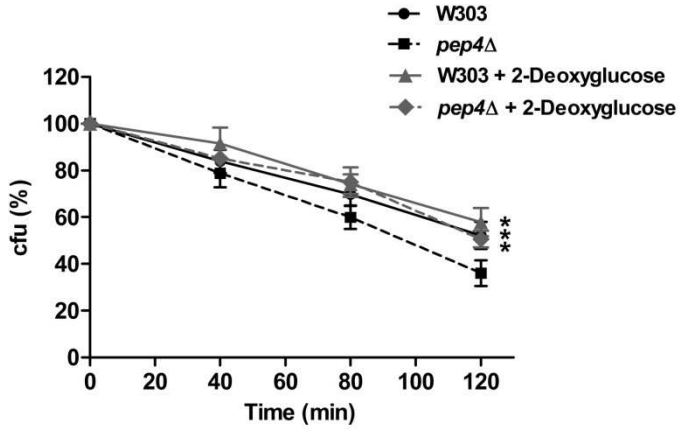

Figure 3.12. Cell death of *PEP4*-deleted cells in response to acetic acid partially depends on aerobic glycolysis. Cell survival was assessed in W303 and *pep4*Δ cells during exposure to 120 mM acetic acid after pre-incubation with 2-deoxyglucose or solvent alone (water). Cell survival was determined by standard dilution plate counts and expressed as a percentage of c.f.u in relation to time 0. Data represent means ± SD (n=3). $^{*}P < 0.05$ compared to *pep4*Δ.

3.2.4. The pro-apoptotic role of Pep4p in acetic acid-induced cell death depends on its catalytic activity

We previously demonstrated in subchapter 3.1 that the protective role of Pep4p in W303-1A cells during acetic acid-induced cell death depends on its proteolytic activity [1]. We therefore also investigated if the observed pro-death role of Pep4p in BY4741 cells in acetic acid-induced cell death is also dependent on Pep4p catalytic activity. To this effect, we transformed the BY4741 strain with the empty vector (pESC) and the isogenic *pep4*Δ mutant with the empty vector (pESC), pESC-*PEP4* (for WT-Pep4p expression) or pESC-*DPM* (for double point mutant-Pep4p expression), and assessed cell viability in response to acetic acid. We observed that expression of WT-Pep4p decreased cell survival in *pep4*Δ cells, while the expression of DPM-Pep4p, deficient in proteolytic activity, did not (Figure 3.13). These observations indicate that a proteolytically active Pep4p is required for its pro-apoptotic role in the BY4741 strain in response to acetic acid.

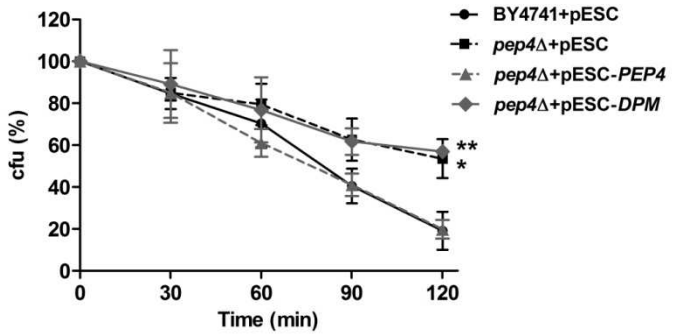

Figure 3.13. Expression of a proteolytically inactive Pep4p does not revert the resistance phenotype of *pep4*Δ cells to acetic acid. The BY4741 strain transformed with the empty vector (pESC) and *pep4*Δ strains transformed with the empty vector (pESC), pESC-*PEP4* (expressing WT-Pep4p) or pESC-*DPM* (expressing DPM-Pep4p) were incubated with 120 mM acetic acid for up to 120 min. Cell survival was determined by standard dilution plate counts and expressed as a percentage of c.f.u. in relation to time 0. Data represent means ± S.D. (n = 3). $^{*}P < 0.05$ comparing *pep4*Δ + pESC-*DPM* to both BY4741 + pESC and *pep4*Δ + pESC-*PEP4*, $^{**}P < 0.01$ comparing *pep4*Δ + pESC to both BY4741 + pESC and *pep4*Δ + pESC-*PEP4*.

3.2.5. The role of Pep4p in acetic acid-induced mitochondrial degradation in BY4741 cells is dependent on its proteolytic activity

Since Pep4p has an opposite role in BY4741 and W303 cells undergoing acetic acid-induced apoptosis, we next investigated whether Pep4p has also a role in mitochondrial degradation in this background. As shown in Figure 3.14A, after 120 min of acetic acid treatment, the BY4741 *pep4*Δ strain had a higher percentage of cells exhibiting mtGFP fluorescence than the wild type strain, like previously described in W303 *pep4*Δ cells [24]. This indicates that deletion of *PEP4* in the BY4741 background, as in W303-1A, results in a delay in acetic acid-induced mitochondrial degradation. Furthermore, the involvement of Pep4p in acetic acid-induced mitochondrial degradation seems to have a pro-death role in BY4741 background, instead of the protective role observed in the W303-1A background [24]. As the pro-apoptotic role of Pep4p in acetic acid-induced cell death of BY4741 cells depends on its proteolytic capacity, we tested whether the role of Pep4p in mitochondrial degradation in this strain does as well. Thus, as previously described [24], we assessed the loss of mitochondrial mass induced by acetic acid in BY4741 pESC, *pep4*Δ pESC, *pep4*Δ pESC-*PEP4* and *pep4*Δ pESC-*DPM* strains

expressing mtGFP by flow cytometry. After 120 min of acetic acid treatment, expression of WT-Pep4p reverted the delay in mitochondrial degradation of the *pep4*Δ strain, whereas expression of the proteolytically inactive mutant did not (Figure 3.14B). These observations indicate that the proteolytic activity of Pep4p in the BY4741 background is also required for its role in acetic acid-induced mitochondrial degradation, as previously observed in subchapter 3.1 for W303-1A cells. In conclusion, Pep4p is involved in mitochondrial degradation, which decreases the survival of BY4741 cells to acetic acid, in a manner dependent on its proteolytic activity.

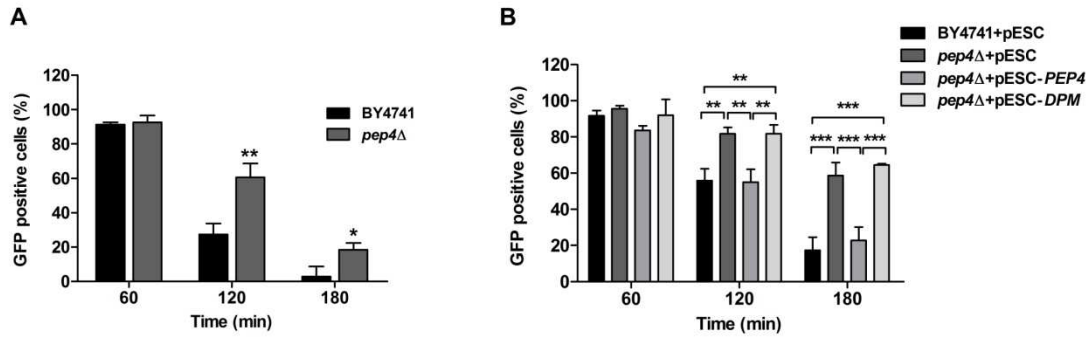

Figure 3.14. Expression of a Pep4p catalytically inactive mutant does not revert the delay in mitochondrial degradation of *pep4*Δ cells. Mitochondrial degradation was assessed in BY4741, BY4741 *pep4*Δ, BY4741 pESC, BY4741 *pep4*Δ pESC, BY4741 *pep4*Δ pESC-*PEP4* and BY4741 *pep4*Δ pESC-*DPM* expressing mitochondrial GFP (pGAL-CLbGFP), by measuring the loss of mtGFP fluorescence during exposure to 120 mM acetic acid, for up to 180 min (100% corresponds to the number of GFP positive cells at time 0). Data represents means ± S.D. (*n = 3*). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Subchapter 3.3

Identification of protein substrates/partners of Pep4p by physical interaction studies

Identification of protein substrates/partners of Pep4p by physical interaction studies

In previous subchapters we show that the role of Pep4p in acetic acid-induced mitochondrial degradation depends on its proteolytic activity, as well on mitochondrial respiration. In this subchapter, we proposed to elucidate novel Pep4p-mediated pathways, namely those involved in mitochondrial degradation, through the identification of physical interactions of cytosolic Pep4p with other proteins, in order to clarify the role of this protease in acetic acid-induced mitochondrial degradation. To this effect we used the Split-Ubiquitin Membrane Yeast Two-Hybrid System from Dualsystems Biotech. In contrast with the conventional yeast-two hybrid system, in which interactions occur in the nucleus, the membrane-based yeast two-hybrid system represents an *in vivo* system that detects interactions between integral membrane proteins, membrane-associated proteins and soluble proteins in their natural environment.

This system uses the split-ubiquitin approach based on the detection of the *in vivo* processing of a reconstituted split ubiquitin [113]. On interaction of X (bait) and Y (prey) proteins, ubiquitin reconstitution occurs and leads to the proteolytic cleavage and subsequent release of a transcription factor that triggers the activation of a reporter system enabling easy detection (Figure 3.15). First, we tested whether Pep4p is able to interact with the outer mitochondrial membrane protein, ortholog of the mammalian VDAC, Por1p, and then we tried to find new protein substrates/partners of Pep4p, as well as to assess the involvement of the identified proteins in acetic acid-induced mitochondrial degradation. Central genes from the pathways identified would be replaced with a selectable auxotrophic marker in strains W303 and *pep4*Δ by homologous recombination. Cells would then be treated with acetic acid and cell viability and mitochondrial degradation after transformation with a plasmid expressing mitochondrial GFP determined along time.

Unfortunately, the objectives proposed have not been achieved due to problems optimizing the split-ubiquitin two-hybrid assay.

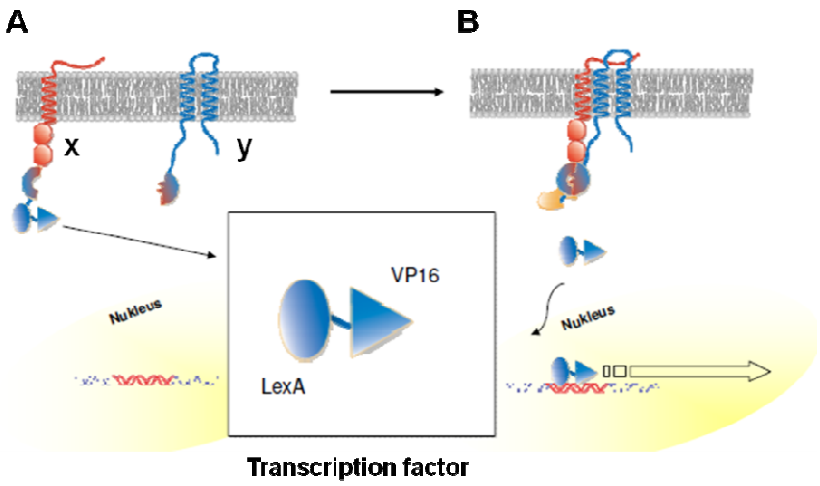

Figure 3.15. Principle of the split-ubiquitin membrane yeast two-hybrid system. (A) No interaction: the transcription factor (LexA-VP16) is kept out of the nucleus by its fusion to a membrane protein (the bait). (B) Interaction of the bait with a NubG-fused prey: formation of split-ubiquitin, cleavage by ubiquitin-specific proteases and release of the transcription factor. The transcription factor relocates to the nucleus and activates the reporter genes. Scheme adapted from Dualsystems Biotech [www.dualsystems.com].

3.3.1. Analysis of an interaction between Pep4p and Por1p by split-ubiquitin membrane yeast two-hybrid

To assess whether Pep4p is able to interact with the yeast voltage dependent channel (Por1p), a mitochondrial membrane protein previously implicated in acetic acid-induced cell death, a split-ubiquitin membrane two-hybrid assay was performed as represented in Figure 3.16. The DSY-1 strain bearing the bait (pBT3-Por1p) was transformed with the pDL2-Pep4p prey vector, empty prey vector (as a negative control), pAlg5-NubI (positive control prey vector) or pAlg5-NubG (negative control prey vector). The DSY-1 reporter strain was also co-transformed with both pMBV-Alg5 (negative control bait vector) and pDL2-Pep4p prey vector. The plasmid pAlg5-NubI encodes a fusion of the resident endoplasmatic reticulum protein Alg5 with NubI and was used as a control prey (positive control). Due to its strong affinity for CUB, NubI will bind to a coexpressed bait, activating the LexA-dependent reporter genes of the yeast host strain. The plasmid pAlg5-NubG encodes a fusion of the resident endoplasmatic reticulum protein

Alg5 with NubG and was used as a negative control prey, and the plasmid pMBV-Alg5 expresses a fusion of the entire open reading frame of the yeast resident ER protein Alg5 to Cub-LexA-VP16 and was used as a negative control bait. Next, the different strains were grown and spotted, in triplicate, on selective plates. As shown in Figure 3.17, all strains grew on selective medium w/o leucine and tryptophan indicating that they contain both the bait and the prey plasmids. In addition, expression of Por1-Cub with the empty vector, as well as co-expression of Por1-Cub with Alg5-NubI, Alg5-NubG and Pep4-NubG resulted in growth of cells on plates lacking histidine, whether in the presence or absence of 3-AT, as well as in blue cells in the presence of X-Gal. In contrast, co-expression of Alg5-Cub with Pep4-NubG did not lead to growth of cells on plates lacking histidine and nor to a ble color in the presence of X-Gal. Growth on selective medium w/o histidine and the blue colour in the presence of X-Gal indicates the transcription of the reporter genes, *HIS3* and *lacZ*, respectively. Unfortunately, it was observed in cells co-expressing Por1-Cub and Alg5-NubG (negative control) and in cells expressing Por1-Cub with the empty vector (negative control), indicating self-activation of the Por1 bait and making impossible to test its interaction with Pep4p.

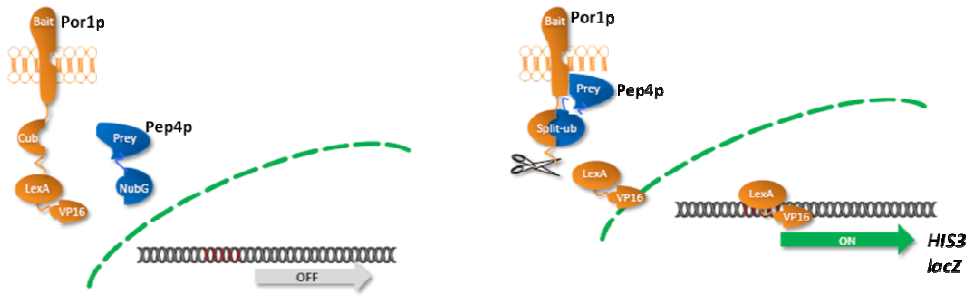

Figure 3.16. Diagrammatic representation of the split-ubiquitin two-hybrid system to detect an interaction between Pep4p and Por1p. The bait is the mitochondrial membrane protein Por1p fused to the C-terminal half of ubiquitin (Cub) followed by a transcription factor (LexA-VP16). The prey is the cytosolic Pep4p fused to the modified N-terminal half of ubiquitin (NubG). If bait and prey interact, Cub and NubG complement to form split ubiquitin, which attracts cleavage by ubiquitin proteases. As a result, the transcription factor is released and translocates to the nucleus, where it can activate transcription of reporter genes (*HIS3* and l*acZ*). This results in blue cells in the presence of X-Gal and growth of the cells on agar plates lacking histidine. Scheme adapted from Dualsystems Biotech [www.dualsystems.com].

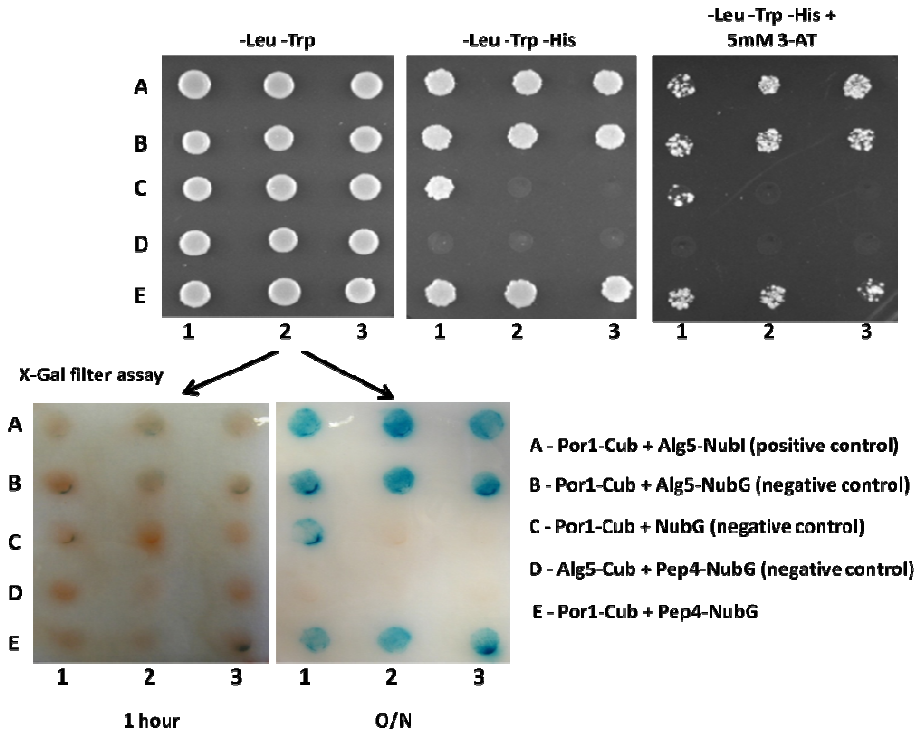

Figure 3.17. Analysis of a Pep4p-Por1p interaction by split-ubiquitin two-hybrid assay. Growth of yeast DSY-1 cells expressing both Por1-Cub, together with Alg5-NuI, Alg5-NubG, NubG, or Pep4-NubG, and Pep4-NubG together with Alg5-Cub on selective plates with or without 3-aminotriazole (3-AT), as well as X-Gal filter assay to detect β-galactosidase activity in response to *lacZ* reporter gene activation (blue colonies). Leu, leucine; Trp, tryptophan; His, histidine.

3.3.2. Identification of new proteins that interact with Pep4p by split-ubiquitin membrane yeast two-hybrid screen

In order to find new protein substrates/partners of Pep4p, we performed the split-ubiquitin two-hybrid assay as shown in Figure 3.18. First, the bait vector was constructed in order to express Ost4-Pep4-Cub, as described in materials and methods (chapter 2), and the correct expression of the bait protein was verified. To this effect, the DSY-1 strain bearing the bait (pCMBV4-Ost4-Pep4) was transformed with the positive control prey vector (pAlg5-NubI) or the negative control prey vector (pAlg5-NubG). After, the two strains were grown and spotted, in triplicate, on selective plates. As observed in Figure

3.19, coexpression of Ost4-Pep4-Cub with Alg5-NubI or Alg5-NubG grew on selective medium w/o leucine and tryptophan indicating that strains contain both plasmids. In contrast, they did not grow on selective medium w/o histidine and did not turn blue in the presence of X-Gal (data not shown). Coexpression of Alg5-NubI with the bait results in reconstitution of split-ubiquitin and the activation of reporter genes only if the Cub-LexA-VP16 reporter moiety is present on the cytosolic side of the membrane. Our results suggest that Ost4-Pep4-Cub is not correctly expressed, since the activation of *HIS3* and *lacZ* reporter genes was not observed in strains coexpressing Ost4-Pep4-Cub and Alg5-NubI. For that reason, we were unable to perform the split-ubiquitin two-hybrid screen in order to detect new protein partners of Pep4p.

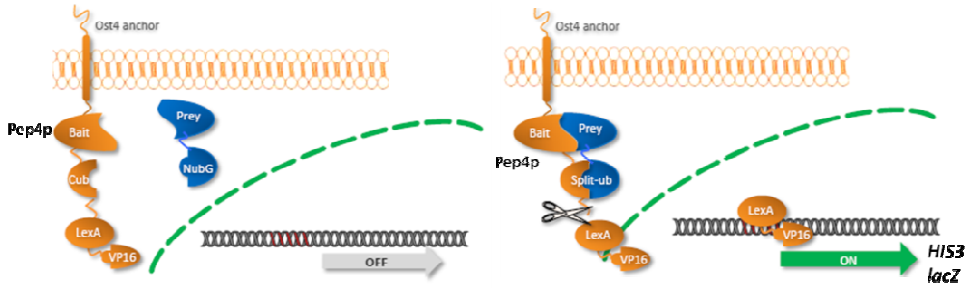

Figure 3.18. Diagrammatic representation of the split-ubiquitin two-hybrid screen. To screen for new interactors of Pep4p, the cytosolic Pep4p was inserted between the membrane protein Ost4p, which anchors Pep4p in the membrane, and the C-terminal half of ubiquitin (Cub) followed by the transcription factor. Potential interactors are expressed from a cDNA library as fusions to the N- terminal half of ubiquitin (NubG). If bait and prey interact, Cub and NubG complement to form split ubiquitin, which attracts cleavage by ubiquitin proteases. As a result, the transcription factor is released and translocates to the nucleus, where it can activate the transcription of reporter genes (*HIS3* and l*acZ*). This results in blue cells in the presence of X-Gal and growth of the cells on agar plates lacking histidine. Scheme adapted from Dualsystems Biotech [www.dualsystems.com].

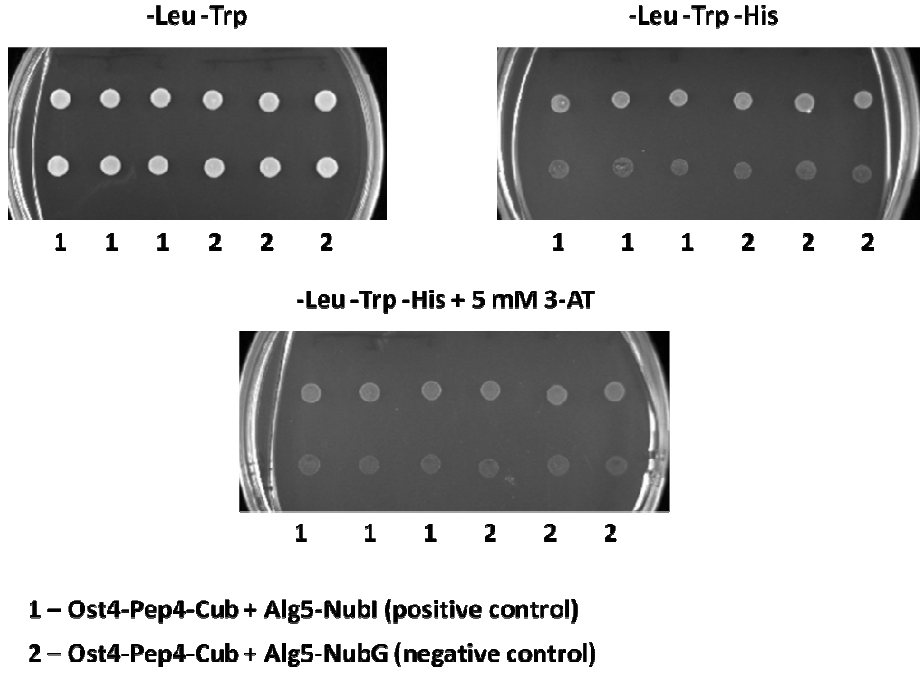

Figure 3.19. Verification of correct expression of the Pep4p bait protein. Growth of yeast DSY-1 cells expressing Ost4-Pep4-Cub, together with Alg5-NubI or Alg5-NubG on selective plates with or without 3-aminotriazole (3-AT). Leu, leucine; Trp, tryptophan; His, histidine.

Chapter 4

General discussion

This chapter comprises parts from the following publications:

Pereira H, Azevedo F, Rego A, Sousa MJ, Chaves SR, Côrte-Real M (2013). The protective role of yeast Cathepsin D in acetic acid-induced apoptosis depends on ANT (Aac2p) but not on the voltage-dependent channel (Por1p). *FEBS Lett* 587(2):200-205.

Oliveira CSF, Pereira H, Alves S, Castro L, Baltazar F, Chaves SR, Preto A, Côrte-Real M (2015). Cathepsin D protects colorectal cancer cells from acetate-induced apoptosis through autophagy-independent degradation of damaged mitochondria. Submitted manuscript.

4.1. Discussion

In previous studies, it was demonstrated that Pep4p (yeast CatD) is released from the vacuole during hydrogen peroxide- or actin stabilization-induced apoptosis [22,23]. It also translocates into the cytosol during acetic acid-induced apoptosis, and is required for efficient mitochondrial degradation and for increased cell survival in response to this acid [24]. Indeed, *PEP4*-disrupted cells display higher susceptibility to acetic acid associated with decreased mitochondrial degradation [24]. However, the precise role of this protease in mitochondrial degradation and its relation with other events and components of the yeast apoptotic cascade remain unclear. Therefore, we have proposed with this work to study the role of Pep4p in mitochondrial degradation and its involvement in the course of apoptosis.

To gain further insight into this process, in subchapter 3.1 we assessed if the increased sensitivity of *PEP4*-deleted mutants to acetic acid was dependent on two mitochondrial proteins involved in acetic acid- induced cell death, Por1p and AAC proteins. For these assays, we deleted *PEP4*, *POR1* or both in the W303-1A strain, as well as *PEP4* in the previously described *aac1/2/3*Δ strain, thus obtaining new isogenic mutants to ensure minimal strain variance. Our results showed, for the first time, that the protective role of Pep4p in acetic acid-induced cell death depends on the presence of AAC proteins but not of Por1p. In agreement with our previous reports [15,24], deletion of *PEP4* or *POR1* decreased the cell survival of yeast cells exposed to acetic acid, associated with an increase in loss of plasma membrane integrity, suggesting that absence of Pep4p or Por1p accelerates apoptosis and secondary necrosis. Under our new experimental conditions, we could now detect an increase in ROS accumulation in both *pep4*Δ and *por1*Δ cells, when compared to the wild type strain, which was not detected in previous studies [15,24]. More importantly, absence of both *PEP4* and *POR1* sensitized cells to acetic acid to a greater extent than either individual mutation, associated with an increase in the loss of plasma membrane integrity and in ROS accumulation. Taken together, these observations suggest that the pro-survival roles of Pep4p and Por1p in acetic acid-induced apoptosis are independent.

Similar to what has been described in mammalian apoptotic scenarios, the typical yeast mitochondrial morphology changes from a tubular network to a punctuate pattern in response to acetic acid [107]. In particular, it was found that absence of AAC proteins and

of Pep4p is associated with the formation of mitochondrial clusters during this process [24]. In addition, Por1p appears to play a role in mitochondrial dynamics in non-treated cells, since its absence is associated with a fragmented morphology [111]. Here, we show that absence of Pep4p in untreated cells does not affect mitochondrial morphology, since mitochondrial fragmentation was increased only in *por1*Δ and *pep4*Δ*por1*Δ cells, which showed similar high fragmentation levels. This observation reinforces the idea that the involvement of Pep4p in mitochondrial morphology is due to its release from the vacuole to the cytosol as a result of an apoptotic stimulus.

Acetic acid-induced yeast apoptosis has also been linked with mitochondrial degradation [107,114,115], and it was previously shown that both Pep4p and AAC proteins are involved in this process [24]. Therefore, we questioned whether Por1p could also have a role in mitochondrial degradation, in a Pep4p-dependent or independent manner. We found acetic acid-induced mitochondrial degradation was delayed only in *pep4*Δ and *pep4*Δ*por1*Δ cells, which displayed similar degradation kinetics, indicating that although involved in acetic acid-induced apoptosis, Por1p does not play a role in mitochondrial degradation.

It was also previously determined that AAC-deficient cells are not defective in Pep4p release from the vacuole but still show a decrease in mitochondrial degradation in response to acetic acid [24]. Therefore, AAC proteins seem to affect mitochondrial degradation at a step subsequent to Pep4p release, possibly triggering degradation through their involvement in mitochondrial permeabilization. In the present study, we show that the protective role of Pep4p in acetic acid-induced apoptosis depends on the presence of AAC proteins. Deletion of *PEP4* in the AAC-deficient strain did not affect cell viability, loss of membrane integrity and ROS production in response to acetic acid, in contrast with the sensitization observed when it is deleted in a wild type strain. Additionally, in contrast with previous studies showing that overexpression of Pep4p decreased acetic acid-induced death in wild type cells [24], overexpression of Pep4p did not affect cell viability and loss of membrane integrity of AAC-deficient cells. Furthermore, expression of a mutated translocation-deficient form of Aac2p (op1), which reverts the resistance phenotype of *aac1/2/3*Δ [15], also reverted the resistance of the *aac1/2/3*Δ*pep4*Δ strain to acetic acid. These results further strengthen the idea that AAC proteins act downstream of Pep4p release in the apoptotic cascade and confirm that the protective role of Pep4p in acetic acid-induced cell death is critical only when AAC proteins are present.

Though the precise mechanism underlying the anti-apoptotic role of Pep4p is still elusive, we have now determined that it depends on its proteolytic activity, by assessing cell survival of *PEP4*-deficient cells expressing wild type Pep4p or a double-point mutant form of Pep4p, deficient in proteolytic activity, and comparing it with that of wild type cells. While expression of wild type Pep4p reverts the sensitivity phenotype of *PEP4*-deficient cells to acetic acid, the expression of double point mutant Pep4p does not. Since autophagy is not active in cells undergoing acetic acid-induced apoptosis, vacuolar membrane permeabilization associated with the release of Pep4p may act as an alternative mitochondrial degradation process [24].

In mammalian cells, when the lysosomal CatD is released into the cytosol, it often triggers a mitochondrial apoptotic cascade. Nevertheless, CatD can also have anti-apoptotic effects in some cellular types and specific contexts. Accordingly, it was demonstrated that acetate-induced apoptosis involves LMP with CatD release into the cytosol in CRC cells [102]. That study indicated that CatD, like Pep4p, has a protective role in this process. However, the mechanisms by which CatD protects CRC cells from acetate exposure are still unknown. Nevertheless, it is generally accepted that CatD is overexpressed and plays an important role in cancer cells [55]. Therefore, targeting this apoptosis regulator in therapies for apoptosis deficiency-associated diseases, such as cancer, requires detailed elucidation of its mechanisms of action. Mitochondrial degradation following apoptosis induction is a common feature of mammalian cells [116], generally mediated by lysosomes and usually occurring through an autophagic process that shows selectivity for mitochondria, termed mitophagy [117]. However, removal of mitochondria is not always dependent on the autophagic machinery [118], implying the existence of alternative pathways. Recently, we found that, like acetic acid in yeast, acetate-induced apoptosis is not associated with autophagy induction in CRC cells. Moreover, inhibition of CatD with siRNA or pepstatin A enhanced apoptosis associated with higher mitochondrial dysfunction and increased mitochondrial mass. These observations in CRC cells suggest that a proteolytically active CatD is involved in the degradation of damaged mitochondria during acetate induced-apoptosis through an autophagy-independent process, allowing the cell to dispose of dysfunctional mitochondria and delaying cell death.

In this thesis, we also now show that a catalytically active Pep4p is required for efficient mitochondrial degradation during acetic acid-induced apoptosis, since expression

of the wild type Pep4p, but not of the double point mutant Pep4p reverts the delay in mitochondrial degradation observed in *PEP4* disrupted cells. Notably, we also found that heterologous expression of human CatD in yeast *PEP4*-deficient cells reverts the delay of mitochondrial degradation during acetic acid-induced apoptosis, as previously observed for expression of wild type Pep4p, providing evidence that the role of CatD in mitochondrial degradation is conserved through evolution.

In addition, it was reported that Pep4p has a pro-death role in BY4741 cells undergoing acetic acid-induced apoptosis. In fact, absence of *PEP4* resulted in increased resistance to acetic acid [86], in contrast with what was previously described in W303 cells [24]. These observations suggested that Pep4p may play a dual function in acetic acid-induced RCD. Since the BY4741 strain has a reduced respiratory capacity, as well as a lower mitochondrial mass than the W303-1A strain [87,88], the resistance of BY4741 *pep4*Δ cells to acetic acid-treatment indicated that mitochondrial respiratory activity might be required for the protective role of Pep4p in acetic acid-induced cell death. Importantly, it was demonstrated that mitochondrial respiration is required for cell death in BY4741 cells, as abrogation of respiration suppressed apoptosis and ROS production [112]. Thus, in subchapter 3.2 we investigated the importance of mitochondrial respiratory function in the protective role of Pep4p during acetic acid-induced apoptosis.

First, to confirm the pro-death role of Pep4p in BY4741 cells, we assessed acetic acid-induced cell death in both wild type BY4741 and *pep4*Δ mutant strains. In agreement with the previous study [86], we confirmed that deletion of *PEP4* increased the cell survival of BY4741 cells to acetic acid. In contrast, the same strain had decreased chronological lifespan, in agreement with a study by Carmona-Gutiérrez *et al*. demonstrating that a *PEP4*-deleted mutant has decreased chronological lifespan when compared with the wild type BY4741 strain, and that Pep4p overexpression protects BY4741 cells fom H_2O_2-induced cell death [85]. In that case, it had also been shown that Pep4p translocates from the vacuole to the cytosol during H_2O_2-induced apoptosis in BY4741 cells, though not affecting cell survival [22].

Consequently, to investigate whether mitochondrial respiration is required for the protective role of Pep4p in acetic acid-induced cell death, we assessed cell viability in W303 and *pep4*Δ deficient respiratory cells (rho^0), as well as in W303 and *pep4*Δ strains co-treated with oligomycin. Our results showed that rho^0 strains are more resistant to acetic

acid than rho$^+$ cells, and that cell death of wild type cells is not affected by oligomycin, as previously demonstrated [13]. More importantly, absence of Pep4p increased resistance of both rho^0 and oligomycin-treated cells to acetic acid, suggesting that the protective role of Pep4p in acetic acid-induced cell death may be dependent on mitochondrial respiratory activity. Therefore, we also questioned whether mitochondrial respiration could also affect the role of Pep4p in acetic acid-induced mitochondrial degradation and its consequence in cell survival. We found acetic acid-induced mitochondrial degradation was similar in both W303 rho^0 and *pep4*Δ rho^0 cells, which also displayed a degradation kinetic similar to that of W303 rho$^+$ cells, while *pep4*Δ rho$^+$ displayed a delay, indicating that although mitochondrial degradation in respiratory-deficient strains continues to occur, Pep4p does not play a role in this process. Taken together, these results suggest that the role of Pep4p in acetic acid-induced mitochondrial degradation, which protects cells from cell death, is critical only when mitochondrial respiratory function is present, implying the existence of alternative degradation pathways when mitochondrial respiration is deficient.

Saccharomyces cerevisiae has the capacity to turn on and off respiration in response to alterations in the carbon source. In addition, aerobic glycolysis is the main metabolic pathway when fermentable carbon sources are available. In the present study, we show that cell death of *pep4*Δ cells in response to acetic acid is dependent on aerobic glycolysis. While untreated *pep4*Δ cells displayed higher sensitivity to acetic acid, cell survival of the *pep4*Δ strain was similar to that of the wild type strain in the presence of 2-deoxyglucose. In addition, cell death of wild type cells in response to acetic acid was not affected by the inhibitor.

Since the BY4741 *pep4*Δ strain exhibited an increased resistance to acetic acid-induced cell death, we also questioned whether this pro-apoptotic role of Pep4p could also depend on its proteolytic activity and found that it does. Indeed, we found that Pep4p has a role in acetic acid-induced mitochondrial degradation in the BY4741 background, since mitochondrial degradation was delayed in *pep4*Δ cells, which was also dependent on its proteolytic activity. Taken together, these observations suggest that catalytically active Pep4p is required for its role in mitochondrial degradation, which in turn decreases the survival of BY4741 cells to acetic acid. Since the involvement of Pep4p in mitochondrial degradation protects W303 cells during acetic acid-induced cell death [24] a lower mitochondrial mass may explain the pro-apoptotic role of Pep4p in BY4741 cells.

In subchapter 3.1, we demonstrated that the catalytic activity of Pep4p is required for its role in mitochondrial degradation, as well as for its anti-apoptotic role in acetic acid-induced cell death in yeast. Whether Pep4p is directly involved in mitochondrial degradation, or acts through downstream substrates has not been determined and warrants further elucidation. Therefore, in subchapter 3.3, we proposed to find new protein substrates/partners of Pep4p, as well to assess the involvement of the identified proteins in acetic acid-induced mitochondrial degradation, to identify Pep4p-mediated pathways, namely those involved in mitochondrial degradation. For this purpose, we intended to use the split-ubiquitin two-hybrid screen to detect physical interactions of Pep4p with other proteins. Unexpectedly, we were unable to perform the screen because our bait protein (Ost4-Pep4-Cub) was not correctly expressed on the cytosolic side of the membrane, as shown in subchapter 3.3. Hence, it was not possible to achieve the objectives proposed in subchapter 3.3.

In summary, in this thesis we show that the protective role of Pep4p in acetic acid-induced apoptosis is independent of the yeast voltage dependent channel Por1p (which has no role on mitochondrial degradation) but dependent on AAC proteins, the yeast adenine nucleotide translocator. In addition, we show that both the Pep4p anti-apoptotic function and its role in mitochondrial degradation depend on Pep4p proteolytic activity. Furthermore, we demonstrate that the pro-survival role of Pep4p in acetic acid-induced apoptosis is dependent on mitochondrial respiratory function, and that deficiency in mitochondrial respiration suppresses its role in mitochondrial degradation. This work therefore shed more light on the role of yeast CatD in mitochondrial degradation, its dependence on apoptotic mitochondrial proteins/events and consequences for cell survival.

4.2. Concluding remarks

CatD has attracted increased attention in recent years since, besides its physiological roles, it has been associated with several human pathologies, including cancer. Indeed, this protease can have both pro- and anti-survival functions depending on its proteolytical activity, cell type, stress stimulus and context. Our previous studies have implicated yeast and human CatD in acetate/acetic acid-induced apoptosis, triggering LMP/VMP, CatD/Pep4p release and mitochondria-dependent apoptosis, and more recently we found specific biochemical processes that are targeted by CatD, namely autophagy impairment and degradation of damaged mitochondria, which render cells more resistant to apoptosis induced by acetate.

The findings in this thesis using the yeast model, combined with the evidence provided by mammalian CRC cell lines, contributed to unveil a novel pro-survival function of CatD in autophagy-independent mitochondrial degradation, which can lead to enhanced cell survival in CRC cells undergoing acetate-induced apoptosis. Understanding the molecular mechanisms underlying the involvement of CatD in mitochondrial degradation will be crucial to develop novel strategies to specifically inhibit this protease in apoptosis deficiency-associated diseases, such as cancer.

Additionally, these studies with yeast reinforce the use of this eukaryotic organism as a valuable model to identify and characterize novel RCD processes, and open the door to new clinical opportunities, with a substantial impact in public health.

4.3. Future perspectives

Although this study brings more understanding on the role of yeast CatD in acetic acid-induced mitochondrial degradation, as well as on the interplay between the vacuole and the mitochondria during this process, some aspects remain to be elucidated and new questions were also raised. In the future, it will be necessary to carry out further experiments to complement the work here presented, such as:

- ascertain if the observed *pep4*Δ increased sensitivity to acetic acid is dependent on the apoptotic factor Aif1p, previously involved in mitochondrial acetic acid-induced cell death, by assessing cell survival and mitochondrial alterations in *aif1*Δ and *pep4*Δ*aif1*Δ mutants;
- investigate whether autophagy is involved in mitochondrial degradation in respiratory-deficient yeast cells (rho^0) and which protease(s) mediate this process, since we observed that Pep4p is not involved;
- identify new protein substrates/partners of Pep4p, as well as CatD interactors in yeast by physical interaction studies by optimizing a yeast two-hybrid screen, as previously described in subchapter 3.3;
- identify new components of the Pep4p-mediated mitochondrial degradation pathway in the course of acetic acid-induced apoptosis. Pep4p interactors identified by physical interaction will be deleted in W303 and *pep4*Δ strains and cell survival and mitochondrial degradation in response to acetic acid will be determined;
- identify yeast proteins involved in Pep4 release from the vacuole during acetic acid treatment. These experiments may provide important clues into the mechanism involved in CatD release from the lysosome in mammalian cells;
- ascertain whether CatD expressed in yeast is proteolytically active, as well as its cellular localization in yeast. If CatD is localized in the vacuole, it would be interesting investigate if it is released into the cytosol in response to acetic acid;
- investigate whether the role of CatD in acetate-induced mitochondrial degradation in CRC cells is dependent on mitochondrial respiration.

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