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Proteases and caspase-like activity in the yeast *Saccharomyces cerevisiae*

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Key words: Programmed cell death, apoptosis, necrosis, proteases, caspases, *Saccharomyces cerevisiae*.

Abbreviations used: PCD, programmed cell death; ROS, reactive oxygen species; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ER, endoplasmic reticulum; MS, mass spectrometry.

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Abstract

A variety of proteases have been implicated in yeast PCD including the metacaspase, Mca1 and the separase Esp1, the HtrA-like serine protease Nma111, the cathepsin-like serine carboxypeptideases and a range of vacuolar proteases. Proteasomal activity is also shown to have an important role in determining cell fate, with both pro- and anti-apoptotic roles. Caspase-3, -6- and -8 like activities are detected upon stimulation of yeast PCD, but not all of this activity is associated with Mca1, implicating other proteases with caspase-like activity in the yeast cell death response. Global proteolytic events that accompany PCD are discussed alongside a consideration of the conservation of the death-related degradome (both at the level of substrate choice and cleavage site). The importance of both, *gain-of-function* changes in the degradome, as well *as loss-of-function* changes are highlighted. Better understanding of both death related proteases and their substrates may facilitate the design of future antifungal drugs or the manipulation of industrial yeasts for commercial exploitation.

Introduction

Understanding the molecular mechanisms regulating and executing programmed cell death (PCD) in fungi is important from a cell biology perspective as some mechanisms may be common to all eukaryotes [1]). It may also facilitate the design of future antifungal drugs [2] or the manipulation of industrial yeasts. The model yeast *Saccharomyces cerevisiae* has the capacity to undergo PCD in response to a range of environmental triggers, or endogenous signals [3]. According to the nature and strength of the death inducing stimulus, PCD in yeasts can be primarily apoptotic, though secondary necrosis invariably follows [4]. Programmed necrosis has also been reported in response to ROS, ER stress and depletion of polyamines [5]. PCD is a complex process, often involving the controlled de-construction of the cellular machinery in a manner regulated and implemented by proteases. A variety of proteases have been implicated in yeast PCD and these are the subject of this paper.

The yeast degradome and the proteolytic landscape

Currently 112 yeast proteases and 60 non-protease homologues of proteases are listed in the MEROPS database (http://merops.sanger.ac.uk). Lineage specific expansion is apparent for some families of protease in yeast including; calpains, ubiquitin-specific peptidases, amidophosphoribosyltransferases, pitrilysin-like metalloproteases, aminopeptidase Ys, subtilisins, and S59 family nucleopore associated endopeptidases, making them attractive targets for antifungal research. Numerous studies have focused on yeast homologues of known mammalian or plant PCD regulators. In most cases these studies have confirmed a role for the proteases in yeast PCD, highlighting the functional conservation of cell death mechanisms across the tree-of-life.

Proteases associated with PCD in yeast

Mca1: Many forms of metazoan apoptosis are dependent upon caspases [6]. True caspases appear to be restricted to the metazoan, but distant homologues, paracaspases and metacaspases, exist in fungi, plants and protists [7]. Caspases, legumains, separases, clostripains and gingipains are MEROPS C14-family (clan CD) proteases. Yeast possesses a single metacaspase encoding gene *MCA1* [8] widely

reported to participate in PCD [9]. Metacaspases are believed to lack true caspase activity and specificity [10-12], preferring arginine and lysine to aspartate at the P1 position. Most studies have focussed on substrates of type II metacaspases, whereas yeast Mca1 is a type I metacaspase. Heterologous Mca1 expression and *in vitro* cleavage of synthetic peptides may also have affected specificity. Lee et al [13] reported that some native substrates of yeast Mca1 are probably cleaved at aspartate residues, a characteristic of true caspases. Additionally, metacaspases and true caspases can share common death-related substrates, with both caspase-3 and mcII-Pa from *Picea abies* cleaving Tudor staphylococcal nuclease [14]. Although hydrogen peroxide, osmotic stress and numerous other stimuli induce metacaspase-dependent cell death in yeast [9, 15], there are many reports of Mca1-independent PCD as well [16].

Esp1: Hydrogen peroxide-induced apoptosis triggers cleavage of Mcd1, a yeast homolog of the Rad21 cohesin required for dsDNA repair, sister chromatid cohesion and chromosome condensation [17]. Cleavage is dependent upon Esp1, a caspase-like separase normally associated with an inhibitory securing, Pds1. During normal growth Pds1 is degraded by the anaphase-promoting complex, releasing Esp1 which cleaves Mcd1 (and substrates such as Slk19 and Scc1) - the fragments remain in the cytoplasm. However in apoptotic cells, a 40 amino acid C-terminal peptide fragment translocates from the nucleus to the mitochondria. This is a gain-of-function cleavage, since the mitochondrial import of the Mcd1 fragment leads to a loss of mitochondrial membrane potential, contributing to the onset of apoptosis. Over-expression of Esp1 causes accumulation of Mcd1 in mitochondria, even without external stimuli. Many cells produce endogenous ROS which is required for mitochondrial import. Esp1 has caspase-1 like activity [18] and caspase-1 inhibitors reduce Mcd1 degradation.

Nma111: Nma111 is an HtrA-like serine protease with a PDZ (post-synaptic density 95/disc/*zona occludens*) domain, which in response to cellular stresses forms intranuclear aggregates [19]. Nma111 contains HtrA-like sequence repeats, but only the N-terminal repeat has a functional catalytic domain. As with many trypsin-like serine proteases, the active site contains a GGSGS consensus motif and also contains a nuclear localization signal, with nuclear import mediated by Kap95 [19]. Cells lacking Nma111 survive heat-shock better than WT cells and fail to undergo hydrogen peroxide-induced apoptosis. Over-

expression enhances apoptosis under stress conditions. Nma11 can cleave the only yeast IAP, Bir1 [20] though Bir1 cleavage *per se* is not sufficient to induce PCD. Bir1 itself does not however appear to interact with Mca1 as might be expected of a *bona fide* IAP.

Kex1: Disruption of N-glycosylation of proteins within the ER leads to apoptosis in yeast [21]. *MCA1* deletion does not abrogate this response [21], however loss of Kex1 decreases caspase-like activity within dying cells and improves the survival of cells suffering ER-stresses, acetic acid induced PCD or chronological aging [22]. Kex1 is a cathepsin A-like serine carboxypeptidase, preferring basic amino acid residues and C-terminal arginine and lysine residues. It is unclear if Kex1 has caspase-like activity or if downstream proteolytic components include such activity. Purified Kex1 cannot cleave synthetic caspase-6 substrates [22] but other caspase substrates have not yet been tested.

Vacuolar proteases: Vacuoles replace lysosomes in fungi. Yeast vacuoles contain acid proteases including proteinase A (Pep4), proteinase B (Prb1), carboxypeptidase Y (Prc1), carboxypeptidase S (Cps1) and aminopeptidase I (Lap4). Vacuolar and lysosomal proteases are mainly non-specific and are synthesised as inactive zymogens, passing via the ER and Golgi to the vacuoles [23]. Lysosomal endopeptidases are responsible for the majority of bulk protein breakdown in yeast, dispose of protein aggregates and degrade membrane proteins. Aminopeptidases and carboxypeptidases finally reduce the products to free amino acids. Complete digestion is so swift that the degradation intermediates are virtually undetectable [24]. Pep4, the yeast equivalent of cathepsin D is principally located in the vacuole. During hydrogen peroxide and acetic acid induced apoptosis Pep4 is released from the vacuole and relocates to the nucleus and cytoplasm, initiating mitophagy-independent degradation of mitochondria and nucleoporins [25].

Proteasome: The ubiquitin-protease system has long been implicated as a key player in yeast PCD [26, 27]. Inactivation of *UBP10*, a de-ubiquitinating enzyme, yields a complex phenotype characterized by a subpopulation of cells exhibitingf typical markers of apoptosis. Cells lacking Ubp10 possess endogenous caspase-like activity, which is only partially lost upon deletion of *MCA1.* Loss of Mca1 suppresses the *ubp10* null mutant phenotype, whilst Mca1 over-expression, even without external stimuli impairs growth and viability of *ubp10* cells. Later stages of acetic acid induced PCD in yeast require a transient elevation

of proteasome-related chymotrypsin, trypsin and PGPH-like activities [28]. Levels of proteasomal catalytic components (Pre2, Pre 3) appear to be unchanged, suggesting a role for a proteasome regulatory component, or altered proteasome accessibility/localization. Inhibition of the proteasomal chymotrypsin-like activity significantly reduces acetic acid induced PCD [28]. In many plant and mammalian cell-types, blocking of proteasome function promotes PCD [29]. In yeast, over-expression of Stm1, a known target of the proteasome promotes apoptosis, but shows a deficiency in apoptosis when deleted [30]. Therefore under some cell death scenarios, the activation of the proteasome may be important for the prevention of yeast PCD as well as its activation.

Caspase-like activities associated with yeast PCD

Positional scanning of synthetic peptides [31 and peptide-based synthetic substrate libraries [32] have been used to ascertain caspase substrate specificities and to assess caspase-like activities during yeast and plant PCD [8, 11, 15, 33-39]. Activities against caspase 3, 6 and 8 substrates, but not caspase 1 substrates have been reported in yeast - see Table 1. This contrasts with PCD associated caspase-like activities in plants which commonly display caspase-1-like activity [39]. Plants contain type I and type II metacaspases, whereas fungi only possess type I, which may explain some differences. Over-expression of yeast Mca1 leads to elevated caspase -6 and -8 like activities [8], but deletion of Mca1 does not remove all activity. Since pro-apoptotic stimuli such as ageing and osmotic stress elicit strong caspase-like activities even in the absence of Mca1, other proteases with caspase-like activity must in part be responsible.

Several metacaspase-independent death associated caspase-like activities have been reported in plants. Subtilisin-like serine proteases have caspase-like activity [40] and DEVDase and TATDase activities have been identified [41]. A screen of 119 non-essential yeast proteases reveals 38 that show some defects in either caspase-1, -6 or -8 substrate cleavage and seven (Yps7, Ybl091ca, Map1, Pim1, Lap2, Aap1 and Rbd2) show reduced death upon stimulation with pro-apoptotic doses of acetic acid **(Figure 1A).** These proteases are up-regulated during PCD, but show limited changes in stressed cells (**Figure 1B, C**). The future challenge is to unravel the primary and secondary roles of these caspase-like proteases and to

identify their downstream targets. The challenge is complex since some proteases target multiple proteins and many substrates are degraded by several proteases.

Inhibitors of caspases often block or reduce the level of PCD in yeast [21, 33]. Again plants and fungi differ. Plant PCD is commonly affected by caspase-1 and -3 inhibitors, whilst caspase-3 and -6 inhibitors appear more potent in fungi. The importance of these activities remains to be seen, as general cysteine and serine protease inhibitors also block plant PCD, without affecting overall caspase-like activity.

Yeast degradomics

Protein abundance is a balance between translation rates and degradation rates. Using TAP-tagged protein expression constructs and a translational block with cycloheximide, Belle et al [42] measured the half-lives of 3751 yeast proteins. The median half-life was observed to be 43 min, but many proteins were much more unstable. Sequencing based tandem MS of the yeast degradome gives more detail about yeast protein turnover [24]. During mid-exponential phase, growth 1,111 peptide fragments originating from 205 parent protein substrates were observed. Cytoplasmic and mitochondrial proteins were the largest components. No proteins uniquely associated with the cell wall or nucleus were identified, nor were membrane proteins (perhaps reflecting technical bias in the extraction procedures used). Whilst the authors asserted that “lysosomal” degradation played little role in the breakdown of the substrates, roles for vacuolar proteases cannot be excluded since vacuolar persistence may be transient and yeast PCD is sometimes associated with translocation of peptides between subcellular compartments eg Pep4 [25]. Strong preferences for P1-site cleavages at Lys>Leu>Arg>Ala>Met>Ser>Thr>Phe>Tyr>Asn are observed, whereas cleavages at Asp, Glu, Gly, Ile, Pro and Val are detected at much lower levels than expected. P1’-sites have a preference for amino acids with short side chains eg Gly, Ala, Ser and Thr. Degradation of mitochondrial proteins is slightly different with P1-site cleavages at Lys>Leu>Ser>Phe>Arg=Ala> Val>Gln and P1’-site cleavages at Gly>Arg>Ser>Lys>Glu>Thr=Ala>Asp implying distinct processing routes.

The detection of Arg-Arg, Lys-Arg and Lys-Lys cut sites suggests the activity of pro-protein convertases such a Kex2. Trypsin-like and chymotrypsin-like proteolysis, favouring Lys-Arg and Phe-Tyr cuts was

also highlighted perhaps attributable to the proteasomal peptidases Pup1 and Pre2. Leu and Ala cleavage at P1-sites might equate to the activity of proteasomal components as well [89]. Surprisingly, over half of the degraded cytosolic regulatory proteins and enzymes were not ubiquitinylated, highlighting the importance of non-proteasomal pathways or ubiquitin-independent proteasomal activities in the degradome.

Studies of global proteolysis have been undertaken in human cells undergoing apoptosis [43, 44] facilitating both substrate and cleavage site identification. Dix et al [43] utilized PROTOMAP, a technology combining SDS-PAGE with shotgun LC-MS/MS, to compare control and staurosporine treated cells, whereas Mahrus et al [44] captured novel N-terminal peptides from etoposide induced apoptotic cells. In these studies alone a total of 261 [43] and 292 [44] caspase substrates were reported. The two datasets overlap (64 proteins) and both datasets overlap with substrates listed in the CASBAH database [45]. Substrates specifically cleaved during the cell death response include ROCK1, linked to membrane blebbing, and the CAD/DFF40 endonuclease. Often multiple components of biosynthetic and transcriptional complexes are targeted [44]. Future studies of the degradome in wild-type yeast and protease deficient / over-expression strains could reveal much about fungal PCD.

Evolutionary conservation of caspase substrates and cleavage sites

Some caspase substrates are cleaved in a species-specific manner (eg cyclin A is targeted during apoptosis of Xenopus oocytes, whilst there is no homologous cleavage site in mammals [46]). Nevertheless it seems unlikely that similar death phenotypes arise from proteolysis of completely different complements of target substrates in different species [47]. Indeed, Shao et al [48] revealed that the P4-P3' caspase-1 cleavage site in GAPDH (D189) is actually conserved in organisms as diverse as man, mouse, cow and yeast. The CASBAH database (http://bioinf.gen.tcd.ie/casbah/) describes 793 caspase substrates (mostly from humans, rats and mice), with cleavage site deatils for 586 proteins [47]. A total of 241 yeast homologues of the caspase substrates listed in the database can be found in the yeast proteome. Since only 16 % of human proteins are conserved in *S. cerevisiae* [49], the conservation of 41% of caspase substrates is much higher than expected by chance. Moreover, alignment of the caspase cleavage sites in human substrates with their yeast homologues reveals considerable

conservation (**Figure 2A**). Cluster analysis based on the overall similarity of cleavage sites reveals that the yeast substrates share highest similarity with human caspase-6 and -8 cleavage sites (**Figure 2B**), supporting earlier observations that caspase-like activities in yeast are caspase-6 and -8 associated.

Conclusions about death-related proteases and proteolysis

Proteolysis is not only a simple form of post-translational modification but an important regulatory process. By altering substrate abundance, proteases can elicit *loss-of-function* effects that directly influence the activation state of metabolic and developmental pathways. Furthermore, proteolytic cleavage of substrates can elicit *gain-of-function* changes, with neopeptide fragments showing novel properties. Examples linked to PCD [reviewed in 50] include the cleavage of executioner procaspases-3 and -7 zymogens by initiator caspases, degradation of Bid by caspase-8 and granzyme B and cleavage, destabilization and degradation of the retinoblastoma associated protein relieving its block on S-phase progression. Cleavage of caspases-7 and -9 generates antagonistic neoepitopes that recruit IAPs, removing their inhibition of apoptosis, whilst cleavage of DFF45/ ICAD allows the formation of a catalytically active DFF40/CAD dimer that can degrade DNA during apoptosis. Loss-of-function effects may require degradation of a substantial proportion of a protein. Gain-of-function requires cleavage of only a small proportion to mediate a biological effect.

The identification of yeast protease substrates should promote further understanding of the role of individual proteases in basic biological processes, and may provide novel targets for drug development. Traditional post-genomic screens for antifungal drugs have often focussed on essential genes, however an alternative strategy might be to identify death-related substrates, which at reduced levels, promote the intrinsic fungal PCD response. The roles that substrate level loss-of-function and gain-of-function changes have on fungal PCD remain largely unexplored, and so should be a significant focus for the future.

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Source	*CASP1 (YVAD)*	*CASP3 (DEVD)*	*CASP6 (VEID)*	*CASP8 (IETD)*	*pan-CASP (VAD)*
Watanabe & Lam (2005) [11]	○	○	○	○	
Madeo et al (2002) [8]		●	●	●	
Hauptmann et al (2006) [21]	○	○	●*	●	
Guaragnella et al (2006) [34]			●	●	
Gonzalez et al (2007) [38]		●	●	●	
Guaragnella et al (2010) [35]			●	●	
Herker et al (2004) [36]					●
Vachova & Palkova (2007) [15]					●
Weinberger et al (2005) [37]					●
Fannjiang et al (2004) [33]		●*			

Table1. Summary of caspase-like activities reported in yeast. Synthetic caspase substrates that are cleaved are shown as closed circles (●∗), activities tested but not detected are shown as open circles (○). Inhibitors of caspase activity that prevent PCD are marked (∗).

Figure 1. *Saccharomyces cerevisiae* proteases with caspase-like activity. (**A**) Deletion mutants with reduced caspase-like activity. Highlighted in red are those with reduced sensitivity to acetic acid induced PCD. (**B**) Limited activation of death-related proteases under stress conditions (20 mM acetic acid). (**C**) Rapid and sustained increase in expression of protease:GFP reporter of death-related proteases with caspase-like activity in dying cells (160 mM acetic acid).

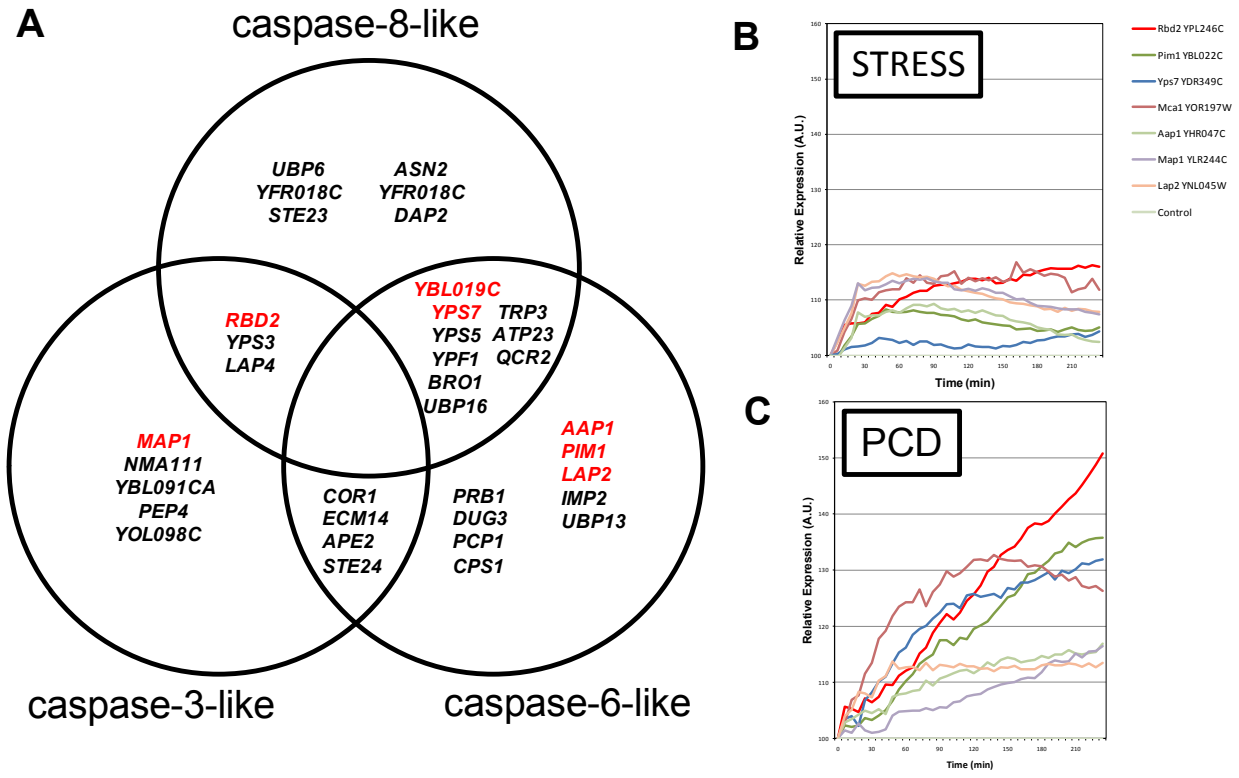

Figure 2. (**A**) Consensus caspase cleavage sites within human caspase substrates. (**B**) Consensus sequence of aligned yeast homologues of human caspase substrates. Amino acids are colored according to their chemical properties: polar amino acids (G,S,T,Y,C,Q,N) are green, basic (K,R,H) blue, acidic (D,E) red and hydrophobic (A,V,L,I,P,W,F,M) amino acids are black. (**C**) Clustered similarity matrix for individual human caspases substrates and yeast homologues with a conserved aspartate residue at P1 (yASP), the complete set of conserved yeast proteins with any amino acid at the P1 position (Yorig) and a set of random 8-amino acid yeast sequence substitutions based on the background amino acid frequencies (ysub).