Programmed Cell Death Initiation and Execution in Budding Yeast

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ABSTRACT Apoptosis or programmed cell death (PCD) was initially described in metazoans as a genetically controlled process leading to intracellular breakdown and engulfment by a neighboring cell . This process was distinguished from other forms of cell death like necrosis by maintenance of plasma membrane integrity prior to engulfment and the well-defined genetic system controlling this process. Apoptosis was originally described as a mechanism to reshape tissues during development. Given this context, the assumption was made that this process would not be found in simpler eukaryotes such as budding yeast. Although basic components of the apoptotic pathway were identified in yeast, initial observations suggested that it was devoid of prosurvival and prodeath regulatory proteins identified in mammalian cells. However, as apoptosis became extensively linked to the elimination of damaged cells, key PCD regulatory proteins were identified in yeast that play similar roles in mammals. This review highlights recent discoveries that have permitted information regarding PCD regulation in yeast to now inform experiments in animals.

KEYWORDS cyclin C; apoptosis; oxidative stress; mitochondria; signal transduction

TWO types of regulated cell death, necrosis and programmed cell death, have been described in budding yeast (Lin and Austriaco 2014). Necrotic cell death was originally characterized as a simple collapse of the cell leading to cell wall breakdown and ultimately lysis. However, more recent studies report the existence of a regulatory network governing necrotic cell death (Eisenberg *et al.* 2010). This review concentrates on programmed cell death (PCD) in yeast, which closely resembles the intrinsic or mitochondrial-derived apoptosis in multicellular organisms (Perrone *et al.* 2008). Mammalian apoptosis is initiated by accumulation of **B**cl2 **h**omology 3 (BH3) containing proteins such as Bax on the mitochondrial outer membrane. Bax induces pore formation leading to the release of cytochrome *c*, which stimulates a cascade of proteases termed **c**ysteine-dependent **asp**artate-specific prote**ases** or caspases (Danial and Korsmeyer 2004). Plants and fungi possess a related protease family called metacaspases (Uren *et al.* 2000). Metacaspases share sequence and functional similarities but differ with respect to substrate recognition sites (asparagine/lysine rather than aspartic acid). Budding yeast possesses a single metacaspase (Yca1) and BH3 domain protein (Ybh3), which are both required for oxidative stress-induced PCD. Standard assays for PCD, such as double strand breaks or phophatidylserine externalization (Annexin V staining), routinely used to monitor apoptosis in metazoans, are also employed to assay PCD in yeast (Madeo *et al.* 1997). However, following excessive damage, these PCD hallmarks may be joined by necrotic markers (*e.g.*, propidium iodide permeability) (Yamaki *et al.* 2001). Therefore, it is important to note that these different cell-death modes can be observed simultaneously within a population and care should be used when judging the contribution that each death pathway has on overall cell viability.

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Oxidative Stress, a Common Denominator for PCD Initiation

There are many stimuli, either externally or internally derived, able to induce PCD in yeast. For example, aging (Corte-Real and Madeo 2013), extreme pH environment (Ludovico *et al.* 2001), plant toxins (Narasimhan *et al.* 2001), defects in actin function (Gourlay and Ayscough 2006), osmotic stress (Silva *et al.* 2005), acetic acid (Ludovico *et al.* 2002), the presence of lipid hydroperoxides (Alic *et al.* 2003), and prolonged mating-factor exposure (Severin and Hyman 2002) (although the exact nature of this cell death

is in question) (Zhang *et al.* 2006) all stimulate PCD. Although these stressors appear different, many have in common the ability to generate internal reactive oxygen species (ROS). For example, a specific mutation in Cdc48 induces PCD in yeast (Madeo *et al.* 1997) due to elevated ROS (Madeo *et al.* 1999) produced from defective mitochondria (Braun *et al.* 2006; Braun and Zischka 2008). Similarly, defects in endoplasmic reticulum (ER)-dependent protein folding also produces ROS (Tu and Weissman 2004) to levels sufficient to induce PCD (Haynes *et al.* 2004). In addition, defects in the electron transport chain (ETC) lead to ER-produced ROS through hyperactivation of the ER NADPH oxidase Yno1 (Leadsham *et al.* 2013). These findings demonstrate the intricate relationships that have evolved between organelles that produce and respond to ROS-induced damage. The transcriptional response to, and the macromolecular damage caused by, oxidative stress in yeast are the subject of several excellent reviews (Avery 2011; Farrugia and Balzan 2012; Morano *et al.* 2012) and will not be detailed here. Rather, given the universal nature of the oxidative stress response from yeast to humans, this review focuses on recent insights into the signaling systems that transduce the ROS signal and the effector proteins that coordinate the response between organelles in budding yeast.

External origins of ROS

The cell maintains redox homeostasis by balancing low-level ROS produced by organelles or exogenous sources with an arsenal of antioxidant enzymes that neutralize reactive oxygen (*e.g.*, superoxide dismutase, catalase) or repair oxidative damage (*e.g.*, chaperones, DNA repair enzymes) once it occurs (Perrone *et al.* 2008). However, increased internal ROS concentrations above a certain threshold lead to an accumulation of oxidized lipids, proteins, and DNA, collectively termed oxidative stress (Tsuzi *et al.* 2004; Drakulic *et al.* 2005; Temple *et al.* 2005). Exogenous sources of ROS occur in many forms including prooxidants such as H_2O_2 (Veal *et al.* 2007), exposure to heavy metals that stimulate superoxide production through the Fenton reaction (Liang and Zhou 2007; Nargund *et al.* 2008), or treatment with certain anticancer drugs (Almeida *et al.* 2008) (Figure 1). Exogenous ROS can alter plasma membrane characteristics that trigger sensors able to induce signal transduction pathways such as the cell-wall integrity pathway (Levin 2011) or the osmolarity-sensing pathway (Singh 2000; Bilsland *et al.* 2004) resulting in dramatic changes in the transcriptome. In addition, direct oxidation of transcription factors (*e.g.*, Yap1) promotes stress-responsive gene transcription (Delaunay *et al.* 2000; Kuge *et al.* 2001).

Internal sources of ROS

Internal ROS is mostly derived from organelles performing their normal functions. The best studied and perhaps most important of these are the mitochondria (Figure 1). The mitochondrial function of ATP synthesis inherently produces

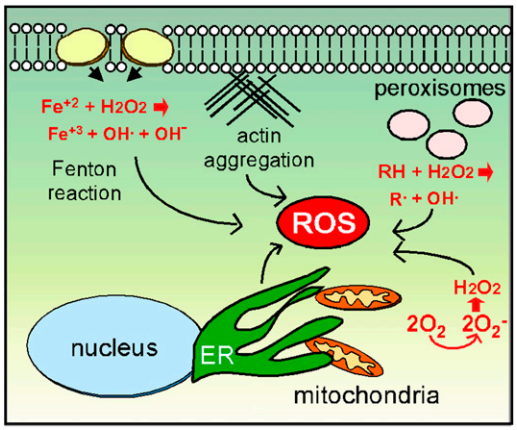

Figure 1 Sources of reactive oxygen species (ROS). External sources of ROS can be derived from prooxidants like H_2O_2 or heavy metals that form reactive oxygen as a byproduct of the Fenton reaction. Internal sources of ROS sufficient to induce an oxidative stress response are the result of organelle dysfunction including the peroxisomes, mitochondria, and the ER. Fatty acid oxidation in the peroxisome leads to reactive oxygen intermediates. In the mitochondria, NADPH oxidase converts molecular oxygen to reactive species that are converted to H_2O_2 by super oxide dismutase. Actin aggregation indirectly induces ROS through elevated RAS signaling and regulation of mitochondrial dynamics.

reactive oxygen through the leakage of electrons from the ETC. This amount of ROS is limited and thought to represent a signaling molecule affecting many cellular processes (Guaragnella *et al.* 2012). However, mitochondrial dysfunction via mutations in ETC components, compounds that inhibit ETC function, or loss of mitochondrial inner membrane integrity, can generate sufficient ROS concentrations to induce the oxidative stress response (Eisenberg *et al.* 2007). For example, cytochrome *c* mutants display ETC defects that generate H_2O_2 (Barros *et al.* 2003). In addition, stimulating Ras signaling induces high protein kinase A (PKA) activity, leading to loss of mitochondrial integrity and elevated internal ROS (Hlavata *et al.* 2003; Hlavata *et al.* 2008; reviewed in Perrone *et al.* 2008).

In addition to defects in internal processes, mitochondrial-derived ROS can be caused via indirect mechanisms as well. For example, mutations or drugs that reduce actin dynamics cause elevated mitochondrially derived ROS (Gourlay *et al.* 2004). Interestingly, enhancing actin dynamics by deleting a gene (*SCP1*) encoding a bundling protein reduces ROS (Gourlay *et al.* 2004). A second connection between actin and mitochondrial fitness is observed during partitioning of this organelle to daughter cells. Myosin motors direct mitochondria toward the bud along F-actin cables to facilitate organelle partitioning (Mishra *et al.* 2014). In addition, a retrograde actin cable force is present that directs cargo toward the mother. Healthy mitochondria can bind the motors with sufficient strength to navigate to the bud despite the retrograde force moving in the opposite direction (McFaline-Figueroa *et al.* 2011). Pon and coworkers have likened this phenomenon to salmon swimming upstream against the river current (Higuchi *et al.* 2013). This

process assures healthy mitochondria migrate to the bud while defective and ROS leaking mitochondria remain in the mother. As described later, this phenomenon may have consequences in aging-induced PCD.

In addition to the mitochondria, the ER is also a source of reactive oxygen in the cell. The ER provides the critical function of folding newly synthesized proteins and then sorting them for various cellular addresses (Chen *et al.* 2013). ER protein folding utilizes specialized chaperones (protein disulfide isomerases and Ero1) and an oxidative environment (Pollard *et al.* 1998) resulting in conversion of oxygen to H_2O_2 (Zito 2015). Defects in protein folding trigger the well-studied unfolded protein response (UPR) that induces *ERO1* transcription. Prolonged Ero1p expression elevates ROS concentrations, ultimately leading to cell death (Haynes *et al.* 2004). Interestingly, the UPR leads to ROS generation by both the ER and the mitochondria. For example, Yno1/Aim14, a NADPH-oxidase found in the ER, generates ROS and promotes PCD (Rinnerthaler *et al.* 2012). Normally, Yno1-generated ROS concentrations are low and considered a signaling molecule in other fungi (Malagnac *et al.* 2004). However, yeast strains overexpressing Yno1 produce sufficient ROS to induce PCD. Although Yno1 is not part of the ER stress response, cytochrome oxidase *c*-defective mitochondria also raise Yno1 activity by preventing its normal turnover (Leadsham *et al.* 2013). Similar to the engineered overexpression studies, elevated Yno1 levels produce sufficient ROS to induce cell death. These studies highlight the intimate relationship between the ER and mitochondria with respect to ROS homeostasis.

Other organelles also contribute to oxidative stress. The peroxisome is important for β-oxidation of fatty acids that produce oxygen radicals and hydroperoxides (Manivannan *et al.* 2012). In addition, ROS are generated from peroxisomes that are defective in either form or function. For example, loss of Pex6 activity, a protein involved in peroxisome import, results in cells accumulating ROS to levels sufficient to induce cell death (Jungwirth *et al.* 2008). However, these cells show hallmarks of necrosis rather than PCD, indicating that internally produced ROS can induce multiple types of cell death. As discussed below, signaling systems that transduce the ROS signal have been identified. It will be interesting to determine if ROS generated from the mitochondria, ER, or peroxisomes activate similar or different pathways to trigger the oxidative stress response.

Aging and PCD

Two types of aging, chronological and replicative, are studied in yeast. Chronological aging examines how long cells can remain alive in stationary phase and is thought to be analogous to quiescent, postdifferentiated mammalian cells (Braun and Westermann 2011; Corte-Real and Madeo 2013). Conversely, replicative aging determines the number of cell divisions an individual mother cell can undergo and has been proposed to serve as a model for stem cell-like divisions. Both aging types are controlled by genetic factors as well as nutritional conditions, many of which impact mitochondrial function (Kaeberlein 2010; Corte-Real and Madeo 2013). Both replicative and chronological aging processes in budding yeast are driven by ROS accumulation that ultimately results in PCD (Laun *et al.* 2001; Fabrizio *et al.* 2004; Herker *et al.* 2004). For example, mother cells age through accumulation of oxidatively damaged proteins or mitochondria that are not passed on to their daughters (Aguilaniu *et al.* 2003; McFaline-Figueroa *et al.* 2011). In addition, protein aggregates are retained in aging mothers (Rujano *et al.* 2006; Spokoini *et al.* 2012) thus allowing daughter cells to start with a clean aging slate. The cell has multiple avenues to counteract these aging hallmarks. For example, protein aggregates are recognized as cellular damage and are degraded through the activity of protein chaperones and the metacaspase Mca1/Yca1 (Hill *et al.* 2014). Similarly, chronologically aged cells induce the NADP-dependent glutamate dehydrogenase Gdh3 that detoxifies ROS and prevents PCD initiation (Lee *et al.* 2012). These studies, as well as many others, provide a direct link between ROS accumulation, PCD initiation, and longevity. In mammals, this question is more complex as oxidative stress-induced pathology is influenced by the presence of cellular damage, and by other confounding factors including tissue type, stage in development, and the subcellular compartment in which the ROS originated (Cunningham *et al.* 2015). Therefore, the utility of yeast or mammalian tissue culture as a model to investigate some aspects of the free radical theory of aging may be limited.

Role of the mitochondrial dynamics in PCD execution

Mitochondria are dynamic organelles undergoing constant fusion and fission during normal cell division. The proper balance between these activities is required for normal mitochondrial function and to minimize ROS leakage (Ishihara *et al.* 2009; Wakabayashi *et al.* 2009). Under normal growth conditions, the mitochondria are elongated and interconnected. The high surface-to-volume ratio of this structure supports maximum ATP generation capacity and allows repair of damaged organelles through mixing of membrane components and recombination between nucleoids (Braun and Westermann 2011). Conversely, fission enhances the removal of damaged mitochondria via a specialized form of autophagy (mitophagy) and distribution of the organelle to daughter cells (Muller and Reichert 2011; Kurihara *et al.* 2012). The equilibrium between fission and fusion is controlled by the activity of conserved molecular machines driven by dynamin-like GTPases (see Westermann 2010 for review). In budding yeast, the fusion of the inner and outer mitochondrial membranes requires the Mgm1 and Fzo1 GTPases, respectively (Rapaport *et al.* 1998; Meeusen *et al.* 2006). Mitochondrial fission requires the GTPase Dnm1, which forms atypical helical filaments that first encircle, then constrict, mitochondria until scission is achieved (Otsuga *et al.* 1998; Bleazard *et al.* 1999; Sesaki and Jensen 1999). Recruitment of Dnm1 to the mitochondrial outer

membrane (MOM) requires the receptor Fis1 (Mozdy *et al.* 2000; Tieu *et al.* 2002) and one of two adaptors, Mdv1 (Mozdy *et al.* 2000; Tieu *et al.* 2002; Cerveny and Jensen 2003) or Caf4 (Schauss *et al.* 2006; Motley *et al.* 2008). Interestingly, peroxisome fission also requires Fis1 and one of two dynamin-like proteins, Vps1 or Dnm1; the latter seems only important in cultures grown on oleic acid (Hoepfner *et al.* 2001; Kuravi *et al.* 2006). For mitochondria, fission often occurs at sites of interaction with the ER (Friedman *et al.* 2011). Many roles have been described for these junctions including sites of lipid transfer and Ca^{++} signaling (Michel and Kornmann 2012). Therefore, mitochondria–ER communication appears to be important for mitochondrial fission as well.

Of particular interest for this review, extensive mitochondrial fragmentation is a common feature following exposure to many types of damage including oxidative stress. Stress-induced mitochondrial hyperfission is conserved from yeast to mammals and represents an early morphological adaptation of the stress response (Youle and van der Bliek 2012). Mitochondrial hyperfission has been associated with the release of sequestered apoptotic factors (Frank *et al.* 2001; Breckenridge *et al.* 2003) while preventing fission protects cells from PCD. For example, mutants lacking Dnm1 or Fis1 are resistant to ROS-induced PCD (Fannjiang *et al.* 2004).

Although the basic fission machinery is required for stress-induced hyperfission, how their activity is enhanced occurs through an unlikely mechanism. In all eukaryotes examined, cyclin C (Ssn8) and Cdk8 (Ssn3) form a protein kinase that associates with the RNA polymerase II holoenzyme to control transcription (Bourbon 2008) (Figure 2). In budding yeast, this kinase represses ~100 genes that are induced in response to environmental stress (Cooper *et al.* 1997; Holstege *et al.* 1998; van de Peppel *et al.* 2005). To relieve cyclin C–Cdk8 repression, stressed cells translocate cyclin C from the nucleus to the cytoplasm where it is ultimately destroyed through activity of the Not4 ubiquitin ligase (Cooper *et al.* 2012). However, cyclin C has a second function independent of Cdk8. Prior to its destruction in the cytoplasm, cyclin C associates with Mdv1 to induce extensive mitochondrial fragmentation (Cooper *et al.* 2014; reviewed in Strich and Cooper 2014). Deletion of its nuclear anchor, *MED13*, allows aberrant entry of cyclin C into the cytoplasm where it can induce fission in the absence of stress (Khakhina *et al.* 2014). These results indicate that cyclin C is both necessary and sufficient for hyperfission. Cyclin C-dependent hyperfission is directly related to the ability of the cell to induce PCD. Mutants lacking cyclin C are protected from ROS-induced PCD, whereas *med13Δ* mutants, in which the mitochondria are continuously fragmented, are hypersensitive to oxidative stress (Khakhina *et al.* 2014). It is important to note that continuous mitochondrial fission on its own is insufficient to induce cell death, although the health of this organelle suffers under these conditions through loss of mtDNA (Khakhina *et al.* 2014). These observations indicate that mitochondrial fragmentation potentiates the cell toward PCD initiation, but another stress signal is required to initiate this process. The role cyclin C plays in mitochondrial fission and PCD is remarkably well conserved. Mammalian cyclin C also translocates from the nucleus to the mitochondria in response to stress (Wang *et al.* 2015). Knockout mouse embryonic fibroblast (MEF) cells revealed that cyclin C is required for stress-induced mitochondrial fission and apoptotic cell death. Finally, the yeast cyclin C is able to induce complete mitochondrial fragmentation when purified protein is added to permeabilized MEF cultures. In the other direction, the human cyclin C complements the cell-death-resistance phenotype in *cnc1Δ* yeast mutants but not the transcriptional repression defect (Krasley *et al.* 2006). This analysis represents an example in which the direction of information understanding apoptotic control flowed from yeast to mammalian studies.

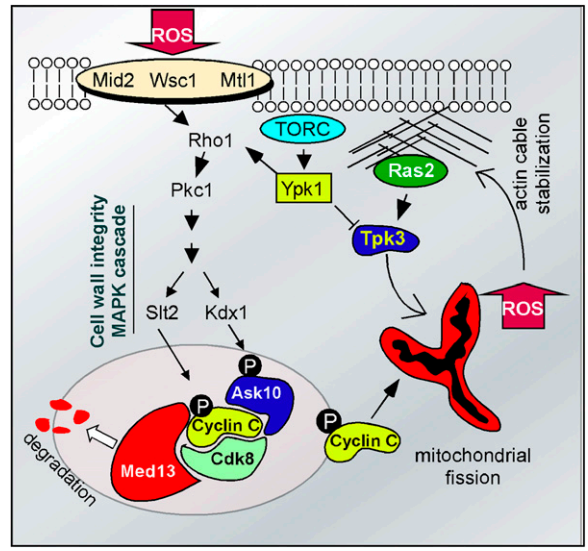

Figure 2 Signal transduction pathways and the oxidative stress response. Exposure to ROS-generating compounds activates cell wall sensors (Mid2, Wsc1, Mtl1) connected to the cell wall integrity MAP kinase pathway. Activation of the MAP kinase (Slt2) and the pseudokinase (Kdx1) result in cyclin C and Ask10p phosphorylation, respectively. Phosphorylation of cyclin C initiates its relocation to the mitochondria. The role of Ask10 modification is currently unknown. Cyclin C relocalization also requires destruction of Med13, the anchor protein that tethers cyclin C to the RNA polymerase II holoenzyme. TORC signaling restricts ROS accumulation by regulating cyclin C levels through the CWI pathway and inhibiting the protein kinase A subunit Tpk3. When activated by Ras due to actin aggregation, Tpk3 causes accumulation of mitochondrial-dependent ROS that can result in more actin filament aggregation due to oxidation of conserved cysteine residues.

Signaling Pathways Directing ROS-Induced PCD: The Cell-Wall Integrity Pathway Controlling Cyclin C Nuclear Release

As indicated above, the failure to translocate cyclin C into the cytoplasm protects the cell from H_2O_2-induced PCD, while its aberrant release from the nucleus causes hypersensitivity to oxidative damage. Given the importance of this

Table 1 Signaling molecules

Yeast proteins	Mammalian orthologs	Function	Reference
Cdc48	VCP/p97	Protein turnover, ER stress response.	Madeo *et al.* (1997)
Mtl2, Mid2, Wsc1	Unknown	Transmembrane receptors sensing oxidative stress. Required for cyclin C nuclear release.	Jin *et al.* (2013)
Ras2	Ras	Relays plant antifungal and aging signals to stimulate PCD.	Narasimhan *et al.* (2005); Gourlay and Ayscough (2006)
Ste20	Mst	Signals excessive mating pheromone response. Ca^{++} mobilization, phosphorylates histone H2B.	Ahn *et al.* (2005); Severin (2002)
Slt2/Mpk1	MAP kinase	Downstream effector of cell-wall integrity. MAP kinase pathway. Required for ROS-induced cyclin C nuclear release.	Jin *et al.* (2014)
Kdx1	MAP kinase pseudokinase	Downstream effector of cell-wall integrity. MAP kinase pathway. Required for ROS-induced cyclin C nuclear release.	Jin *et al.* (2014)

decision, it is not surprising that the switch controlling cyclin C release is complex and appears to be composed of at least two arms. First, the nuclear anchor, Med13 is destroyed in response to oxidative stress with kinetics similar to cyclin C release (Khakhina *et al.* 2014). This destruction is dependent on the 26S proteasome maturation factor Ump1, suggesting the involvement of ubiquitin-mediated proteolysis. Consistent with this model, the SCF ubiquitin ligase is required for ROS-induced Med13 destruction manner (K. F. Cooper, unpublished results). This result parallels a previous study in mammalian cells revealing a role for the SCF ligase in normal Med13 turnover (Davis *et al.* 2013). Currently, it is not known whether the yeast Med13 degradation is essential for cyclin C nuclear release or whether its proteolysis serves to prevent retention of the cyclin if it reenters the nucleus.

The second arm of the cyclin C control pathway is mediated by the cell-wall integrity MAP kinase pathway and includes a bifurcation at the MAP kinase step (Figure 2, see Table 1). The cell-wall integrity pathway transduces the oxidative stress signal from the cell wall to the nucleus to affect changes in transcription (Alic *et al.* 2003; Staleva *et al.* 2004; Vilella *et al.* 2005; Krasley *et al.* 2006; Petkova *et al.* 2010) and actin remodeling (Pujol-Carrion *et al.* 2013). Treating cells with H_2O_2 activates two cell-wall receptor groups containing Wsc1 and either Mid2 or Mtl1 (Vilella *et al.* 2005; Petkova *et al.* 2010; Jin *et al.* 2013). The receptors in turn stimulate the Rho1p GTPase, which activates Pkc1 and the cell-wall integrity MAP kinase pathway (Levin 2011). Recent studies revealed that the Slt2 MAPK directly phosphorylates cyclin C at Ser266 (Jin *et al.* 2014). Eliminating this phosphorylation site prevents cyclin C cytoplasmic translocation, while a phosphomimetic mutation enhances its translocation (Strich and Cooper 2014). The other branch contains the pseudokinase Kdx1 that associates with Ask10 (Jin *et al.* 2014), a previously identified cyclin C-associating factor (Cohen *et al.* 2003). Ask10 is required for cyclin C cytoplasmic translocation (Jin *et al.* 2014) and is phosphorylated in response to oxidative stress (Cohen *et al.* 2003). Surprisingly, Ask10p phosphorylation requires the two cell-wall-integrity pathway MAPKKs, Mkk1 and Mkk2, and the pseudokinase Kdx1, but not Slt2 (Cohen *et al.* 2003; Jin *et al.* 2014), suggesting the presence of yet another signaling system controlling cyclin C release.

Signaling Systems Directing ROS-Induced PCD: The Ras Signaling Pathway

Ras2 signaling also contributes to PCD initiation but in a manner different from the cell-wall integrity pathway. Rather than sensing and transducing the presence of ROS-induced damage, aberrant Ras signaling causes loss of mitochondrial integrity and subsequent ROS release (Smethurst *et al.* 2014). For example, aberrant actin aggregation, caused by specific actin monomer mutations or drugs that promote filament bundling, stimulates Ras2 signaling leading to activation of protein kinase A subunit Tpk3 (Gourlay *et al.* 2004; Gourlay and Ayscough 2006; Leadsham and Gourlay 2010). Tpk3 activation leads to elevated ROS levels in the cell (Figure 2). This system sets up a potential feedback loop in which the mitochondrial-derived ROS drives more actin aggregation through increased disulfide linkage of actin monomers (Haarer and Amberg 2004). Similarly, stationary-phase cells exposed to continuous Ras2 activation display elevated ROS levels and undergo PCD (Gourlay and Ayscough 2005). In both situations, constitutively active Ras results in a Tpk3-dependent loss of mitochondrial integrity and elevated ROS. These findings are similar to results obtained in mammalian cell culture in which prolonged RAS/RAF/ERK signaling also induces apoptosis (Cagnol and Chambard 2010).

The retrograde signaling pathway transduces information about mitochondrial activity and integrity to the nucleus to affect changes in gene expression (Jazwinski 2013). One component of this pathway is the target of rapamycin complex 2 (TORC2), which responds to cellular redox conditions through activation of Ypk1 protein kinase (Niles *et al.* 2014). Ypk1 stimulation activates the cell-wall integrity pathway through maintenance of sphingolipid levels required for proper localization of Rom2, the GAP activator of Rho1 (Niles *et al.* 2014) (Figure 2). This pathway keeps internal ROS levels in check, thus preventing cyclin C cytoplasmic relocalization and destruction (Niles and Powers 2014). Ypk1 also inhibits Tpk3 activity thereby maintaining normal

mitochondrial function and reducing excessive ROS production (Niles *et al.* 2014). Thus, internal reactive oxygen levels are constantly monitored and adjusted to allow ROS to serve as a signaling molecule under certain situations.

Chromatin Modification and PCD Execution

Other, less-well-understood signaling pathways also play a conserved role in PCD execution. Ste20 is the founding member of the PAK (p21 activated protein kinase) protein family (Dan *et al.* 2001). A mammalian homolog of Ste20 (Mst1) (Creasy *et al.* 1996) is activated by caspase cleavage and phosphorylates histone H2B on serine 14 (Cheung *et al.* 2003). This modification promotes chromatin condensation and apoptotic cell death. Similarly, in response to oxidative stress, yeast Ste20 also phosphorylates the analogous H2B residue (Ser10) (Ahn *et al.* 2005) (Figure 3). Mutating H2B Ser10 to alanine protects the cell from H_2O_2-induced PCD, indicating an important role for this modification in the stress response. However, Ser10 phosphorylation occurs following H_2O_2 exposure only when the adjacent lysine (Lys11) is deacetylated by the Hos3 deacetylase (Ahn *et al.* 2006). Therefore, the stress signal must integrate both Hos3 and Ste20 activity. Conversely, monoubiquitylation of Lys123 on histone H2B by the Bre1 ligase prevents Yca1-dependent H_2O_2-induced PCD induced by chronological aging (Walter *et al.* 2010). In addition to H2B, histone H3 is also a target of posttranslational modifications that control PCD execution. Methylation of H3 Lys4 (H3K4) by Set1 reduces aging-dependent PCD (Walter *et al.* 2014). Consistent with this finding, deleting *SET1* renders cells more sensitive to oxidative stress, whereas mutating the demethylase, *JHD2*, makes cells more resistant. These results reveal a chromatin-based tug-of-war between opposing signals that promote or inhibit PCD execution.

Regulators of mitochondrial outer membrane permeability

Similarly to mammalian cells, mitochondrial outer membrane permeabilization represents the commitment step to PCD execution (Green and Kroemer 2004; Antignani and Youle 2006). The loss of inner and outer mitochondrial membrane integrity is required for release of proapoptotic factors such as cytochrome *c* and two nucleases, Nuc1 and Aif1 (see Table 2). In mammalian cells, mitochondrial permeability is regulated through the competing activities of prosurvival (*e.g.*, Bcl-2) and proapoptotic (*e.g.*, Bax, Bak) Bcl-2 homology (BH) family members (Green and Kroemer 2004). In response to proapoptotic stimuli, Bax is recruited to the mitochondrial outer membrane, where it, in conjunction with Bak, forms pores that permeabilize the outer membrane. Therefore, the proper control of Bax and Bcl-2 activity is critical for the correct response to cellular damage. In yeast, loss of the BH-homology protein Ybh3 function reduces PCD efficiency in response to oxidative stress or aging, whereas its overexpression increases H_2O_2 sensitivity

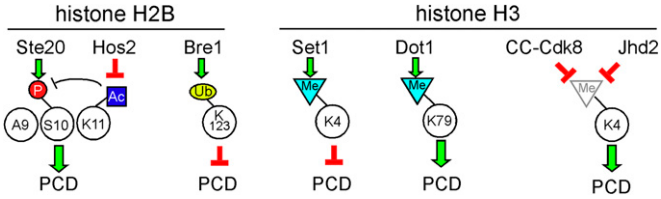

Figure 3 Chromatin modification and PCD regulation. H2K11 acetylation prevents H2S10 phosphorylation and chromatin condensation. Activation of Hos2 deacetylase removes the acetylation mark, allowing Ste20-dependent phosphorylation of H2S10 and PCD completion. Separate methylation marks at H3K4 and H3K79 have opposite impacts on PCD progression. Independent cyclin C–Cdk8 and Jhd2 demethylase activities reduce H3K4 methylation antagonizing Set1 activity and promoting cell death.

(Buttner *et al.* 2011). In addition, Ybh3 function requires the proposed BH3 domain and its activity is suppressed by expression of the prosurvival human Bcl-X_L (Buttner *et al.* 2011). Finally, similarly to mammalian Bax, which relocalizes from the cytoplasm to the mitochondria (Lovell *et al.* 2008; reviewed in Renault *et al.* 2013), Ybh3 translocates from the vacuole to the mitochondria in response to stress (Buttner *et al.* 2011). Interestingly, Ybh3 function requires two associated proteins, Cor1 and Mir1, a ubiquinol–cytochrome *c* oxidoreductase subunit, and a mitochondrial phosphate carrier, respectively (Buttner *et al.* 2011). A similar function was confirmed for the mammalian orthologs of these proteins, QCR1 and PHC (Buttner *et al.* 2011). These results illustrate that, as with the cyclin C studies, the information obtained in analyzing yeast PCD is helping to instruct similar studies in mammalian cells.

Executioners of the programmed cell death pathway

The ultimate goal of mitochondrial outer membrane permeability is the release of proapoptotic proteins including cytochrome *c* and two nucleases (Aif1 and Nuc1, see Table 2). Genetic studies have verified their role in PCD. Deleting these nucleases increases resistance to ROS-induced cell death, whereas their overexpression causes hypersensitivity (Wissing *et al.* 2004; Buttner *et al.* 2007). Similar to their mammalian counterparts Aif1 and EndoG, the yeast Aif1 and Nuc1 enter the nucleus and fragment chromatin. In mammalian cells, Aif1 activity requires association with the peptidylprolyl *cis-trans* isomerase cyclophilin A (Cande *et al.* 2004). Likewise, yeast Aif1 function is dependent on the yeast homolog of cyclophilin A (Cpr1) but not cyclophilin B (Cpr2). Taken together with the chromatin modification similarities, the nuclear changes in response to PCD execution are remarkably similar in yeast and mammals.

In mammalian cells, release of cytochrome *c* from mitochondria activates the caspase 9 initiator protease, which resides in the Apaf-1 apoptosome complex (Riedl and Salvesen 2007). Yeast genetic evidence indicates that cytochrome *c* is partially required for efficient PCD (Ludovico *et al.* 2002; Giannattasio *et al.* 2008), although no Apaf-1 ortholog has been identified. Genetic studies have identified

Table 2 Executioner molecules

Yeast protein	Mammalian orthologs	Function	Reference
Yca1/Mca1	Metacaspase	Cleave proteins	Madeo *et al.* (2002)
Nma111	HtrA2/Omi	Nuclear serine protease required for ROS-induced PCD, cleaves Bir1.	Fahrenkrog *et al.* (2004)
Bir1	IAP	Inhibitor of apoptosis. Substrate of Nma111.	Walter *et al.* (2006)
Aif1, Ndi1	Aif/AMID	Mitochondrial nuclease released following permeability. Required for chromatin destruction.	Wissing *et al.* (2004)
Esp1	Separin	Caspase-like protease, cleaves the cohesion Mcd1.	Yang *et al.* (2008)
Nuc1	EndoG	Mitochondrial nuclease released following permeability. Required for chromatin destruction.	Buttner *et al.* (2007)
Kex1	Caspase-like	Required for PCD in response to glycosylation defects, acetic acid, aging.	Hauptmann and Lehle (2008)
Cyclin C/Ssn3p	Cyclin C	Translocates to mitochondria following stress. Associates with mitochondrial fission machinery, required for mitochondrial fragmentation and permeability.	Cooper *et al.* (2012, 2014)
Ybh3	Bax	Translocate to the mitochondria following stress. Induce mitochondrial outer membrane permeability.	Buttner *et al.* (2011)

several proteases that are required for PCD execution. Similarly to the caspase cascade in mammalian cells, Yca1 is activated by proteolysis and required for H_2O_2 and acetic acid-induced PCD (Madeo *et al.* 2002). Esp1 cleaves the cohesin Mcd1 in response to H_2O_2 treatment (Yang *et al.* 2008). Nma111, an ortholog of the human HtrA protease (Belanger *et al.* 2009), cleaves Bir1, the yeast ortholog of the mammalian inhibitor of apoptosis factor (Walter *et al.* 2006). Interestingly, these proteases exhibit full, partial, or no role in PCD execution depending on the stress (Liang *et al.* 2008; Madeo *et al.* 2009). These results suggest that different stimuli utilize specific caspases to execute the cell-death pathway. Alternatively, these proteases may perform overlapping activities masking their roles. The genetic analyses possible in yeast will be able to address whether functional overlap exists between proteases, or whether additional proteases exist that have not been ascribed a role in PCD control. In support of the latter possibility, protease activities that do not correspond to known caspase-like enzymes have been identified that are able to cleave fluorescent substrates with specificities similar to mammalian caspases (Wilkinson and Ramsdale 2011).

Coordinating the oxidative stress response throughout the cell

The oxidative-stress response is a culmination of changes in gene expression, organelle structure/function, and the cytoskeleton (reviewed in Smethurst *et al.* 2014). The organellar communication between the nucleus and mitochondria has been well studied (Hill and Van Remmen 2014; Shaughnessy *et al.* 2014). One example of this coordination, and insight into the complexity of the regulatory system governing this process, is demonstrated by examining cyclin C–Cdk8 activity in stressed and nonstressed cells. Several studies indicate both a prosurvival and prodeath role for cyclin C translocation from the nucleus to the cytoplasm. As described above, transcriptome analysis and more directed studies indicate that cyclin C-Cdk8 represses genes involved in the stress response (Cooper *et al.* 1997; Holstege *et al.* 1998). Therefore, the stress-induced nuclear release of cyclin C inactivates Cdk8, which remains nuclear (Cooper *et al.* 2012). The inactivation of Cdk8 allows complete and timely induction of meiotic (Cooper *et al.* 1997) or stress-responsive (Cooper *et al.* 2012) genes. In addition, cyclin C–Cdk8 restricts H3K4 methylation (Law and Ciccaglione 2015), a chromatin mark that prevents PCD-induced chromatin condensation (Walter *et al.* 2014). As H3K4 methylation is associated with transcriptional activation, these processes may well be related. Finally, cyclin C translocation to the cytoplasm induces extensive mitochondrial fragmentation, which may aid in the removal of damaged, ROS-leaking organelles (Youle and van der Bliek 2012). Therefore, derepressing stress response genes, enhancing H3K4 methylation, and removing damaged mitochondria all protect cells from PCD-inducing insults. These findings would explain why *cnc1Δ* mutants are more resistant to oxidative stress than either *fis1Δ* or *dnm1Δ* mutants (Cooper *et al.*

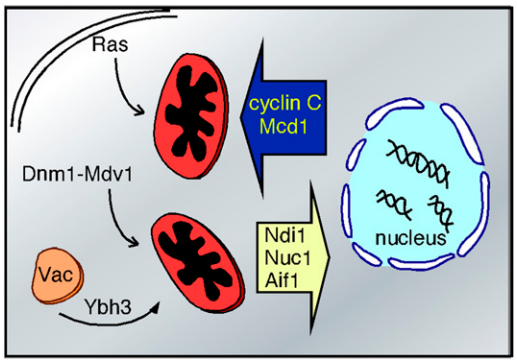

Figure 4 Stress-induced relocalization of PCD regulators. The direction of protein translocation is depicted in stressed cells. Ras2 and Ybh3 transit to the mitochondria from the plasma membrane and vacuole (Vac), respectively. Mdv1 and Dnm1 relocalize from the cytoplasm to the mitochondria to induce fission. Cyclin C and Mcd1 relocalization from the nucleus to the mitochondria induces fission and loss of mitochondrial outer membrane integrity. Translocation of mitochondrial proteins Aif1, Nuc1, and Ndi1 stimulate chromatin breakdown in the nucleus.

2014). However, the tipping point toward PCD is not mitochondrial fission. Therefore, the cell requires an additional signal, perhaps mitochondrial recruitment of Ybh3, to initiate the cell death pathway. In this process, the cell has utilized cyclin C relocalization to affect changes in gene expression, chromatin remodeling, and mitochondrial dynamics.

Physiological role for PCD in a single-celled organism

Due to the lack of obvious counterparts (*e.g.*, p53, Bcl-2), many early studies considered yeast an *in vivo* test tube in which to analyze mammalian apoptotic regulators free of complications from yeast-based PCD (Manon *et al.* 1997; Ligr *et al.* 1998; Lisa-Santamaria *et al.* 2009; Greenwood and Ludovico 2010; reviewed in Silva *et al.* 2011; Clapp *et al.* 2012). However, extensive studies in budding yeast, fission yeast, and other single-cell eukaryotes challenge this view (Shemarova 2010). The identification of conserved regulatory proteins such as cyclin C, Ybh3, and Yca1 in budding yeast argues that PCD regulation is an ancient process. Several models have been put forth to explain the early evolutionary appearance of both pro- and antiapoptotic proteins (Taylor-Brown and Hurd 2013). Given the prominent role of the mitochondria in PCD regulation, it is not surprising that many models start at the conception of the eukaryotic cell with a bacterial parasite that eventually became endosymbiotic with its host. As cellular stress is a universal PCD initiator, one possibility is that ancient parasites recognized that their host was compromised and elicited cell death. This provided the bacteria a last gasp of nutrients as well as a free path to find another host (Nedelcu *et al.* 2011). As this relationship evolved to be less selfish and more mutually beneficial, the health of the newly identified mitochondria became coordinated with the rest of the cell.

As the eukaryotic cell and its symbiont became more intertwined, regulatory systems evolved to take advantage of this unique situation (Ameisen 2002). For example, proteins that regulate the newly evolving PCD would be predicted to have "day jobs" required for normal cellular growth and development (Kroemer 1997). As described earlier, the yeast metacaspase Yca1 helps resolve protein aggregates. Nuc1 and Aif1 are important for mitochondrial RNA processing (Zassenhaus and Denniger 1994), while cyclin C regulates transcription in unstressed cells. However, it would be important for the cell to prevent the precocious activation of the PCD pathway until the proper stress signal occurs. To separate their cell death functions from their important day jobs, the cell utilizes regulated subcellular relocalization. For example, Ybh3 is found on the vacuole in unstressed cells but relocalizes to the mitochondria following stress (Figure 4). Likewise, cyclin C translocates from the nucleus to the mitochondria upon stress. Conversely, Nuc1 (Buttner *et al.* 2007), Aif1 (Wissing *et al.* 2004), and the AMID ortholog Ndi1 (Li *et al.* 2006) leave the mitochondria and are targeted to the nucleus where they fragment chromatin. In addition, Ras2 translocates from the plasma membrane in response to loss of mitochondria activity (Amigoni *et al.* 2013) or defects in ETC function (Leadsham *et al.* 2013). Mitochondrial Ras2 accumulation increases ROS production and sensitizes cells to PCD (Amigoni *et al.* 2013; Leadsham *et al.* 2013). In addition, H_2O_2 treatment induces the Esp1-dependent cleavage of the chromosomal cohesion Mcd1, resulting in a carboxyl terminal fragment that relocalizes to the mitochondria to drive loss of mitochondrial integrity (Yang *et al.* 2008). Therefore, the increased compartmentalization of the eukaryotic cell stages proteins at one address but allows their translocation to a different location in response to stress.

Why would yeast maintain a cell death pathway? Altruism has been argued to provide a selective pressure to maintain PCD based on the normal colony mode for yeast growth. For example, colonies contain regions of young and old cells (Vachova and Palkova 2005; reviewed in Gourlay *et al.* 2006) with the death of older cells no longer capable of cell division providing metabolites for the younger cells. Sporulating colonies also provide evidence for more complex architecture in that zones of sporulating cells are separated by vegetative layers (Piccirillo and Honigberg 2010). This patterning is consistent with cells possessing different "identities" based on their age, location within the colony, and environmental signals. Therefore, recycling the components of severely damaged or nonreplicative cells within a colony would maximize growth chances for younger, reproductive cells.

Future challenges for the singled-cell model community

As the single-celled eukaryotic community moves past the "if" and "why" questions concerning PCD, attention can now be focused on "how." It seems clear that as eukaryotes became more complex, additional layers of regulation were required to fulfill the requirements for tissue sculpting and removal of unwanted immune cells and damaged cells. Although some of these regulatory systems may be missing in single-celled organisms, the basic switches that recognize damage, transmit the signals, and coordinate the responses between the different organelles appear well conserved. Therefore, understanding how organelle-to-organelle communication coordinates both the stress response and ultimately PCD initiation represents a key challenge for the community in the near future.

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