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YEAST PROGRAMED CELL DEATH AND AGING

Topic Editors
Manuela Côrte-Real and Frank Madeo

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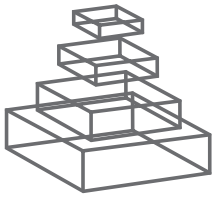

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YEAST PROGRAMED CELL DEATH AND AGING

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Similarly to metazoans, yeast cells can exhibit several characteristics of apoptosis, including chromatin condensation, DNA breakage, flipping of phosphatidylserine to the outer leaflet of the plasma membrane, accumulation of reactive oxygen species (ROS), and release of pro-death factors such as cytochrome c or Endonuclease G from mitochondria. Yeast programed cell death has been shown to occur in response to a variety of stimuli, such as oxidative stress, exposure to acetic acid, and expression of mammalian pro-apoptotic proteins. This program is also inherent to the yeast life cycle, as aged mother cells and cells exposed to pheromone also display an apoptotic and necrotic phenotype. Yeast therefore comprises a conserved core programed cell death process that shares several regulators with mammalian cells, which play major roles in the pathogenesis of human diseases. At the same time, it lacks many of the cell death regulators that have evolved in higher eukaryotes, probably due to the invention of multicellularity. The simplicity of the yeast model allows elucidating the basic molecular pathways of programed cell death without interference from multifaceted regulation, due to various protein isoforms or cellular specificity often observed in studies using mammalian systems. In addition, yeast heterologous expression systems offer the opportunity to exploit the individual functional and mechanistic properties of mammalian apoptotic regulators.

This e-book gathers recent studies enhancing the understanding of PCD and its deregulation, relevant in human pathologies and aging. These include review, mini-review, original research, perspective, and hypothesis and theory articles dealing with the identification of previously uncharacterized proteins and the function of different cellular compartments and organelles involved in PCD and aging, as well as the exploitation of humanized yeasts to untangle the role of apoptotic regulators.

Yeast has long been established as a valuable model system to study conserved biological processes of relevance to human health, and several reviews address the importance of studying endogenous yeast mechanisms to understand human pathologies, particularly cancer and aging. Mollinedo stresses the relevance of lipid rafts in cell physiology and the advantages of the yeast model system to address unsolved questions regarding their role in

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EDITORIAL
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Yeast programed cell death and aging

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Keywords: programmed cell death, apoptosis, necrosis, yeast, cell model system

Similarly to metazoans, yeast cells can exhibit several characteristics of apoptosis, including chromatin condensation, DNA breakage, flipping of phosphatidylserine to the outer leaflet of the plasma membrane, accumulation of reactive oxygen species (ROS), and release of pro-death factors such as cytochrome *c* or Endonuclease G from mitochondria. Yeast programed cell death has been shown to occur in response to a variety of stimuli, such as oxidative stress, exposure to acetic acid, and expression of mammalian pro-apoptotic proteins. This program is also inherent to the yeast life cycle, as aged mother cells and cells exposed to pheromone also display an apoptotic and necrotic phenotype. Yeast therefore comprises a conserved core programed cell death process that shares several regulators with mammalian cells, which play major roles in the pathogenesis of human diseases. At the same time, it lacks many of the cell death regulators that have evolved in higher eukaryotes, probably due to the invention of multicellularity. The simplicity of the yeast model allows elucidating the basic molecular pathways of programed cell death without interference from multifaceted regulation, due to various protein isoforms or cellular specificity often observed in studies using mammalian systems. In addition, yeast heterologous expression systems offer the opportunity to exploit the individual functional and mechanistic properties of mammalian apoptotic regulators.

This special issue gathers recent studies enhancing the understanding of PCD and its deregulation, relevant in human pathologies and aging. These include review, mini-review, original research, perspective, and hypothesis and theory articles dealing with the identification of previously uncharacterized proteins and the function of different cellular compartments and organelles involved in PCD and aging, as well as the exploitation of humanized yeasts to untangle the role of apoptotic regulators.

Yeast has long been established as a valuable model system to study conserved biological processes of relevance to human health, and several reviews address the importance of studying endogenous yeast mechanisms to understand human pathologies, particularly cancer and aging. Mollinedo stresses the relevance of lipid rafts in cell physiology and the advantages of the yeast model system to address unsolved questions regarding their role in survival and cell death signaling in mammalian cells, which will impact the design of lipid raft-mediated approaches to treat human pathologies caused by dysfunction of survival and cell death processes (1).

Tosato and co-workers review two yeast models relevant for cancer formation and progression, one mimicking genome instability, a hallmark of cancer, and another metabolic features of cancer cells, including the Warburg effect (2), whereas Mazzoni and colleagues hypothesize that their newly developed yeast clonal life span assay will provide a valuable complement to aging studies (3).

Kitanovic and co-workers showed that intracellular acidification resulting from accumulation of acetic acid in exhausted medium, causes cellular energetic deficiency and nutrient starvation (4). The role of acetic acid, one the main alcoholic fermentation sub-products, as an extrinsic mediator of both processes, and the key function of acetic acid detoxification enzyme Ach1p for mitochondrial functionality, is discussed by Orlandi and co-workers (5).

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Lipid raft involvement in yeast cell growth and death

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The notion that cellular membranes contain distinct microdomains, acting as scaffolds for signal transduction processes, has gained considerable momentum. In particular, a class of such domains that is rich in sphingolipids and cholesterol, termed as lipid rafts, is thought to compartmentalize the plasma membrane, and to have important roles in survival and cell death signaling in mammalian cells. Likewise, yeast lipid rafts are membrane domains enriched in sphingolipids and ergosterol, the yeast counterpart of mammalian cholesterol. Sterol-rich membrane domains have been identified in several fungal species, including the budding yeast *Saccharomyces cerevisiae*, the fission yeast *Schizosaccharomyces pombe* as well as the pathogens *Candida albicans* and *Cryptococcus neoformans*. Yeast rafts have been mainly involved in membrane trafficking, but increasing evidence implicates rafts in a wide range of additional cellular processes. Yeast lipid rafts house biologically important proteins involved in the proper function of yeast, such as proteins that control Na^+, K^+, and pH homeostasis, which influence many cellular processes, including cell growth and death. Membrane raft constituents affect drug susceptibility, and drugs interacting with sterols alter raft composition and membrane integrity, leading to yeast cell death. Because of the genetic tractability of yeast, analysis of yeast rafts could be an excellent model to approach unanswered questions of mammalian raft biology, and to understand the role of lipid rafts in the regulation of cell death and survival in human cells. A better insight in raft biology might lead to envisage new raft-mediated approaches to the treatment of human diseases where regulation of cell death and survival is critical, such as cancer and neurodegenerative diseases.

Keywords: lipid rafts, membrane domains, ergosterol, yeast, *S. cerevisiae*, ion homeostasis, nutrient transporters, cell death

INTRODUCTION

Apoptosis is an intrinsic cell death process that plays critical roles in the normal development and health of multicellular organisms. However, in the last years, growing evidence suggests that apoptosis-like cell death also occurs in a number of unicellular organisms, including yeast (Madeo et al., 2002, 2004; Wissing et al., 2004; Pereira et al., 2008; Carmona-Gutierrez et al., 2010; Sousa et al., 2011). Some features of apoptotic-like cell death can be induced in yeast following stress conditions, such as acetic acid (Ludovico et al., 2001, 2002), membrane-permeable C2-ceramide (Carmona-Gutierrez et al., 2011), hydrogen peroxide (H_2O_2; Madeo et al., 1999; Ribeiro et al., 2006), hyperosmotic (Silva et al., 2005; Ribeiro et al., 2006), and NaCl (Huh et al., 2002; Wadskog et al., 2004) stress.

Cell membranes are structurally heterogeneous, composed of discrete domains with unique physical and biological properties. The concept of the organization of membrane lipid components into domains (Karnovsky et al., 1982), and the subsequent demonstration of the existence in the plasma membrane of a particular type of microdomain enriched in sterols and sphingolipids, named as lipid rafts (Simons and Ikonen, 1997; Brown and London, 2000; Simons and Toomre, 2000; Ikonen, 2001; Maxfield, 2002), have profoundly changed our view of membrane organization and membrane-regulated processes. Membrane domains can form through a number of mechanisms involving lipid–lipid and protein–lipid interactions.

Despite lipid rafts have different sizes depending on the membrane composition, a consensus definition of a lipid raft developed at the 2006 Keystone Symposium of Lipid Rafts and Cell Function, held in Steamboat Springs (CO, USA), concluded that "membrane rafts are small (10–200 nm), heterogenous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein–protein and protein–lipid interactions" (Pike, 2006). In resting cells, rafts appear small and unstable, and the current consensus is that their sizes are smaller than the optical diffraction limit (250 nm). Following stimulation, raft-preferring proteins are clustered, inducing larger and stabilized rafts, likely through lipid lateral diffusion and coalescence of small raft units.

A critical issue in the studies of raft biology lies in the difficulty to visualize rafts in living cells, unless they coalesce in large raft platforms. Thus, most of the evidence for their identification relies on indirect methods, such as the use of non-ionic detergent extraction. On these grounds, rafts are usually defined as the insoluble fraction or detergent-resistant membrane (a.k.a., DRM) after non-ionic detergent solubilization at 4°C, which can be isolated by flotation in density gradients. The association

of a protein with cholesterol-rich rafts is strengthened when it becomes detergent-soluble after depletion of cholesterol from the membrane by the use of methyl-β-cyclodextrin or other agents. Nevertheless, the physiological existence of rafts has been challenged by a number of criticisms (Munro, 2003; Lichtenberg et al., 2005; Lingwood and Simons, 2007; Simons and Gerl, 2010), in particular regarding the use of detergents that could lead to artifacts and misinterpretations since, for instance, Triton has been shown to promote the formation of ordered domains in model bilayers (Heerklotz, 2002). Also, a main concern has been raised on the diverse effects that might be expected by depleting cholesterol from the membrane because cholesterol has important functions in the whole plasma membrane, apart from forming lipid rafts. On these grounds, the functional involvement of rafts as well as the raft localization of proteins based only on the use of detergents and cholesterol depletion has been challenged, and therefore caution should be taken before assigning a role of rafts in different biological processes. In addition, the evidence for the presence of rafts in the plasma membrane of living cells has, until recently, not been compelling, thus raising some doubts about the physiological existence of rafts. However, the advent of new microscopy techniques has finally demonstrated the existence of rafts in the cell. The use of stimulated emission depletion (STED) microscopy has proved that sphingolipids and glycosylphosphatidylinositol (GPI)-anchored proteins are transiently trapped in cholesterol-dependent molecular complexes in live cells (Eggeling et al., 2009). In this regard, the application of novel technologies, such as fluorescence resonance energy transfer (FRET), fluorescence polarization anisotropy (FPA), total internal reflection fluorescence (TIRF) microscopy, single quantum dot tracking, single particle tracking (SPT), and fluorescence correlation spectroscopy (FCS), have provided evidence for the localization of GPI-anchored proteins and other lipid-modified proteins in cholesterol-dependent clusters (Kusumi et al., 2004; Sharma et al., 2004; Lenne et al., 2006; Marguet et al., 2006; Vyas et al., 2008; Pinaud et al., 2009). In addition, near-field scanning optical microscopy (NSOM) in combination with quantum dots showed that T cell stimulation triggers the organization of T cell receptors in nanodomains in live cells (Zhong et al., 2009; van Zanten et al., 2010). The development of the above high temporal and spatial resolution techniques has allowed to locate different molecular constituents in membrane domains with reduced mobility. The combined use of different biophysical, biochemical, and genetic technologies are now providing evidence demonstrating the existence of sterol-dependent membrane raft domains as well as their role in critical physiological functions. Despite some technical and conceptual limitations, as stated above, resistance to non-ionic detergent solubilization, together with flotation in gradient density centrifugation and manipulation of sterol, remain as the most widely used techniques for studying lipid rafts.

There are a wide number of reports showing that rafts in mammalian cells house proteins involved in cell survival and growth, as well as in the proper functioning of immune system receptors (Simons and Toomre, 2000; Szoor et al., 2010). In this regard, cancer cells, usually showing an increased ability to proliferate and survive, have been found to have higher levels of cholesterol (Dessi et al., 1994; Kolanjiappan et al., 2003; Freeman and Solomon, 2004; Tosi and Tugnoli, 2005) and cholesterol-rich lipid rafts (Li et al., 2006) than their normal counterparts. Nevertheless, in the last decade, a number of death receptors and downstream apoptotic signaling molecules have also been localized in cholesterol- and sphingolipid-rich lipid rafts in cancer cells (Gajate and Mollinedo, 2001, 2005, 2007; Gajate et al., 2004; Mollinedo and Gajate, 2006; Reis-Sobreiro et al., 2009; Mollinedo et al., 2010a). The localization of the death-inducing signaling complex (DISC), a major apoptotic complex containing Fas/CD95 death receptor, Fas-associated death domain-containing protein (FADD) and procaspase-8, in lipid rafts has been shown by electron microscopy in T cell leukemic cells, when they are engaged to undergo apoptosis (Gajate et al., 2009a). In addition, raft nanodomains have been shown, following a FCS strategy, to be present in both the outer and inner leaflets of the plasma membrane and to play a crucial role in triggering the survival phosphatidylinositol-3 kinase/Akt signaling pathway, by facilitating Akt recruitment and activation upon phosphatidylinositol-3,4,5-triphosphate accumulation in the plasma membrane (Lasserre et al., 2008). On these grounds, mammalian cell lipid rafts behave as platforms that can house different, and even opposite signaling processes, such as survival and apoptosis, and therefore these membrane domains play a critical role in the modulation of cell signaling that regulates cell fate.

Lipid rafts have also been identified in yeast as membrane domains enriched in ergosterol and sphingolipids (Wachtler and Balasubramanian, 2006). The budding yeast *Saccharomyces cerevisiae* is one of the best characterized eukaryotic organisms. In spite of its simplicity as a free-living unicellular fungus, yeast cells are similar to higher eukaryotes regarding the cell structure and several physiological processes. Due to its genetic tractability and increasing wealth of accessible data, yeast has become a model system of choice for the study of different physiological processes occurring in mammalian cells. In this regard, yeast could be an interesting biological system to analyze the role of lipid rafts in both survival and cell death responses, despite yeast lack death receptors and most of the typical apoptotic signaling molecules present in mammalian cells.

RAFTS IN YEASTS

Regarding the lipid constituents of lipid rafts, and although the lipid levels vary between different cell types, the plasma membrane of the mammalian cell usually contains, on a molar basis, about 30–40% cholesterol and 10–20% sphingomyelin of plasma membrane lipids, while glycosphingolipids are usually present at low levels (Lange et al., 1989; van Meer, 1989). However, yeasts do not have sphingomyelin, but instead have inositol phosphosphingolipids, which may function as orthologs of mammalian sphingomyelin (Matmati and Hannun, 2008). In addition, unlike mammalian cells that have cholesterol, yeast contain ergosterol, serving the same function as cholesterol in animal cells. Ergosterol is even a better raft former than cholesterol (Xu et al., 2001). Studies on the generation of model membranes from yeast total lipid extracts led to the conclusion that formation of membrane domains depended on specific interactions between yeast sphingolipids and ergosterol

(Klose et al., 2010). This selective interaction between yeast sphingolipids and ergosterol results in phase separation into membrane domains with liquid ordered- and liquid disordered-like properties, that is in raft formation (Klose et al., 2010). Thus, whereas lipid rafts in mammalian cells contain cholesterol and sphingomyelin or glycosphingolipids (Simons and Ikonen, 1997), raft domains in *S. cerevisiae* contain ergosterol and complex sphingolipids (Kubler et al., 1996; Bagnat et al., 2000), including inositol-phosphoceramide (IPC), mannose-inositol-phosphoceramide (MIPC), and mannose-(inositol phosphate)$_2$-ceramide (M(IP)2C) (Dickson et al., 2006).

Lipid rafts are enriched in sterols, composed of a four-ring structure with an aliphatic tail that can pack tightly with the lipid acyl chains of sphingolipids to create a compacted region of condensed bilayer termed the liquid-ordered state (Munro, 2003; Megha et al., 2006). Because of its rather unique lipid composition, lipid rafts are more resistant to extraction with cold non-ionic detergents, and therefore they were originally defined as DRMs, due to their relative insolubility in cold non-ionic detergents (London and Brown, 2000; Maxfield, 2002; Simons and Gerl, 2010). In the yeast, rafts have also been defined biochemically as DRMs and proven to be critical for protein sorting through the endoplasmic reticulum and Golgi apparatus (Bagnat et al., 2000, 2001; Bagnat and Simons, 2002). The terms lipid raft, liquid-ordered domain, and DRM are widely used indistinctly and are suggested to refer to the same chemico-biological entity. However, in a very strict way, they might have different implications and caution should be taken (Lichtenberg et al., 2005).

The yeast sphingolipid is peculiar in that it contains a saturated very long-chain fatty acid with 26 carbon atoms (Schneiter et al., 1999, 2004), which is synthesized and coupled to raft-located proteins, like the proton pumping ATPase Pma1p, already in the endoplasmic reticulum. Then, the resulting protein–lipid complex is transported and sorted as an entity to the plasma membrane. This rather long C26 fatty acid is required for proper assembly of the protein–lipid complex and transport to the membrane, as a shortening to C22 fatty acid by mutations impairs raft association of Pma1p, and induces Pma1p degradation by rerouting the ATPase enzyme from the plasma membrane to the vacuole (Gaigg et al., 2006; Toulmay and Schneiter, 2007).

Another important difference between mammalian cells and yeast cells lies in that lipid raft formation occurs primarily in the Golgi apparatus in mammals (Brown and Rose, 1992), whereas it takes place in the endoplasmic reticulum in yeast, where is suggested that proteins associate with yeast rafts (Bagnat et al., 2000). In both yeast and mammalian cells, sphingolipids and sterols are mainly present in the plasma membrane (Lange et al., 1989; Patton and Lester, 1991; Zinser and Daum, 1995), but these molecules are synthesized in compartments of the early secretory pathway (Daum et al., 1998; Futerman and Riezman, 2005), and therefore raft-located proteins could be recruited into these domains in other subcellular structures distinct from the plasma membrane. Thus, in yeast newly synthesized Gas1p, a GPI-anchored protein, and Pma1p have been found to be recruited to lipid rafts in the endoplasmic reticulum (Bagnat et al., 2000; Lee et al., 2002).

Because a major constituent of lipid rafts is sterol, the naturally fluorescent sterol-binding antibiotic filipin has been widely used to detect regions with high sterol content in the plasma membrane of fungi. The use of this compound has led to the identification of large sterol-rich domains (SRDs) in the plasma membranes of fungi (Wachtler et al., 2003; Alvarez et al., 2007), ranging from about 3 to 15 μm^2. Due to the dynamic nature of lipid rafts and to their ability to aggregate, clusters of rafts in mammalian cells can be formed under different stimuli to lead to raft platforms as big as the ones reported for some fungi (Ausili et al., 2008). In this regard, it might be envisaged that SRDs in yeasts correspond to clusters of sterol-rich rafts or raft platforms, which are required for the accomplishment of specific functions. This clustering of rafts may be critical for the proper onset of certain cell functions, which might require the concentration of a great amount of proteins in specific sites of the cell, thus leading to cell movement, cytokinesis, etc. On these grounds, lipid rafts act as dynamic and mobile platforms that transport the required proteins at the proper place to act.

Sterol-rich membrane domains have been identified in several fungal species, including the budding yeast *S. cerevisiae* (Kubler et al., 1996; Bagnat et al., 2001), the fission yeast *Schizosaccharomyces pombe* (Wachtler et al., 2003), as well as the pathogen yeasts *Candida albicans* (Martin and Konopka, 2004; Alvarez et al., 2007) and *Cryptococcus neoformans* (Siafakas et al., 2006; Alvarez et al., 2007), having being involved in a number of important processes like mating (Nichols et al., 2004; Proszynski et al., 2006), cytokinesis (Rajagopalan et al., 2003; Wachtler et al., 2003), and hyphal formation (Martin and Konopka, 2004).

Sterol-rich domains are polarized in the rod-shaped *S. pombe* throughout the vegetative life cycle in a cell cycle-dependent way, namely they are located to the growing cell ends during interphase, and to the medial zone where cells undergo cytokinesis, as well as at the tips of mating projections (Rajagopalan et al., 2003; Wachtler et al., 2003; Wachtler and Balasubramanian, 2006; Alvarez et al., 2007). Thus, in fission yeast, rafts localize to regions of polarized growth and to the division site. Unlike to what happens in *S. pombe*, sterols are distributed uniformly throughout the plasma membrane in the vegetative life cycle of the budding yeast *S. cerevisiae*. However, filipin-stained rafts are also detected at the tips of cells induced with mating pheromone (Bagnat and Simons, 2002; Proszynski et al., 2006; Alvarez et al., 2007). In the human pathogens *C. neoformans* and *C. albicans*, sterols are concentrated at the leading edges of mating projections, at the actively growing sites at bud tips, at sites of septation, and at the tip of hyphal growth (Martin and Konopka, 2004; Alvarez et al., 2007).

RAFTS IN YEAST PATHOGENS

Proteomic analysis in DRMs from *C. albicans* led to the identification of 29 proteins, including the well-known lipid raft marker in *S. cerevisiae* Pma1p (Insenser et al., 2006). Surprisingly, only three proteins (∼10%) were typically located in the plasma membrane, whereas most of raft-located proteins were usually present in internal membranes, including proteins located in mitochondrial (31%), Golgi (7%), and endoplasmic reticulum (7%) membranes. This could support the existence of raft domains in the membranes of mitochondria, endoplasmic reticulum, and Golgi, as reported by different researchers (Bini et al., 2003; Mielenz et al., 2005;

Mollinedo et al., 2011). The proteins located in *C. albicans* rafts were involved in a number of biological processes, including lipid metabolism, cell wall biogenesis, protein metabolism, electron transport, and ATP synthesis (Insenser et al., 2006). In addition heat shock proteins were also present (Insenser et al., 2006), similarly to what has been observed in mammalian cells (Nieto-Miguel et al., 2008). Likewise, ATP synthase was also located at the raft membranes in *C. albicans* (Insenser et al., 2006), and a similar raft location for this enzyme has been found in proteomic studies conducted in mammalian cells (Bae et al., 2004), leading to the suggestion that this protein might be located in plasma membrane rafts as well as in mitochondria (Bae et al., 2004). Furthermore, it is worth mentioning the presence in *C. albicans* lipid rafts of a series of proteins involved in lipid metabolism and multidrug efflux, such as: Erg11p and Scs7p, involved in the lipid metabolism of major raft components (ergosterol and ceramide; Insenser et al., 2006); Rta2p, a translocase that moves sphingolipid long chain bases from the inside to the outside membrane (Wang et al., 2012); and the ATP-binding cassette (ABC) multidrug transporter CaCdr1p (Pasrija et al., 2008). The presence of cytosolic proteins in the *C. albicans* rafts suggest that protein–protein interactions could play a major role in bringing soluble proteins to the raft domains.

Candida albicans-associated bloodstream infections are linked to the ability of this yeast to form biofilms (Mukherjee et al., 2005). These latter are aggregates of microbial cells, adhering to each other, which are embedded within a self-produced matrix of extracellular polymeric substances, usually made up of extracellular DNA, proteins, and polysaccharides, and represent a common mode of microbial growth. Microbes growing as biofilm are highly resistant to commonly used antimicrobial drugs. *Candida* biofilms associated with indwelling devices provide a protected niche for the fungal cells, where they can evade the host immune system, and are especially problematic due to their inherent resistance to commonly used antifungal agents (Chandra et al., 2007). The microbial cells growing in a biofilm are physiologically distinct from planktonic cells of the same organism, which are single-cells floating in a liquid medium. Biofilm formation by *Candida* species is believed to contribute to invasiveness of these fungal species, and there is a correlation between *C. albicans* biofilms and fungal pathogenesis. By using lipidomics, a significant difference was observed in the lipid profiles of *C. albicans* biofilms and planktonic cells. Biofilms contained higher levels of phospholipid and sphingolipids than planktonic cells. In the early phase, levels of lipid in most classes were significantly higher in biofilms compared to planktonic cells. The unsaturation index of phospholipids decreased with time, with this effect being particularly strong for biofilms. Inhibition of the biosynthetic pathway for sphingolipid (M(IP)2C) by myriocin or aureobasidin A, and disruption of the gene encoding inositolphosphotransferase 1 (*IPT1*), abrogated the ability of *C. albicans* to form biofilms, suggesting that lipid rafts might be involved in biofilm formation (Lattif et al., 2011). The differences in lipid profiles between biofilms and planktonic *Candida* cells may have important implications for the biology and antifungal resistance of biofilms (Lattif et al., 2011).

In addition, lipid rafts have been found to be important platforms for the concentration of certain virulence factors at the cell surface of pathogenic fungi, to allow efficient access to enzyme substrate and/or to provide rapid release to the external environment. Thus, rafts from the fungal pathogen *C. neoformans* contain the virulence determinant phospholipase B1 (Plb1p), a GPI-anchored protein, and the antioxidant virulence factor Cu/Zn superoxide dismutase (Sod1p; Siafakas et al., 2006). The enzyme Plb1p contains phospholipase B (PLB), lysophospholipase (LPL), and LPL transacylase (LPTA) activities (Chen et al., 1997a,b), and therefore it might affect raft lipid composition.

LIPID RAFTS AND ION HOMEOSTASIS IN YEAST

The maintenance of ion homeostasis in response to changes in the environment is vital to all living cells. In yeast cells, the active transport of inorganic ions and nutrients relies on the existence of an electrochemical gradient of protons across the plasma membrane. In *S. cerevisiae*, this electrochemical gradient is mainly generated by the essential H^+-ATPase gene, *PMA1*, which encodes one of the most abundant proteins in the yeast plasma membrane (Serrano et al., 1986). This Pma1p-mediated electrochemical gradient is balanced by the activity of a number of symporters and antiporters, but the high-affinity potassium uptake through the plasma membrane transporters Trk1p and Trk2p, the former being the most biologically relevant potassium transporter, is the major consumer of the gradient (Gaber et al., 1988; Ko and Gaber, 1991; Madrid et al., 1998). Potassium transport into yeast cells results in plasma membrane depolarization, leading to Pma1p stimulation and a concomitant cytosolic alkalinization (Rodriguez-Navarro, 2000). Thus, the regulation of both Pma1p and Trk1p is critical for the modulation of the electrical membrane potential and intracellular pH. Thus, Pma1p and the high-affinity potassium transporters Trk1p and Trk2p are the major determinants of yeast membrane potential and internal pH, and thus should be co-ordinately regulated. The plasma membrane proton ATPase Pma1p is a resident raft protein (Bagnat et al., 2001). The major K^+ transporters, Trk1p and Trk2p, have also been reported to be present in lipid rafts (Zeng et al., 2004; Yenush et al., 2005). Intracellular pH plays a critical role in modulating the activity of many cellular systems, including those regulating cell death, both in yeasts (Ludovico et al., 2001, 2002; Sokolov et al., 2006) and mammalian cells (Perez-Sala et al., 1995; Gottlieb et al., 1996; Meisenholder et al., 1996).

In *S. cerevisiae*, intracellular pH and K^+ concentrations affect many cellular activities, including cell growth and death, and thereby they must be tightly controlled through the regulation of the H^+-pumping ATPase Pma1p and the major K^+ transporters Trk1p and Trk2p (Yenush et al., 2002, 2005). Pma1p is an electrogenic pump with an optimal pH of 6.5 and therefore is well suited to set the intracellular pH at a neutral value (Portillo, 2000), and together with Trk1p, both systems are the major regulators of cell volume, turgor, membrane potential, and pH in yeast. Potassium accumulation through Trk1p can be harmful to the cell, leading to an increase in turgor pressure and the risk of cell lysis. Trk1p is activated by Hal4p and Hal5p kinases and inhibited by the Ppz1p and Ppz2p phosphatases. Hal3p is a negative regulatory subunit of the Ppz1p Ser/Thr phosphatase (de Nadal et al., 1998), and it has been shown that the inhibition of Ppz1p by Hal3p is pH dependent (Yenush et al., 2005). Interestingly, Ppz1p–Hal3p

interaction would act as an intracellular pH sensor, and a model has been proposed for the modulation of H^+ and K^+ homeostasis through the regulation of Trk1p activity by intracellular pH (Yenush et al., 2005). At a relatively alkaline pH the interaction between Hal3p and Ppz1p would be destabilized, and hence the Ppz1p phosphatase would act on Trk1p to decrease the potassium uptake into the cell. Thus, following accumulation of K^+, cells use then a concomitant increase in intracellular pH through the extrusion of protons by Pma1p to downregulate potassium transport.

Ppz1p has been mostly located at the plasma membrane, although some of this protein was also present in internal non-vacuolar membranes, likely the endoplasmic reticulum and/or the nuclear membrane (Yenush et al., 2005). However, unlike Trk1p, Ppz1p was not found in DRMs (Yenush et al., 2005). Nevertheless, because the raft-located Trk1p has been shown to interact with Ppz1p (Yenush et al., 2005), it cannot be ruled out that lipid rafts might transiently be a place where these proteins could interact each other, as shown for other non-raft proteins in mammalian cells that are translocated and recruited in lipid rafts to regulate cell death signaling (Gajate et al., 2004; Gajate and Mollinedo, 2007; Nieto-Miguel et al., 2008; Reis-Sobreiro et al., 2009).

The above data on yeast are of major importance for mammalian cells, as regulation of potassium transport and intracellular pH homeostasis is implicated in many diseases (Shieh et al., 2000), including cancer, and it is plausible to envisage that similar transduction pathways connecting pH and potassium homeostasis might play a critical role in human disease and constitute interesting therapeutic opportunities.

In addition, the plasma membrane Na^+/H^+ antiporter Nha1p from the budding yeast *S. cerevisiae*, which plays an important role in intracellular Na^+ as well as pH homeostasis, by mediating the exchange of Na^+ for H^+ across the plasma membrane, has also been shown to associate with lipid rafts (Mitsui et al., 2009). In *lcb1-100* mutant cells, which are temperature-sensitive for sphingolipid synthesis, newly synthesized Nha1p failed to localize to the plasma membrane at the non-permissive temperature, but the addition of phytosphingosine or the inhibition of endocytosis in *lcb1-100* cells restored the targeting of Nha1p to the plasma membrane (Mitsui et al., 2009).

Thus, Pma1p, Trk1p, and Nha1p, regulating membrane potential and intracellular pH, are located in lipid rafts in *S. cerevisiae* (**Figure 1**), and they are critical in the modulation of ion homeostasis, by keeping the major monovalent cations (H^+, K^+, and Na^+), mainly through proteins that extrude H^+ and Na^+ and import K^+ ions (**Figure 1**), at the appropriate narrow range of ion concentrations for the proper function of distinct biological processes.

NUTRIENT TRANSPORTERS IN YEAST LIPID RAFTS

Table 1 shows a number of proteins that have been located in lipid rafts in *S. cerevisiae*, including proteins involved in ion homeostasis, nutrient transport, mating, stress response, and actin cytoskeleton organization.

Several nutrient transporters have been located in lipid rafts in *S. cerevisiae* (**Table 1**). The arginine/H^+ symporter Can1p (arginine permease) has been found to be present in lipid rafts in *S. cerevisiae* (Malinska et al., 2003). Double labeling experiments with Can1p-GFP and Pma1p-RFP-containing yeast cells showed that these proteins were located in two different non-overlapping membrane domains (Malinska et al., 2003), suggesting the presence of distinct rafts in the same yeast cell. The general amino acid permease Gap1p present at the plasma membrane is also associated with DRMs, and in the absence of sphingolipid synthesis Gap1p fails to accumulate at the plasma membrane and is missorted to the vacuole (Lauwers and Andre, 2006). Likewise, the hexose

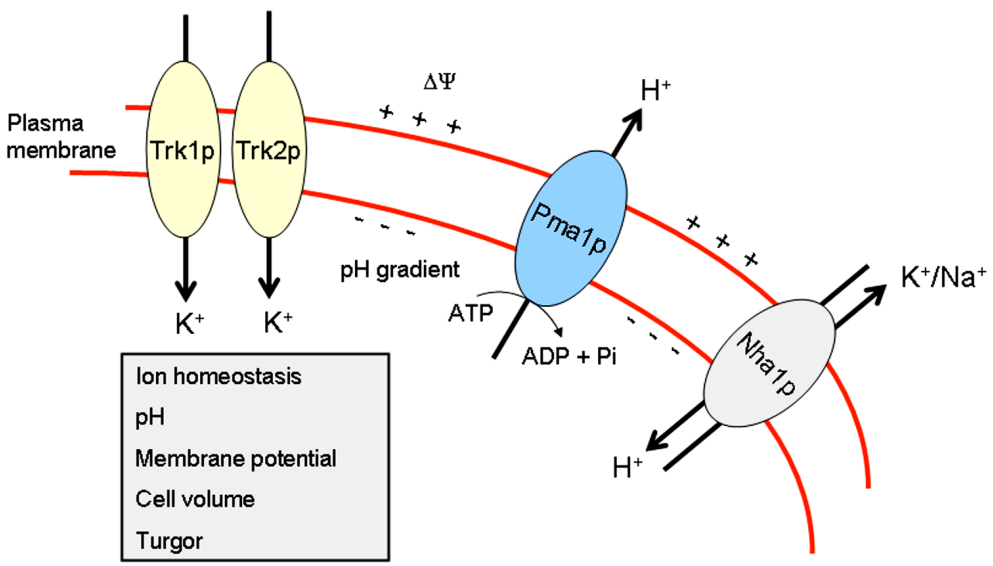

FIGURE 1 | Ion homeostasis in yeast. This scheme portrays the major proteins, Pma1p, Trk1p, Trk2p, and Nha1p, involved in maintaining ion homeostasis in *S. cerevisiae*.

Table 1 | Proteins associated with lipid rafts in *S. cerevisiae*.

Gene	Description	Biological process	Major localization	Technical approach	Reference
PMA1	Plasma membrane ATPase	Proton transport, pH regulation	Plasma membrane	DRMGC	Bagnat et al. (2001)
TRK1	Potassium transporter	Cellular potassium ion homeostasis	Plasma membrane	DRMGC	Yenush et al. (2005)
TRK2	Potassium transporter	Cellular potassium ion homeostasis	Plasma membrane	RTE	Zeng et al. (2004)
NHA1	Na^+/H^+ antiporter	Ion homeostasis	Plasma membrane	DRMGC	Mitsui et al. (2009)
TAT2	Tryptophan transporter	Tryptophan transport	Plasma membrane	DRMGC	Umebayashi and Nakano (2003)
CAN1	Arginine permease	Arginine transport	Plasma membrane	DRMGC	Malinska et al. (2003)
GAP1	General amino acid permease	Amino acid transport	Plasma membrane	DRMGC	Lauwers and Andre (2006)
HXT1	Low-affinity glucose transporter	Glucose transport, hexose transport	Plasma membrane	DRMGC	Lauwers and Andre (2006)
FUR4	Uracil permease	Uracil transport	Plasma membrane	DRMGC	Hearn et al. (2003)
FUS1	Cell FUSion	Mating	Mating projection tip	DRMGC	Bagnat and Simons (2002)
FUS2	Cell FUSion	Mating	Mating projection tip	DRMGC	Bagnat and Simons (2002)
FIG1	Factor-induced gene	Mating	Mating projection tip	DRMGC	Bagnat and Simons (2002)
SHO1	Transmembrane osmosensor	Osmosensor activity, mating	Plasma membrane, mating projection tip	DRMGC	Bagnat and Simons (2002)
STE6	Plasma membrane ATP-binding cassette (ABC) transporter	Peptide pheromone export, mating	Plasma membrane, mating projection tip	DRMGC	Bagnat and Simons (2002)
PRM1	Pheromone-regulated membrane protein	Response to pheromone, mating	Mating projection tip	DRMGC	Bagnat and Simons (2002)
SLG1/WSC1	Sensor-transducer of the stress-activated PKC1-MPK1	Response to heat and osmotic stress, actin depolarization	Plasma membrane, mating projecting tip	RTE	Lodder et al. (1999)
RVS161	Amphiphysin-like protein	Actin cytoskeleton organization, response to osmotic stress and starvation	Actin cortical patch, mating projecting tip	RTE	Balguerie et al. (2002)
RVS167	Amphiphysin-like protein	Lipid tube assembly	Actin cortical patch, mating projecting tip	RTE	Germann et al. (2005)
GAS1	Glycophospholipid-anchored surface protein/β-1,3-glucanosyltransferase	Fungal-type cell wall organization	Plasma membrane, cellular bud scar	DRMGC	Bagnat et al. (2000)
HSP30	Heat shock protein	Stress response, negative regulation of Pma1p	Plasma membrane	DRMGC	Bagnat et al. (2000)
MRH1	Membrane protein related to Hsp30p	Unknown	Plasma membrane, mitochondrion	DRMGC	Bagnat et al. (2000)
NCE2/NCE102	Non-classical export	Plasma membrane organization	Plasma membrane	DRMGC	Bagnat et al. (2000)

DRMGC, non-ionic detergent-resistant membrane (DRM) fractions at 4°C followed by flotation in density gradient centrifugation; RTE, resistance to Triton X-100 extraction.

transporter Hxt1p (low-affinity glucose permease) present at the cell surface was also associated with DRMs (Lauwers and Andre, 2006). The plasma membrane protein uracil/H^+ symporter Fur4p (uracil permease) has also been reported to be associated with lipid rafts in *S. cerevisiae* (Hearn et al., 2003). The amount of this protein in plasma membrane is highly regulated. Under stress conditions, including heat stress and high concentrations of uracil in the culture medium, Fur4p is degraded by a process that includes phosphorylation, ubiquitination, endocytosis, and transport to the vacuole where the protein is eventually hydrolyzed (Galan et al., 1998; Marchal et al., 2002). Because rafts act as platforms for the integration and modulation of signaling pathways and processes, it could be envisaged that the raft location of Fur4p might be critical for its regulation.

The plasma membrane localization of the tryptophan permease Tat2p is regulated by the external tryptophan concentration and is dependent on lipid rafts. In wild-type cells, Tat2p is transported from the Golgi apparatus to the vacuole at high tryptophan level, and to the plasma membrane at low tryptophan level. However, Tat2p is missorted to the vacuole at low tryptophan concentration in the *erg6*Δ deleted mutant (*ERG6* gene encodes *S*-adenosylmethionine Δ24 methyltransferase, acting in the last steps of ergosterol biosynthesis by converting zymosterol to fecosterol; Umebayashi and Nakano, 2003), and following yeast treatment with the ergosterol biosynthesis inhibitor zaragozic acid, that inhibits squalene synthetase, which catalyzes the first committed step in the formation of cholesterol/ergosterol (Daicho et al., 2007). Likewise, additional proteins, such as Fus1p, a plasma membrane protein required for yeast mating, is excluded from rafts and missorted to the vacuole in the *erg6*Δ mutant (Bagnat and Simons, 2002). These evidences support the view that several plasma membrane proteins can be missorted in *erg6*Δ mutants due to impaired raft association, and this might underlie the mating deficiency (Gaber et al., 1989) and drug hypersensitivity (Kaur and Bachhawat, 1999; Gupta et al., 2003; Maresova et al., 2009) of *erg6*Δ mutants.

Furthermore, as shown in **Table 1**, mating projection-localized proteins Fus2p, Fig1p, Sho1p, Ste6p, and Prm1p have been found to be associated with DRMs (Bagnat and Simons, 2002), supporting a critical role for lipid rafts in the mating process.

RAFTS AND CELL DEATH IN YEAST

Dysregulation of ion homeostasis mediates cell death, and this represents the mechanistic basis by which a growing number of amphipathic but structurally unrelated compounds elicit antifungal activity (Zhang et al., 2012). Pma1p is displaced from lipid rafts and delivered and degraded to the vacuole upon *S. cerevisiae* incubation with edelfosine (Zaremberg et al., 2005), an amphipathic antitumor ether phospholipid that affects and reorganizes lipid rafts (Gajate et al., 2004, 2009a; Ausili et al., 2008). Because Pma1p is mainly involved in maintaining ion homeostasis and membrane potential in yeast, its displacement from lipid rafts, following treatment of *S. cerevisiae* with the ether phospholipid edelfosine, has been shown to lead to yeast cell death (Zaremberg et al., 2005). Edelfosine has been reported to have affinity for cholesterol, and for cholesterol-rich membranes such as rafts (Ausili et al., 2008; Busto et al., 2008), because of the complementarity of the molecular geometrics of sterols and edelfosine (Busto et al., 2008). Edelfosine induces apoptotic cell death in a wide number of human cancer cells (Mollinedo et al., 1997, 2004, 2010a,b; Gajate and Mollinedo, 2002, 2007; Gajate et al., 2012) through raft reorganization and redistribution of the raft protein content (Gajate and Mollinedo, 2001, 2007; Gajate et al., 2004, 2009a). In human hematopoietic cancer cells, edelfosine treatment leads to the recruitment of apoptotic molecules into raft platforms, thus leading to the emerging concept of an apoptotic "liquid-ordered" plasma membrane platform named as "cluster of apoptotic signaling molecule-enriched rafts" (CASMERs; Gajate and Mollinedo, 2005; Gajate et al., 2009b; Mollinedo and Gajate, 2010a,b). These CASMERs may reduce the apoptotic signal threshold by facilitating and stabilizing protein–protein interactions and cross-talk between signaling pathways, and thereby act as a membrane signaling platforms to launch and catalyze the transmission of apoptotic signals (Mollinedo and Gajate, 2010a,b). The protein composition of these CASMERs includes death receptors and downstream signaling molecules (Gajate and Mollinedo, 2005; Gajate et al., 2009b; Mollinedo and Gajate, 2010a,b). On these grounds, lipid rafts play a major role in the generation of apoptotic signals in mammalian cells. In contrast to mammalian cells, yeasts lack death receptors and most of the typical apoptotic molecules, so the involvement of rafts in the cell death process might be less obvious in yeast. However, lipid rafts seem to be critical structures and scaffolds for the proper function of proteins whose activities are required for the normal function of a yeast cell, including yeast survival and growth (**Figure 2**), such as proteins involved in ion homeostasis and nutrient transport (**Table 1**). In this regard, it might be envisaged that redistribution and displacement of raft-located proteins to non-raft domains might lead to a failure in yeast functioning and eventually to cell death.

In mammalian cells, lipid rafts usually house proteins involved in survival signaling and growth, and thereby their presence is expected to play a role in the proliferation and survival of cancer cells. However, as indicated above, recent evidence in the last few years has also shown the presence of rafts enriched in death receptors and apoptotic molecules, leading to the emerging concept of the proapoptotic CASMER. Thus, at least two major different raft domains leading to survival and cell death can apparently be formed in mammalian cells. However, because yeast cells lack death receptors and the classical mammalian-like caspases, yeast rafts are supposed to be involved only in rather positive outcomes that keep the yeast cell alive. According to this notion, it might be envisaged that lipid raft disruption could facilitate and prompt yeast cell death.

The cationic amphipathic and antiarrhythmic drug amiodarone interacts with lipid membranes to exert their biological effect. In *S. cerevisiae*, toxic levels of amiodarone trigger a transient membrane hyperpolarization, likely through its ability to intercalate into the lipid bilayer (Herbette et al., 1988) altering lipid fluidity (Rosa et al., 2000), which is followed by depolarization, coincident with influx of Ca^{2+} and H^+ that can overwhelm cellular homeostasis and lead to cell death (Maresova et al., 2009). Amiodarone has been shown to have potent fungicidal activity

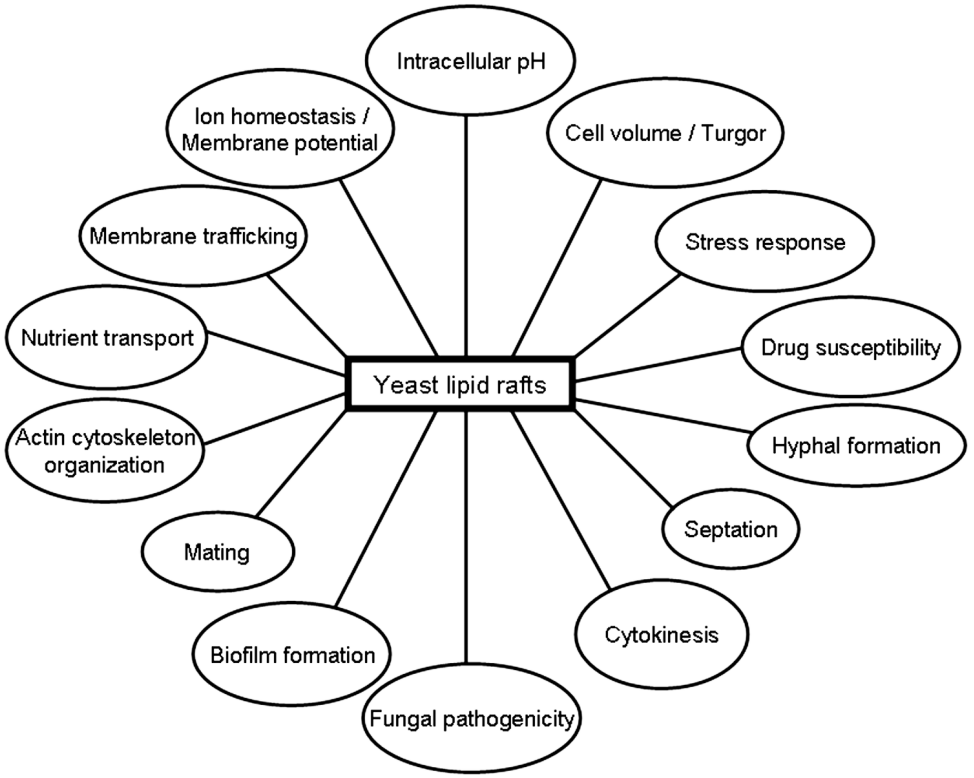

FIGURE 2 | Putative involvement of lipid rafts in different yeast functions.

against not only *S. cerevisiae*, but also for species of *Cryptococcus*, *Candida*, *Fusarium*, and *Aspergillus* (Courchesne, 2002). Using a genome-wide screen in a *S. cerevisiae* single-gene deletion library, 36 yeast strains with amiodarone hypersensitivity were identified, including mutants in transporters (*PMR1*, *PDR5*, vacuolar H^+-ATPase), ergosterol biosynthesis (*ERG3*, *ERG6*, *ERG24*), intracellular trafficking (*VPS45*, *RCY1*), and signaling (*YPK1*, *PTC1*; Gupta et al., 2003). The fact that azole resistant mutants in the ergosterol biosynthesis pathway of *S. cerevisiae* (*erg3*Δ, *erg6*Δ, and *erg24*Δ) exhibited hypersensitivity to amiodarone, suggests that the drug may be particularly effective for treatment against azole-resistant fungal strains (Gupta et al., 2003), which might be defective in raft-mediated processes. In addition, low doses of amiodarone and an azole (miconazole, fluconazole) are strongly synergistic and show potent fungicidal effects in combination (Gupta et al., 2003). These data suggest that lipid raft disruption might favor amiodarone cytotoxic action against yeast.

The initial hyperpolarization seems to be critical for the amiodarone cytotoxic effect. Glucose increases membrane potential by increasing H^+ pumping activity of the plasma membrane ATPase Pma1p (Serrano, 1983). Downregulation of the H^+ pump activity of the yeast plasma membrane upon glucose removal (Serrano, 1983) was accompanied by an attenuation of amiodarone-induced Ca^{2+} burst, thus protecting against drug toxicity (Muend and Rao, 2008). Amiodarone-induced hyperpolarization was lower in the mutant strain *pma1-105* that has 65% reduction in activity (Perlin et al., 1989). Furthermore, a decrease in membrane potential by glucose removal, addition of salts or in *tok1*Δ, *ena1-4*Δ, or *nha1*Δ mutants, involved in ion homeostasis, protected against amiodarone toxicity, suggesting that initial hyperpolarization was important in the mechanism of antifungal activity (Maresova et al., 2009). Thus, there is a link between membrane hyperpolarization and amiodarone toxicity. Because ion homeostasis and membrane potential is mainly regulated by raft-located proteins, these data also involve these membrane domains in the cytotoxicity of certain fungal drugs.

Interestingly, a recent work by Tulha et al. (2012) has shown that deletion of *GUP1* in *S. cerevisiae* leads to hypersensitivity to acetic acid treatment, high levels of reactive oxygen species (ROS) and reduced lifespan, leading to yeast cell death, likely through necrosis rather than apoptosis. Gup1p is a membrane-bound *O*-acyltransferase involved in remodeling GPI anchors (Bosson et al., 2006), playing an important role in the assembly/integrity of lipid rafts (Ferreira and Lucas, 2008). Because *gup1*Δ mutant is affected in lipid raft integrity/assembly, lipid metabolism, and GPI-anchor remodeling, the above data (Tulha et al., 2012) suggest a role of lipid rafts in yeast cell death process, and in the type of cell death process that ensues reorganization of plasma membrane domains.

Cytotoxicity of the antitumor ether phospholipid edelfosine against *S. cerevisiae* has been shown to be enhanced in yeast mutants defective for *LCB1*, an essential serine palmitoyltransferase that catalyzes the first step in sphingolipid synthesis,

and *ERG3*, a sterol C-5 desaturase involved in the final steps of ergosterol synthesis (Zaremberg et al., 2005). On the other hand, *S. cerevisiae* mutants affected in sphingolipid and ergosterol biosynthesis, namely *ipt1*Δ, *sur1*Δ, *skn1*Δ, and *erg3*Δ deletion mutants, are resistant to the azole antimycotics miconazole, mainly due to the role of lipid rafts in mediating intracellular accumulation of miconazole in yeast cells (Francois et al., 2009). Taken together these data suggest a major role of lipid rafts in the cytotoxicity of drugs in yeast.

In this regard, a number of data support that absence of ergosterol, which is one of the major constituents of membrane rafts, has a direct effect on drug susceptibility and morphogenesis of *C. albicans*. Low doses of amiodarone have been reported to be synergistic with fluconazole in fluconazole-resistant *C. albicans* (Gamarra et al., 2010). Ergosterol deficiency in *erg1*Δ mutants led to defects in growth and increased susceptibilities to drugs, including fluconazole, ketoconazole, cycloheximide, nystatin, amphotericin B, and terbinafine in *C. albicans* (Pasrija et al., 2005a,b). Reduced drug efflux activity of the *erg1*Δ mutant was associated with poor surface localization of Cdr1p, suggesting that enhanced passive diffusion and reduced efflux mediated by the ABC transporter Cdr1p increases drug susceptibility. Additionally, conditional *erg1*Δ mutant strains were unable to form hyphae in various media in *C. albicans* (Pasrija et al., 2005a,b). Likewise, the loss of (M(IP)2C) in the *C. albicans ipt1*Δ mutant, a sphingolipid biosynthetic gene, resulted in increased sensitivity to drugs like 4-nitroquinoline oxide, terbinafine, *o*-phenanthroline, fluconazole, itraconazole, and ketoconazole. The increase in drug susceptibilities of *ipt1*Δ mutant cells was linked to an altered sphingolipid composition, which appeared to be due to the impaired functionality of Cdr1p, a major drug efflux pump of *C. albicans* that belongs to the ABC superfamily (Prasad et al., 2005). Taken together, the above data indicate that an altered composition of sphingolipid or ergosterol, the major constituents of membrane rafts, affects drug susceptibility and morphogenesis in *C. albicans*.

ACTIN CYTOSKELETON, RAFTS, AND STRESS RESPONSE

Wild-type *S. cerevisiae* cells depolarize actin following salt stress and repolarize after a period of adaptation (Chowdhury et al., 1992; Balguerie et al., 2002). Two proteins are mainly involved in this process, namely Wsc1p for actin depolarization and the amphiphysin-like protein Rvs161p for actin repolarization (Balguerie et al., 2002). Thus, *rvs161*Δ mutant was able to depolarize actin in response to NaCl stress, but was unable to repolarize afterward, whereas *wsc1*Δ mutants was impaired in depolarizing actin (Balguerie et al., 2002). *RVS161/END6* gene, the budding yeast homolog of amphiphysin (Sivadon et al., 1995; Youn et al., 2010), is associated, in part, with lipid rafts (Balguerie et al., 2002), and co-localizes with actin patches (Balguerie et al., 1999), thus suggesting a link between rafts and actin cytoskeleton in *S. cerevisiae*. Rvs161p is suggested to locate in rafts through a putative association with a raft-bound protein, as Rvs161p has no GPI signal anchor or transmembrane domain, and therefore it cannot be directly integrated in rafts. The *SLG1/WSC1* gene product has also been reported to be partially present in DRMs (Lodder et al., 1999). Clustering of Slg1p/Wsc1p is enhanced under stress conditions, either heat or hypo-osmotic shock, as assessed by single-molecule atomic force microscopy, suggesting its relevance in stress response (Heinisch et al., 2010). Thus, lipid rafts could function as platforms for actin depolarization and actin repolarization in response to stress in *S. cerevisiae*.

In yeast, nutrient starvation leads to entry into stationary phase. *RVS161* (RVS for Reduced Viability upon Starvation) was identified as a critical gene to respond properly to carbon, nitrogen, and sulfur starvation conditions in *S. cerevisiae*, and it has been implicated in the control of cellular viability. Thus, *rvs161*Δ mutant cells die during the stationary phase and show sensitivity to high salt concentrations (Crouzet et al., 1991). *Rvs161*Δ displays a phenotype similar to that shown for the actin mutants: actin cytoskeleton disorganization, random budding of the diploids, loss of polarity, and sensitivity to salt. In addition, *rvs161*Δ, together with mutations in the actin gene, *ACT1*, leads to synthetic lethality (Breton and Aigle, 1998), thus suggesting that actin and Rvs161p are linked in a common functional pathway that is critical for yeast viability under stress conditions. These data might suggest a role of lipid rafts as platforms for the interaction of proteins that are required for yeast survival.

Rvs167p, another amphiphysin-like protein that interacts with Rvs161p to regulate actin cytoskeleton, endocytosis, and viability following starvation and osmotic stress, has been reported to localize to Rvs161p-containing lipid rafts (Germann et al., 2005). Several protein networks involved in diverse cellular functions, including endocytosis/vesicle traffic, converge on Rvs161p-Rvs167p complex (Bon et al., 2000), and thereby Rvs167p-Rvs161p complex might act as a docking platform for proteins involved in the regulation of different biological processes requiring actin cytoskeleton (Germann et al., 2005). In addition, the *rvs* mutants accumulate late secretory vesicles at sites of membrane and cell wall construction, and are synthetic-lethal with the *slt2/mpk1*Δ mutation, which affects the MAP kinase cascade controlled by Pkc1p and is required for cell integrity (Breton et al., 2001). These data support the idea that the RVS proteins, and thereby lipid rafts, are involved in the late targeting of vesicles whose cargoes are required for cell wall construction.

Actin-linking proteins ezrin, moesin, RhoA, and RhoGDI were shown to be recruited into clusters of Fas/CD95-enriched rafts in human leukemic cells upon treatment with the anticancer drug aplidin (Gajate and Mollinedo, 2005). Disruption of lipid rafts and interference with actin cytoskeleton prevented Fas/CD95 clustering and apoptosis, suggesting a major role of actin cytoskeleton in the formation of Fas/CD95 clusters and in the aggregation of proteins in lipid raft clusters in human cancer cells, thus regulating raft-associated signaling events (Gajate and Mollinedo, 2005). There is increasing of evidence of structuring of rafts by the cortical actin cytoskeleton, including evidence that the actin cytoskeleton associates with rafts, and that many of the structural and functional properties of rafts require an intact actin cytoskeleton (Chichili and Rodgers, 2009; Ayling et al., 2012).

In yeast, stabilization of actin by addition of jasplakinolide, by point mutations in the *ACT1* gene, or by deletion of certain genes that regulate F-actin, leads to cell death. Yeast mutant lacking the gene *END3* shows stabilized actin and elevated levels of ROS, this phenotype being dependent on downstream elements of

the Ras/cAMP pathway (Gourlay and Ayscough, 2006). Following yeast treatment with methyl-β-cyclodextrin, which depletes sterols from plasma membrane and disrupts lipid rafts, and manumycin A, that blocks prenylation, Ras2 membrane association and the level of ROS were reduced, and cell death progression was inhibited (Du and Ayscough, 2009). These data suggest that lipid rafts in yeast could be somehow related to providing platforms for the generation of stable complexes that could launch pro-cell death signals. This could open the possibility that lipid rafts in yeast could provide appropriate membrane domains for pro-cell death signaling molecules, as it has been recently described in mammalian cells, and not only for survival and growth signaling. However, this pro-cell death view of rafts in yeast remains to be elucidated, and further studies as well as the molecular characterization of the putative processes involved will be required.

LIPID RAFTS AND EISOSOMES

Studies on the budding yeast *S. cerevisiae* have revealed that fungal plasma membranes are organized into different subdomains. Pma1p (plasma membrane H^+-ATPase) and Can1p (H^+-arginine symporter) have been located in lipid raft membrane domains, but, as stated above, these proteins occupy two different non-overlapping membrane microdomains (Malinska et al., 2003). Thus, at least two different types of rafts can be distinguished in the yeast plasma membrane. Similarly to what was observed with Can1p, a family of integral membrane proteins, including Sur7p, Ynl194p, and Ydl222p, were visualized in cortical patches in *S. cerevisiae* (Young et al., 2002). Current evidence suggests the existence of at least two subcellular compartments in the yeast plasma membrane, namely a raft-based membrane compartment represented by a network-like structure housing Pma1p, and another raft-based membrane compartment that houses a number of proton symporters (Can1p, Fur4p, Tat2p). These two raft domains, also named as the membrane compartment occupied by Pma1p (MCP) and membrane compartment occupied by Can1p (MCC; Malinska et al., 2004; Grossmann et al., 2007), apparently require different lipids to keep their respective protein compositions (**Figure 3**). The proper sorting of Pma1p has been reported to be more dependent on sphingolipids, ceramide, and the C26 acyl chain that forms part of the ceramide (Lee et al., 2002; Gaigg et al., 2005, 2006). However, sterols are required for the correct targeting of Tat2p (Umebayashi and Nakano, 2003). Thus, these data suggest the putative existence of raft domains more enriched in either sphingolipids or sterol-rich in the yeast membrane, each one containing a specific set of proteins, and this compartmentalization or lateral segregation seems to be dependent on the membrane potential (Grossmann et al., 2007). Plasma membrane depolarization caused reversible dispersion of the H^+ symporters previously present in 300-nm patches (Grossmann et al., 2007). In addition, yeast plasma membrane seems to contain an additional subdomain named eisosomes (from the Greek "*eis*," meaning into or portal, and "*soma*," meaning body), which are immobile protein complexes, composed mainly of the cytoplasmic proteins Pil1p and Lsp1p at the plasma membrane that mark static sites of endocytosis (Walther et al., 2006). Pil1p and Lsp1p form punctuate clusters (eisosomes) on the cytoplasmic surface of the plasma membrane at MCC sites (Walther et al., 2006) and associate with the plasma membrane via their BAR domains (named for the Bin/Amphiphysin/Rvs proteins), that bind membranes and promote curvature (Zimmerberg and McLaughlin, 2004). Eisosomes form at the sites of invaginations in the plasma membrane, being flanked by Can1p-rich MCC domains at the upper edges of the furrows, whereas Pil1p is located at the bottom of the furrow (Stradalova et al., 2009; Douglas et al., 2011) and Sur7p, a protein involved in endocytosis, seems to be at the boundary between MCC and eisosomes (**Figure 3**). Microscopic and genetic analyses link these stable, ultrastructural assemblies, named eisosomes, to the endocytosis of both lipid and protein cargoes in cells, and are mainly composed of BAR domain proteins (Douglas et al., 2011; Olivera-Couto et al., 2011). Eisosomes have been suggested to function as organizing sites for endocytosis (Toret and Drubin, 2006). Thus, degradation of the arginine permease Can1p induced by excess of its substrate required first Can1p release from MCC patches, and only then it was endocytosed (Grossmann et al., 2008), thus suggesting that the protein is to a large extent unavailable for endocytosis and subsequent degradation as long as it stays in the protective area of MCC (Malinsky et al., 2010). In this regard, rapidly moving endocytic marker proteins avoid raft domains, and consequently the raft domain-accumulated proton symporters show a reduced state of substrate-induced endocytosis and turnover (Malinsky et al., 2010). Genetic analysis of the MCC/eisosome components indicates that these domains broadly affect overall plasma membrane organization (Douglas et al., 2011). The analysis of the major constituents of eisosomes, i.e., BAR proteins, is of major importance also in mammalian cells. BAR family proteins contribute to a range

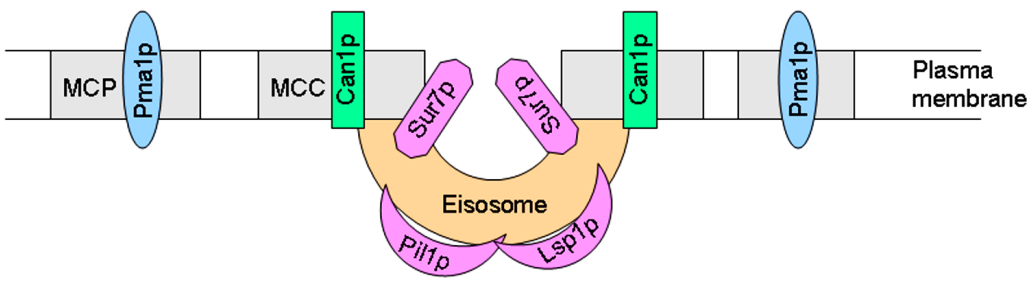

FIGURE 3 | Schematic view of yeast MCP, MCC, and eisosome membrane subdomains.

of cellular functions characterized by membrane and cytoskeletal remodeling, inducing membrane curvature and recruitment of effector proteins, with important consequences in several human disorders, including cancer cell invasiveness, as well as immune and neurologic disorders (Chen et al., 2012).

PERSPECTIVES

Recognition of the presence of distinct domains at the cell membrane has been one of the most significant scientific achievements in the last decades. Lipid raft membrane domains are gaining momentum in current biology, and they seem to regulate a wide number of critical processes. Lipid rafts are variable in size and composition, and can change in a highly dynamic way both by recruiting and expelling components as well as by coalescing smaller raft units, forming large clusters. The presence of these membrane domains in all eukaryotic cells opens new ways to study the physiological role of rafts in distinct biological systems. The existence of lipid rafts in yeast has provided an excellent way to study the role of these membrane domains in different biological processes, due to the remarkable yeast genetic tractability. Thus, changes in ergosterol and sphingolipid composition, the major raft constituents in yeast, by disrupting key metabolic genes lead to improper surface localization of major proteins involved in keeping yeast viable against some external aggression, including proteins involved in ion homeostasis, drug efflux, and stress response. Yeast lipid rafts house proteins that are critical for the regulation of membrane potential, intracellular pH, and nutrient transport. In fact, the plasma membrane potential is mainly considered as the driving force for ion and nutrient translocation in yeast, and the correct location of the proteins involved in these processes in lipid rafts is crucial for the proper functioning of the yeast. Lipid rafts could also behave as scaffolds where proteins dock and concentrate to either launch signals to be transmitted to other parts of the cell or to build new structures required for cell integrity or cell division. Thus, yeast cells constitute an excellent biological system to analyze the role of lipid rafts in both survival and cell death responses, in spite of lacking most apoptotic molecules present in mammalian cells. Key proteins for yeast survival and proper yeast functioning are localized in lipid rafts, and their displacement from raft domains leads to cell death. In addition, drugs interacting with sterols affect lipid raft composition and membrane integrity and are detrimental to yeast. In addition, yeast plasma membrane contains at least three distinct subdomains that appear to have specialized functions, and they could interact each other. Understanding the dynamic structure of lipid rafts in yeast, as well as their mobility, composition, and biological role, will be of an inestimable value in getting a better insight into the role of these membrane domains in survival and cell death signaling. This insight will recognize the importance of membrane lipids in cellular function, highlighting lipid rafts as a new and promising therapeutic target (Mollinedo and Gajate, 2006), and could be useful for the search of novel antifungal agents and, following extrapolation to mammalian cells, to hopefully set up the underlying bases for the treatment of human diseases. Membrane compartmentalization in lipid rafts plays a key role in signaling process in mammalian cells, and this aspect might also be true in yeast as a plethora of processes seem to involve proteins located in these yeast membrane domains. Thus, a better knowledge of the yeast lipid raft composition and function might help us to gain insight in the regulation of critical processes regarding cell fate, which might be extrapolated to other organisms and could be valuable to conceive new approaches in the treatment of human diseases where cell death and survival are critical, such as cancer and neurodegenerative diseases.

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Warburg effect and translocation-induced genomic instability: two yeast models for cancer cells

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Yeast has been established as an efficient model system to study biological principles underpinning human health. In this review we focus on yeast models covering two aspects of cancer formation and progression (i) the activity of pyruvate kinase (PK), which recapitulates metabolic features of cancer cells, including the Warburg effect, and (ii) chromosome bridge-induced translocation (BIT) mimiking genome instability in cancer. *Saccharomyces cerevisiae* is an excellent model to study cancer cell metabolism, as exponentially growing yeast cells exhibit many metabolic similarities with rapidly proliferating cancer cells. The metabolic reconfiguration includes an increase in glucose uptake and fermentation, at the expense of respiration and oxidative phosphorylation (the Warburg effect), and involves a broad reconfiguration of nucleotide and amino acid metabolism. Both in yeast and humans, the regulation of this process seems to have a central player, PK, which is up-regulated in cancer, and to occur mostly on a post-transcriptional and post-translational basis. Furthermore, BIT allows to generate selectable translocation-derived recombinants ("translocants"), between any two desired chromosomal locations, in wild-type yeast strains transformed with a linear DNA cassette carrying a selectable marker flanked by two DNA sequences homologous to different chromosomes. Using the BIT system, targeted non-reciprocal translocations in mitosis are easily inducible. An extensive collection of different yeast translocants exhibiting genome instability and aberrant phenotypes similar to cancer cells has been produced and subjected to analysis. In this review, we hence provide an overview upon two yeast cancer models, and extrapolate general principles for mimicking human disease mechanisms in yeast.

Keywords: aneuploidy, cancer, chromosome translocation, double-strand break, genome stability, pentose-phosphate pathway, Warburg effect, yeast model system

INTRODUCTION

Yeast (*Saccharomyces cerevisiae*) and human split around a billion years ago, therefore a plethora of cellular mechanisms have evolved in parallel. However, at the same time many fundamental processes remain strongly conserved, and thus yeast represents an efficient utility that can help to understand the molecular mechanisms underlying human disease physiology. Although many yeast models for studying cancer have been established (reviewed in Pereira et al., 2012), we here focus on two physiological processes that appear to be deeply similar between yeast and humans: (i) the reprograming of central metabolism during rapid cell growth [glycolysis, the pentose-phosphate pathway (PPP), amino acid metabolism, and respiration], bearing similarities to the Warburg effect in cancer cells (Bayley and Devilee, 2011; Cairns et al., 2011; Grüning and Ralser, 2011), and (ii) the bridge-induced translocation (BIT) system and its genetic and physiological consequences (Tosato et al., 2005, 2009; Nikitin et al., 2008; Rossi et al., 2010), which resemble and perhaps could simulate genomic instability of leukemia cells.

The majority of cancer research institutions around the world use yeast genetics as part of their research strategy and at least two of them are (or were) led by Nobel laureates who achieved their major honors for research accomplished in yeasts (Leland H. Hartwell and Paul Nurse, who together with Tim Hunt were awarded the 2001 Nobel prize in Physiology or Medicine for their work on the eukaryotic cell cycle). *S. cerevisiae* remains prominent in research of basics of eukaryotic molecular cell biology. Undisputedly, this yeast is a very advantageous system for this purpose: *S. cerevisiae* cells are rapidly growing and easy to handle, have a short cell cycle and use a large number (but not all) of the molecular genetic mechanisms known from multicellular organisms. Most importantly, yeast is the most highly developed system amenable to change its genome by genetic engineering, reintroducing precisely engineered genetic changes into the genome and to study the effects of those manipulations *in vivo*, in short, to do "reverse genetics." This is in particular true for cell cycle regulation, mutagenesis, and DNA repair, and the suicide process of apoptosis, all of which have been found to be important for understanding the biology of cancer cells. To give an example and a quote, the motivation of Lee Hartwell to do cell cycle research in yeast was that he wanted to contribute to the understanding of cancer. He started his Nobel lecture with these words: "My research career has

been motivated by a desire to understand cancer. Each time I have identified an intriguing aspect of the cancer problem, I found that it could be approached more effectively in the simple eukaryotic cell, *S. cerevisiae*, than in the human cell" (Hartwell, 2004). The key point is to mimic, if possible, the pathological changes observed in cancer cells in yeast cells and then to manipulate these model phenocopies in order to try to reduce the effects of those changes. Examples would be the loss of cell cycle checkpoints in cancer cells and the loss of pathways to enter the apoptotic program.

COMPARATIVE GENOMICS

The prediction that nearly half (∼3,000) of all yeast genes would have structural or functional homologs in the human genome, prompted many comparative genetic studies between the yeast and mammalian cell systems. Indeed, several yeast orthologs exist of human genes considered tumor suppressors important for tumor initiation and/or progression. For other genes, like the human p53 tumor suppressor and cell cycle checkpoint gene, even if there is no direct yeast ortholog itself, an analogous signal transduction pathway in which it participates does exist. The human protein can be expressed in yeast, where the mutations occurring in this gene and their phenotypic consequences can be studied much easier. In this way, the mutational spectrum of p53 was determined and found to be identical to the one found in human cancers (Brachmann et al., 1996; Inga et al., 1998; Schlichtholz et al., 2004). Since the genetic system of yeast allows for the selection of specific types of mutants, for instance dominant negative mutations, the spectrum of dominant negative mutations of p53 obtained in yeast was then found identical to the mutational spectrum in cancers (Brachmann et al., 1996).

New anti-cancer drugs that ideally should interfere with the special pathological processes of cancer cells without harming normal cells are being tested in yeast cells mimicking "cancer-like" genetic (mutants defective in checkpoints) or environmental (cells under severe oxidative stress) conditions. The efficacy of these drugs often depends on a "synthetic lethal" effect. For instance, as a driving mutation inactivates a certain repair pathway, the drug inhibits the only other remaining parallel repair pathway in the cancer cell (Bjornsti, 2002; Hartwell, 2004). As outlined by Hartwell, these strategies have already proved very valuable for broadening the understanding of cancer development and treatments given to patients. As example, our understanding of the molecular mechanism of action of cancer drugs which inhibit topoisomerase II or the proofreading activity of DNA polymerases has improved greatly using a panel of 70 yeast strains that are defective in exactly those highly conserved cell cycle, checkpoint, or DNA repair functions which are also found in clinical cancer specimens (Hartwell, 2004). Therefore, therapies can be developed and improved, if the relevant biochemical defect of the cancer in question is determined. A more exotic approach is to use yeast cells or substances derived from yeast cells as a cancer cure (Ghoneum and Gollapudi, 2004; Liu et al., 2009) that is, however, not the topic of this review.

GENOME STABILITY AND MAINTENANCE

An important contribution of yeast research to our understanding of cancer arose from genome-wide screenings for mutations that decrease genomic stability (Yuen et al., 2007; Stirling et al., 2011). This concerns missegregation of chromosomes resulting in aneuploidy, chromosome mutations like translocations, inversions and deletions, and also point mutations. Both the mitochondrial and the nuclear genomes were considered for these lines of research. Also included in these attempts are investigations of aging cells that were found to show an increased level of genetic instability. In the last few years, these attempts were supported by an increasing number of whole genome sequences of human tumor cells. These sequences identified hundreds of mutations in those tumors and have resulted in the identification of new cancer-relevant genes, including CIN (chromosomal instability) alleles (Bignell et al., 2010). We believe that the genetic changes that give rise to the genetic instability of tumor cells may provide the key to tumor cell sensitivity (Hartwell et al., 1997).

The occurrence of genomic instability is an almost universal marker of cancer cells, but it is less clear if mutations leading to genomic instability are the most important early "driver" mutations which clonally initiate cancers (Michor et al., 2005). This view is now enlarged by the findings of Marie Hardwick in yeast (personal communication) showing that in a majority of all the deletion mutants which she looked at in their yeast collection, secondary mutations were quite unexpectedly (but rapidly) selected and occurred in the strains as they were distributed out. These secondary mutations were in multiple genes and also represented multiple alleles within the same gene (example: *WHI2*). This genomic instability could be due to the selective forces that apparently work in a genome where a functional gene has been lost.

It is also not clear if a single chromosomal translocation event can be sufficient to trigger the complex chain of events that we call tumor progression. This is the case in certain leukemias, the most well known of which are chronic myelogenic leukemia (CML) and acute myeloid leukemia caused by the translocation event of the Philadelphia chromosome (Sherbenou and Druker, 2007). At the present, even if the earliest direct demonstrations of the role of chromosomal translocations as causative agents of tumors were found in liquid tumors, there are nevertheless numerous examples, even in epithelial tumors, of the pathogenic effect of these translocations in solid tumors as well.

We would like to ask the question as to "what would conceivably be the phenotype of yeast cells that can serve as a model for human cancer cells?" To many people's opinion, unrestricted growth is the most problematic phenotype of cancer cells. Normal wild-type yeast cells recapitulate this phenotype: on rich media, cells multiply and the biomass grows until nutrients, or one essential nutrient, are used up. However, wild-type cells are able to respond to the level of all essential nutrients, and to many other conditions (for instance, the presence of an alpha mating partner in an a-cell) in an ordered and life-promoting fashion. These responses work via signal transduction pathways or rather a network of such signal transduction pathways that regulates the cell cycle. These mechanisms can stop the cell cycle at junctions that were termed "checkpoints" by Hartwell and his coworkers (Hartwell and Weinert, 1989; Weinert, 1997). The final phenotypic outcome of these wild-type checkpoint mechanisms is cell cycle arrest (for instance in response to DNA damage), repair, and if repair does not take

place, either apoptotic cell death or trans lesion DNA synthesis resulting in a permanent damage to the genome, and hence genomic instability. These pathological forms of growth and cell division were not observed frequently in yeast mutants, but they are hallmarks of human cancer cells.

Early yeast models for cancer signaling, the RAS gene

In one of the first valid examples of "cancer phenotypes" in yeast, the oncogenic point mutation Ha-ras-val12 was compared with a homologous mutation in a closely related yeast gene, *RAS2*-val19. It is well known that this single point mutation can transform mouse 3T3 cells from an immortalized but harmless cell line into a highly cancerogenic line leading to numerous cancerogenic *foci* in cell culture and to cancers if transplanted into immune deficient mice (Weinberg, 1983). The same mutation in yeast (*RAS2*-val19) renders yeast cells into being insensitive to the starvation signal(s) and prevents the synthesis of reserve carbohydrates. Eventually, this causes cell death during starvation (in particular nitrogen starvation), a very short mother cell-specific life span even in the presence of nutrients, creates oxidative stress and increases apoptotic death (Tatchell et al., 1985; Toda et al., 1985). Biochemically, the two homologous mutant genes in yeast and human cells render the small G protein RAS insensitive to regulatory proteins (GAPs and GEFs), keep it in a permanently activated state and therefore permanently activate the ensuing signal transduction cascade. Interestingly, although the downstream kinases are different in yeast and human cells, the observed changes resulting from the activating ras mutation are similar in both systems. If the human Ha-ras-val12 mutation is tested in an otherwise wild-type cell, it leads to hypermitogenic arrest and apoptosis, just as in the yeast cell (Serrano et al., 1997). Only if other typical mutations of cancer cells are present, the murine cells are transformed to acquire cancerous growth. This example shows what can be expected from yeast cells that model cancer cells and which cancer phenotypes can be studied in yeast. In our view, two of the most telling and pathogenetically relevant phenotypes of cancer cells are their genomic instability and the remodeling of metabolism to a hypoxic-like state although oxygen is present in those cells at levels comparable to wild-type non-cancerous cells. Little or nothing is presently known about the interrelationship of these two phenotypes. The latter one is also known as the Warburg effect and has recently experienced a renaissance of intensive investigations among cancer researchers. Genomic instability occurs in the majority if not in all cancer cells, but the role and history of subsequent steps of genomic instability during tumor progression is hard to study because early stages of cancers are usually not available in clinical samples. It is unclear how and why a final step in tumor progression results in a highly aneuploid but finally stable endpoint and what the biochemical commonalities are between those vastly different cancer cells, which are all highly aneuploid. For those reasons, a number of groups have tried to define mutations in genes which are normally responsible for genomic stability and lead to strong mutator phenotypes resulting in chromosome loss or gross chromosomal rearrangements (GCR) in certain mutants. It is now possible but very difficult to perform such a study in human cells (Paulsen et al., 2009). However, the yeast genetic system has been exploited for this purpose and has led to a comprehensive set of both non-essential (Yuen et al., 2007) and essential genes (Stirling et al., 2011) which, when mutated, contribute to genomic instability. The yeast system has the additional advantage to define whole sets of genes and physiological pathways through interaction networks, which also contributes to genome maintenance. The endpoints used for the genome-wide screening of both the yeast deletion collection and several collections of conditional mutations in essential genes were (i) loss of a centromeric chromosome fragment and screening for ade2-mutant colony color; (ii) screening for bi-maters; (iii) screening for a-like fakers; and (iv) screening for GCRs by simultaneously scoring forward mutation to canR and loss of *URA3* on 5-fluoroorotic acid (5-FOA) (Stirling et al., 2011). A total of 692 genes was identified in functional classes (GO terms) that are highly plausible based on prior knowledge of genome maintenance (mitosis, replication, repair, DNA modification, telomere maintenance, transcription, RNA processing, nuclear transport, and proteasome) or define peripheral functions, like iron-sulfur cluster biosynthesis (Veatch et al., 2009). Most importantly, some of the most central and most highly conserved of those genes have human orthologs, mutant alleles of which were found in tumor specimens – among them, *SGS1* (human genes *BLM* and *WRN*, repair helicase), *MRE11* (*MRE11A*, the gene of a syndrome related to ataxia telangiectasia), *DUN1* (*CHK2*, one of the genes of familial Li–Fraumeni syndrome, coding for a cell cycle checkpoint protein), and *BUB1* (*BUB1*, frequently mutated in colorectal cancer). These findings are proof of principle for the usefulness of this approach to study genome instability in yeast as an avenue to understand genome instability in cancers. The consensus among the authors in this field is that genome instability can probably be a primary cause creating the other mutations found in cancers and constituting the tumor progression sequence. A similar conclusion was reached by analyzing clinical data with a mathematical model in light of the "two hit" hypothesis (Michor et al., 2005). The activation of error prone DNA synthesis, which then indirectly leads to more mutations including point mutations, is another important consequence of genome instability (Daee et al., 2010). This can happen both by activating error prone DNA polymerases that exist in all cells for different purposes, and also by producing mutant forms of the replicative DNA polymerases, which for instance have lost their proofreading exonuclease functions (Daee et al., 2010).

We believe that BIT in diploid yeast cells (Tosato et al., 2005), which leads to unstable genomes, can be a valid model system to study the role and the physiological consequences of genomic instability. This system consists of the artificial induction of chromosome translocation based upon the DNA transformation of wild-type yeast cells with a linear, double-stranded DNA molecule (cassette) obtained by PCR. The DNA cassette has the two ends with a sequence homologous to two different chromosomal sites of the genome and flank a positively selectable marker such as KAN^R or HYG^R. The integration of the two free DNA cassette ends at their homologous site by homologous recombination, forms a DNA bridge between two different chromosomes, that is, a chromosome translocation between them. This event can be selected screening the cells for the stable appearance of the

phenotype conferred by the selectable marker carried by the cassette.

The connection between unstable genomes and metabolic remodeling can be studied in those cells that have undergone BIT translocation. Indeed, in these cells, a complex genomic rearrangement is triggered after the primary BIT event, leading to a general status of gene de-regulation that slowly settles down, selectively remodeling the metabolism according to the environmental conditions of growth, in what is called the adaptation phase.

STUDYING YEAST PHYSIOLOGY TO EXPLAIN THE WARBURG EFFECT OF CANCER CELLS

The leading biochemist Otto Warburg described as early as in the 1920s that tumor tissue ferments at the expense of respiratory activity (reviewed in Warburg, 1956). He speculated that defects in mitochondria are thus a cause of cancer. From today's perspective, it is known that most cancer cells, with the important exception of oncocytoma (Mayr et al., 2008), possess a functional respiratory chain. However, most of them show increased uptake of glucose (this property is explored in imaging to stain cancer tissue using the glucose analogous probe 2 fluoro-deoxy-glucose (2FDG; Kurtoglu et al., 2007)), although many cancers have reduced activity of oxidative phosphorylation (Ferreira, 2010; Cairns et al., 2011). From the historical view of metabolism as a producer of energy and intermediates, this behavior is counterintuitive as mitochondrial respiration is more efficient in energy production compared to anaerobic glycolysis, and the rapid growth of tumors has a high demand for energy (Cairns et al., 2011; Gruning et al., 2011). Research of the recent years, involving yeast as central model now indicates, that metabolic integrity and homeostasis of the system could explain the necessity of wasting energy, and reconfiguring metabolism when growing rapidly.

Yeast recapitulates features of the Warburg effect. At maximum growth speed, *S. cerevisiae* strongly prefers fermentation to respiration. Hence under conditions where sufficient nutrients are available, energy (in form of ATP) appears not to be a limiting factor for rapid proliferation. After entering the stationary phase, yeast growth is slowed and the demand for energy declines. Interestingly, it is exactly this point where respiration becomes an important source of energy (van Dijken et al., 1993). This important physiological parallelism between most cancer cells and yeast has prompted a vigorous research in this area.

The yeast *S. cerevisiae* is a very useful tool in studying the Warburg effect, as respiratory metabolism can be induced or repressed easily via switching the carbon source (De Deken, 1966; Crabtree effect, described by H. G. Crabtree in the 1920s; Crabtree, 1928). Although they have roughly the same energy content and are both fermented, glucose represses respiration, but galactose does not. Ruckenstuhl et al. (2009) used this yeast property to investigate the effects of respiratory bursts on apoptosis, and the impact of free radicals on this process. Inhibition of respiration, or free radical scavenging conferred a survival advantage during seeding and early development of yeast colonies (Ruckenstuhl et al., 2009). Similarly, cancer cells are reactive oxygen species (ROS) sensitive, and might profit from anti-oxidant therapies (Perera and Bardeesy, 2011).

Recent advances in understanding the Warburg effect in cancer and yeast came from investigation of the enzyme pyruvate kinase (PK), which was recognized as a cellular coordinator of respiration and of the anti-oxidant system. It has been reported that exchanging the human PK isoform pyruvate kinase muscle isozyme 2 (PKM2) with its constitutive isozyme PKM1 dramatically slows growth of xenograft tumors, and reactivates respiratory metabolism (Christofk et al., 2008). In this context, it was assumed that PKM2 is specific to proliferating tissue, found only in the embryo or in cancer cells. The lack of other cancer-specific metabolic enzymes thus placed PKM2 center stage for research on cancer cell metabolism (Bayley and Devilee, 2011; Cairns et al., 2011; Hamanaka and Chandel, 2011). However, a PKM2 cancer specificity could not be confirmed in follow up studies, indeed most adult tissues express PKM2 as their predominant PKM form independent of whether they are cancerous or healthy (an exception is, however muscle, which expresses PKM1; Bluemlein et al., 2011). The interpretation of PKM2 being cancer-specific thus potentially resulted from using mouse muscle tissue as non-cancer control in Western blot experiments (Christofk et al., 2008). Despite this setback, however, PK emerged as a central regulator of glycolysis, with immense importance for cancer (Bayley and Devilee, 2011; Chaneton and Gottlieb, 2012). Yeast models helped in understanding the global function of this enzyme.

These results might further help to solve a seemingly paradox about PK activity in cancer cells: as discussed by Diaz-Ruiz et al. (2011), the inhibition of PKM2 seems contradictory in respect to the high glycolytic flux and increased lactate excretion measured in cancer cells, since an inactive PK would severely impair cell energy production in the cells that depends mainly on glycolysis for ATP synthesis. Indeed however, studying PKM2 expression using absolute quantitative mass spectrometry reveals that PKM2 levels are thoroughly higher in cancer cells than they are in matched control tissues (as determined in Bluemlein et al., 2011; please see **Figure 1** for an illustration). Thus, although *PKM2* is not to 100% active in cancer, its overall activity might still be higher as it is in the corresponding control tissue. Indeed, recent investigations show that *PKM2* knock-down increases flux of the TCA cycle and amino acid metabolism also in cancer cells, indicating that PKM2 is of considerable residual flux in tumors (Chaneton et al., 2012).

Similar to PKM proteins in mammalian cells, yeast possesses two paralogous PK genes, *PYK1* and *PYK2* that encode for enzymes with related properties. *PYK1* encodes for the predominant PK isoform when cells grow on glucose media, whereas Pyk2p has lower specific activity and is induced under respiring conditions (Boles et al., 1997). Creating yeast strains with different PK activities (by ectopic expression of either Pyk1p or Pyk2p at both high and low level in a $\Delta pyk1\Delta pyk2$ strain) we found that a reduced activity of this enzyme is sufficient to increase oxygen uptake and respiratory activity. Unexpectedly however, strains with lower PK activity exhibited an increased resistance to several oxidants. Moreover, although respiring at higher rates, these cells did not show an increase in the concentration of superoxide and hydrogen peroxide, nor did they display features of oxidative stress (Gruning et al., 2011). This indicated that low PK activity does not only lead

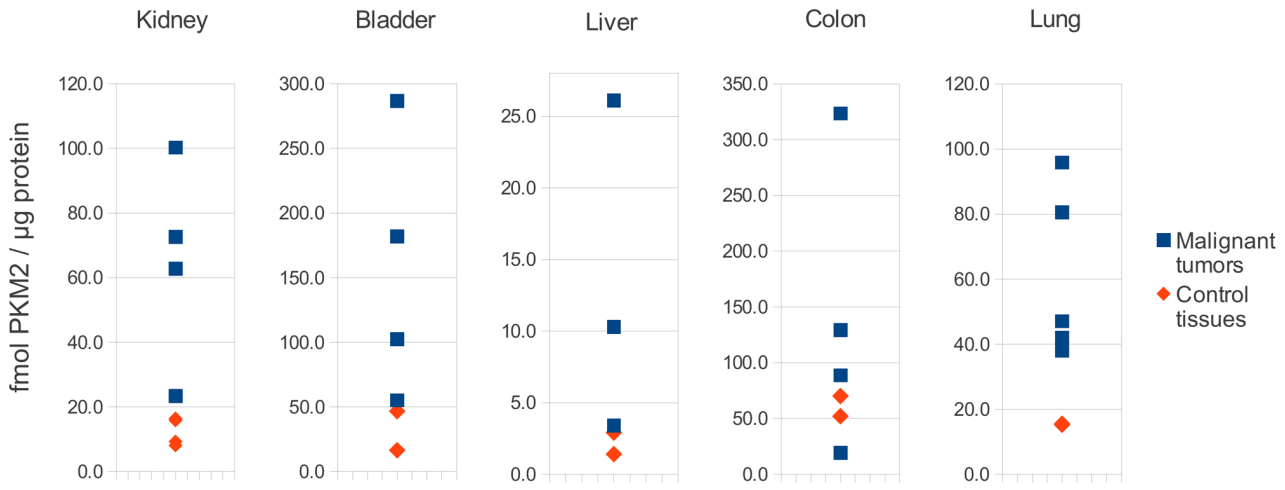

FIGURE 1 | *PKM2* is up-regulated in human cancer. Illustration of the absolute concentrations of PKM2 in several cancer tissues and matched controls, as determined by liquid chromatography-multiple reaction monitoring (LC-MRM) as described in (Bluemlein et al., 2011). PKM2 levels are clearly increased in cancer biopsies compared to the controls.

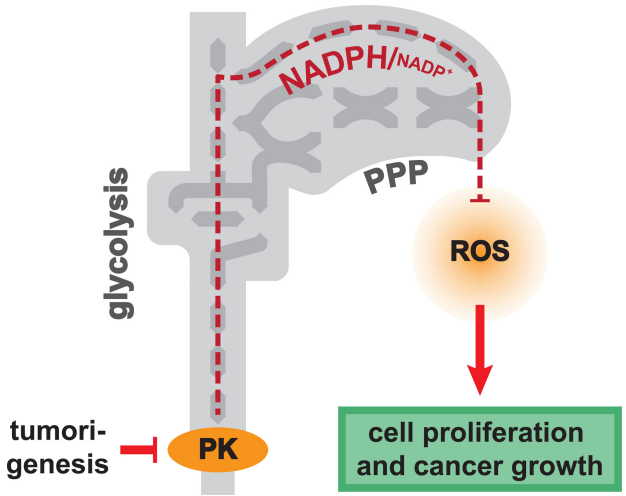

FIGURE 2 | Pyruvate kinase (PK) activity as regulator of anti-oxidant metabolism. Low activity of PK, found in cancer and in respiring yeast, leads to increased flux of the pentose phosphate pathway. Increased PPP activity is required for maintaining the redox balance under conditions with high ROS load: reduced NADPH is required as redox-power for anti-oxidant enzymes, and PPP activity stimulates the anti-oxidative gene expression program.

to increased respiration, it also causes an increase in the anti-oxidant capacity. This physiological reconfiguration eventually compensated for the increased ROS generation during oxidative metabolism (**Figure 2**).

Pyruvate kinase converts phosphoenolpyruvate (PEP) to pyruvate, a reaction which yields one molecule of ATP (Fraenkel, 1986). The substrate PEP is a highly polar sugar phosphate, and accumulates in yeast and *E. coli* when PK activity is low (Emmerling et al., 2002; Gruning et al., 2011). It has been reported that PEP can interfere with more than one reaction of glycolysis, including phosphoglycerate mutase, glucokinase, phosphoglucoisomerase, phosphofructokinase, aldolase, and triosephosphate isomerase (TPI; Ogawa et al., 2007; Fenton and Reinhart, 2009; Vander Heiden et al., 2010). Interestingly, inhibition of the latter was sufficient to increase resistance to oxidants in yeast and *C. elegans*. The increase in stress resistance can be attributed to increased metabolite levels in a metabolic pathway parallel to glycolysis, the PPP (Ralser et al., 2006, 2007). The PPP shares several metabolites with glycolysis, and plays a pivotal role in the oxidative stress response. First, this pathway can quickly and dynamically increase in activity to suffice the increased need for the redox co-factor NADPH upon an oxidative burst (Ralser et al., 2009). Second, it is involved in the induction of the anti-oxidant gene expression program (Kruger et al., 2011). It appears that TPI feedback inactivation by PEP is required for the increase in stress resistance of the PK mutants, as cells expressing a mutant human TPI allele ($TPI^{Ile170Val}$) that is largely robust to PEP inhibition do not show the PK dependent increased resistance to oxidants. *Vice versa*, in cells with low PK activity, deletion of the first enzyme of the PPP, glucose-6 phosphate dehydrogenase (*ZWF1*) causes increased ROS levels, protein oxidation, and mitochondrial damage (Gruning et al., 2011). In sum, a reduction of PK activity increases the flux of the PPP protecting against oxidants, and the feedback inhibition of TPI by the PK substrate PEP is crucial for this adaptation.

Although respiring at moderate rates, also cancer cells suffer from high ROS load (Pelicano et al., 2004; Chandra and Singh, 2011; Israel and Schwartz, 2011; Perera and Bardeesy, 2011). It is assumed that the majority of these ROS are side products of the high metabolic activity of cancer cells, especially beta-oxidation of fatty acids, and the activity of NADPH oxidases (Pelicano et al., 2004; Cairns et al., 2011). However, this information has a high degree of uncertainty, as a reliable genome-wide quantification of ROS contributions in cancer is lacking till the present

day. Nonetheless, maintaining the redox balance appears to be a major issue for mammalian tissue, indicated by the high concentration of the anti-oxidant peptide glutathione, which exceeds the cellular ATP level by an order of magnitude (Meister and Anderson, 1983). In tumors, PKM2 seems to fulfill a similar role in anti-oxidant defense as discovered for yeast *PYK* genes. The low activity of PKM2 in lung cancer cells leads to a higher activity of the NADP reduction in the PPP, and to increased anti-oxidant defense. Expressing of a oxidation-resistant PKM2 mutant in xenograft tumors reduced the activity of the PPP, and markedly slowed tumor growth (Anastasiou et al., 2011).

Overall, these observations indicate that the balancing of the metabolic network (and so maintaining the redox state and metabolite homeostasis) might be more difficult to achieve for rapidly proliferating cells than to guarantee a sufficient supply with ATP. Understanding this principle can be very valuable for developing anti-cancer therapies. For instance, one could imagine inducing a ROS boost into cancer cells for making them vulnerable to chemotherapeutics (Perera and Bardeesy, 2011). The broad experimental possibilities offered by yeast are invaluable help in deciphering these complex questions.

Interestingly, recent results from several laboratories reveal that a redirection of central carbon metabolism by PK does not only change redox metabolism, but is also important for amino acid metabolism (Bluemlein et al., 2012; Chaneton et al., 2012; Kung et al., 2012; Ye et al., 2012). In yeast, a change in the activity or expression level of PK causes a strong reconfiguration of the entire amino acid profile, with seven amino acids (arginine, aspartic acid, histidine, lysine, threonine, valine, and serine) being present a lower concentration, and two amino acids (glutamine and glutamate) being increased when PK activity is low (Bluemlein et al., 2012). Hence, PK seems to link the generation of energy within central metabolism, and the metabolism around energy consumption at the level protein biosynthesis.

In mammalian cells, the function of PKM2 in regulating serine biosynthesis has been studied in detail, and reveals a feedback control system which controls the levels of free amino acids. It has been found that serine is an allosteric activator of human PKM2, and that overall PK activity is reduced when cancer cells are deprived of this amino acid (Chaneton et al., 2012; Ye et al., 2012). At the same time, the glycolytic block caused by reduced PKM2 activity feeds back into serine biosynthesis, preventing serine deprivation during cancer formation (Chaneton et al., 2012). Consistently, in human thyroid follicular adenoma, the expression of the serine-biosynthetic enzyme serine hydroxymethyltransferase (SHMT1) is increased compared to healthy control tissue, and correlates with the absolute PKM2 expression level (Bluemlein et al., 2012). In addition, small molecule activation of PKM2 induces serine auxotrophy in cancer cells, indicating that this control mechanism could be exploited for therapeutic purposes (Kung et al., 2012).

In sum, studies in yeast led to the discovery of redox state control by the enzyme PK (Gruning et al., 2011). This mechanism appears to be of importance for the progression of lung cancer cells, but potentially other cancer types as well (Anastasiou et al., 2011). PK further moonlights to the regulation of protein biosynthesis, and amino acid metabolism in yeast and human cells (Bluemlein et al., 2012; Chaneton et al., 2012). Serine appears to be central for this regulatory mechanism, as it can act as allosteric activator of PKM2 and hence report the concentration of free amino acids to central carbon metabolism (Chaneton et al., 2012; Ye et al., 2012). The PK enzyme is thus a central player in coordinating cellular metabolism. Yeast turned out to be a very effective model in studying the interplay of the involved metabolic pathways.

SPONTANEOUS AND INDUCED CHROMOSOMAL TRANSLOCATIONS

One of the best possibilities offered by the yeast system to model gross genetic alterations known to induce well-characterized cancer forms in humans, is the BIT, bridge-induced chromosomal translocation. Indeed, this technology induces the formation of a translocated chromosome exploiting the yeast natural homologous recombination system (HRS) between the two ends of a DNA bridge molecule harboring a positively selectable marker (i.e., KAN^R). This type of chromosomal aberration has been since long time connected to the insurgence of forms of tumors, like the renowned Philadelphia chromosome, resulting from a translocation between chromosome 9 and 22 in humans, leading to CML. Several other chromosomal translocations are known to promote cancer and their molecular mechanisms of occurrence can be studied efficiently in yeast, using the inducible BIT system. In the following part, chromosomal translocations and BIT will be deeply analyzed with respect to their cellular consequences leading ultimately to cancer.

Chromosomal translocations are rare cellular phenomena in which two chromosomes are interacting with each other either by physical fusions or by copying one chromosome's fragment on another. Depending on the nature of these interactions, chromosomal translocations can have reciprocal or non-reciprocal configuration. Translocations might pass unnoticed by the cell, bringing no consequences; however, in the majority of cases, a translocation's onset has a tremendous impact, limited not only to the single cell, but also to the organism as a whole. Chromosomal translocations yield a variety of effects ranging from distorted transcription patterns to cell death due to increased apoptosis. In multicellular organisms, chromosomal translocations can be related to a systemic death observed in human malignancies, in particular hematological or mesenchymal cancers. As a result, translocations can also be useful markers in the diagnosis of liquid and solid tumors (Herve et al., 2011; Klemke et al., 2011). This fact greatly increases interest in the investigation of all chromosomal translocations aspects: origins, causes, outcomes, and clearly – their association with genetic diseases. Despite the growing number of laboratories working on these subjects and the great efforts made by investigators all over the world, the topic of chromosomal translocation is still not fully covered and the mechanistic molecular factors that elicit these GCRs are still object of investigation. Progresses are greatly impaired by the rare occurrence of spontaneous translocations, in particular in mammalian cells, by the broad panorama of secondary rearrangements and by a lack of effective detection techniques.

Spontaneous chromosomal translocation can arise either from spontaneous recombination between repeated elements dispersed

through a genome or from the free DNA ends originated from double-strand breaks (DSBs), stalled replication forks or dysfunctional telomeres (Jinks-Robertson and Petes, 1986; Loidl and Nairz, 1997; Richardson et al., 1998). Although DSBs are an extreme threat to the cell, they are crucial for its existence and happen very frequently in a programmed manner as a part of specific life cycle processes such as meiosis, mating type switching in fungi or V (D) J recombination during immunoglobulin and T-cell receptors maturation (Bassing et al., 2002; Zhang et al., 2011). DSBs can appear as a result of cellular processes like DNA replication, through single-strand nicks, or elevated levels of ROS, but they can be also induced by exogenous factors. Cell exposition to DSBs-inducing agents (e.g., ionizing radiation, ROS, viruses, some chemotherapeutic drugs, and more) greatly enhance the probability of spontaneous translocations and teratogenicity, as even a single DNA break in the cell can result in GCRs (Kolodner et al., 2002). There exist two major, different DSB repair pathways: homologous recombination (HR) and non-homologous end joining (NHEJ), and both of them can give rise to translocations. HR can occur by means of three sub-pathways: gene conversion (GC), break induced replication (BIR), and single-strand annealing (SSA). For a detailed review of these repair pathways in mammalian and yeast cells the reader is referred to (Aylon and Kupiec, 2004). Genome rearrangements, by definition, are not beneficial for the cell and will be actively prevented by various mechanisms. For example, the choice of the correct repair pathway (HR or NHEJ) at the right moment of the cell cycle is fundamental for the suppression of translocation (Branzei and Foiani, 2008). In budding and fission yeast, HR is the dominating pathway for DSB repair, and NHEJ seems to be restricted only to the G1 phase. In fact, experiments indicate that NHEJ mutants of *S. cerevisiae* are resistant to ionizing radiation, whereas HR mutations severely compromise survival (Siede et al., 1996; Manolis et al., 2001). In mammalian cells the situation is opposite. NHEJ is the dominating repair pathway employed during the entire cell cycle, whereas HR is restricted to the S phase (van Gent and van der Burg, 2007; Shrivastav et al., 2008). In effect, NHEJ pathway was originally identified in mammals, and later its elements were discovered in bacteria and yeasts (Moore and Haber, 1996; Doherty et al., 2001). The prevalence of the HRS in budding yeast suggested its exploitation in the production of "*ad hoc*" translocations in *S. cerevisiae*, as described in the following paragraphs.

The broad spectrum of the effects of translocations and their complex involvement into cancerogenic processes are the reason why chromosomal translocations are so intensively studied. Neoplastic transformation is associated to reciprocal or non-reciprocal translocations that can lead to altered expression of proto-oncogenes and loss of heterozygosity (LOH) of tumor suppressor genes. Proto-oncogenes have their own homolog in budding yeast (the most popular is *SAS3*, ortholog of *MOZ*) as also do tumor suppressors (the yeast genes *TEP1*, *FSH1*, *HNT2*), with the renowned exception of *TP53*.

The genetic mechanism through which chromosome translocations elicit the onset of certain tumors is the fusion of the coding sequence of two non-contiguous genes located at the translocation site, on different chromosomes, with the consequent expression of a novel hybrid protein able to disrupt the correct control of cell proliferation. Usually, two major groups of genes are involved into neoplastic transformation as an indirect result of chromosome translocation: tyrosine kinases and transcription factors. Oncogenic mechanisms of chimeric proteins results from the cancer-promoting nature of such proteins or by disruption of another gene regulation system. Novel gene fusions caused by DSB repair are responsible for around 20% of human cancer morbidity. Until now, 337 genes were identified in 358 gene fusions (Mitelman et al., 2007). These numbers are rapidly increasing due to development of rapid sequencing methods and constantly growing microarray databases. Identification of gene fusions gains remarkable importance as a diagnostic and prognostic marker (Prensner and Chinnaiyan, 2009). The role of chromosomal translocations in neoplasia is so significant, that a specialized database of chromosomal aberrations and gene fusions in cancer has been created. This database can be accessed at: http://cgap.nci.nih.gov/Chromosomes/Mitelman.

However, as mentioned before, one of the main problems that researchers have to overcome is the extremely low frequency of spontaneous translocations arising either in mammals or in model systems. This negative aspect of the parallel between human cancer cells and yeast could be overcome by the induction of chromosome translocation events in both cellular systems with mutagenic agents. Unfortunately, any mutagenic process utilized in mammalian cells would require the appearance of a strong, detectable mutant phenotype to allow the selection of those cells that have undergone a translocation event, and this would occur very slowly and would be difficult to select for. On the contrary, with the model yeast cells, given the possibility to manipulate an almost endless number of them and to intervene more directly on their genome, this can be achieved rather easily. In order to increase the events to reach statistically significant numbers, various methods were developed for induction of chromosomal translocations. Generally, these methods are based on two major principles: artificial induction of DSBs within the desired regions sharing strong homology, or recombination between special elements catalyzed by site-specific recombinases. In both cases, a time-consuming molecular engineering of the sites selected for the translocation, is necessary prior the induction of the translocation event. Recently, a third methodology of induction of chromosomal translocation has been developed. This method, BIT, allows the generation of non-reciprocal translocations in mitosis without pre-modifications of the genome, exploiting the natural HRS of *S. cerevisiae*. The phenotypic changes of yeast cells after a BIT translocation event seem to mimic closely the oncogenic transformation of mammalian cells (Tosato et al., 2005). The most common methodologies to induce GCRs and their implications are discussed extensively in the next sections.

ADVANTAGES AND DRAWBACKS OF THE MAIN MOLECULAR SYSTEMS TRIGGERING TRANSLOCATIONS

Several strategies to introduce DSBs by artificial means, ensuing in chromosome translocations were developed in the last 15 years. Transformation of yeast cells with a chromosomal fragmentation vector (CFV) resulted in the gain of a chromosomal fragment (CF) with or without the loss of the targeted chromosome, following

DSB processing by break-copy duplication (Morrow et al., 1997). The discovery of fragmental duplication in yeast led to demonstrate, 1 year later, that a chromosomal DSB produced by the HO endonuclease could be repaired by BIR, producing NHEJ-mediated reciprocal translocation (Bosco and Haber, 1998). Later on the HO system has been utilized for the production of a DSB on two chromosomes, ensuing in reciprocal translocations by NHEJ (Yu and Gabriel, 2004) after the repair of the broken chromosomes by SSA (Liddell et al., 2011). Other meganucleases, such as I-SCEI, can be used to generate DSBs in higher organisms promoting translocations with a frequency of 1–4% (Egli et al., 2004). More recently, the cre site-specific recombination-based system producing reciprocal translocations at pre-engineered loxP sites has been developed to study speciation in yeasts (Delneri et al., 2003) and successively improved to minimize the occurrence of unwilled, secondary rearrangements (Carter and Delneri, 2010). Finally, transposable elements are regularly utilized to produce chromosomal manipulations with variable efficiencies. Among all these systems, transposons-related methodology are mostly exploited for the development of new variety of plants (Yu et al., 2012), the cre-lox is used to generate animal models of human cancers (Buchholz et al., 2000; Rabbitts et al., 2001; Forster et al., 2005; Yu et al., 2010) although meganuclease-related methods are also utilized to produce DSBs in malignant cell lines (Cheng et al., 2010; Kitao et al., 2011). However, all these experimental systems need preceding modifications of the genome in order to produce translocations. Moreover, almost always they result in reciprocal translocations. It was therefore necessary to develop a simple system that triggers translocations without any prerequisite for strain engineering, without the assistance of any cloned exogenous/endogenous enzyme and that allows also the simultaneous recovery of events such as non-reciprocal translocations, telomeric fusions and deletions usually occurring during neoplastic transformations. For these reasons the BIT methodology was developed.

BIT: BRIDGE-INDUCED TRANSLOCATION

THE BIT SYSTEM

The system consists in the production of selectable translocation-derived recombinants ("translocants") generated at desired chromosomal locations in wild-type yeast strains transformed with a linear DNA cassette carrying a selectable marker (i.e., KanR) flanked by two DNA sequences homologous to two different chromosomes (Tosato et al., 2005; **Figure 3**). The bridge, which is obtained exploiting the endogenous HR machinery of the yeast cell, is obtained with a variable efficiency (typically from 2 to 15%) depending on the length of the homologies, the secondary structures, and the base composition of the target regions, and the strain's genetic background. This last variability is probably due to a different extension of the rDNA region of chromosome XII that may act as recombination hotspot. It was demonstrated that the resulting translocation is non-reciprocal, that it occurs with similar efficiency between heterologous and homologous chromosomes (Tosato et al., 2009) and that it is usually associated with aneuploidy (Rossi et al., 2010). Effectively, we verified that several other GCRs leading to LOH, such as intrachromosomal deletions, DNA duplications, unspecific translocations due to micro-homology, arose after transformation with the linear cassette.

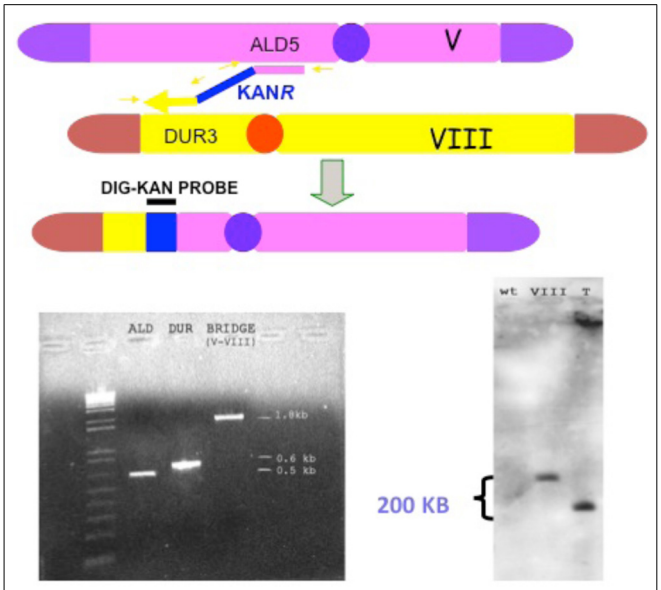

FIGURE 3 | Schematic representation of a BIT chromosome translocation event induced in the yeast *S. cerevisiae*, and its molecular verification by PCR and Southern blot analysis. BIT translocation designed between the *ALD5* locus on chromosome V and *DUR3* on chromosome VIII and obtained by transformation with a linear double-stranded DNA cassette having the two extremities homologous to the two *loci*, flanking the positively selectable marker KAN^R. The translocation between the two top chromosomes, catalyzed by the DNA cassette functioning as a bridge, produces the translocated chromosome below the big gray arrow. Verification of the correct chromosome translocation by gel electrophoresis analysis of PCR amplification (bottom, left) of the two DNA junctions at the *ALD5* and *DUR3* *loci* (between primers indicated by the two small yellow arrows on the right and the left, respectively), lanes ALD and DUR of the gel. Verification of the formation of the DNA bridge between the two chromosomes by PCR amplification of the region between the two external primers indicated by the two small external yellow arrows, lane BRIDGE of the gel. Bottom, right: Southern hybridization with a DNA probe corresponding to the KAN^R gene, of a contour-clamped homogeneous electric field (CHEF) electrophoresis spread of chromosomes from a wild-type strain (lane wt), a strain with chromosome VIII previously marked with KAN^R (lane VIII) and a strain subjected to BIT translocation at the same *loci* (lane T).

GENERAL CELLULAR EFFECTS OF BIT TRANSLOCATION

As a consequence of a single translocation event produced via BIT, the yeast cell exhibits an abnormal phenotype characterized by elongated buds, nucleated pseudo-hyphae, karyokinetic defects and nuclear fragmentation (Nikitin et al., 2008; Rossi et al., 2010). Moreover, the metabolism of the translocants was severely impaired; they show, in particular, altered fitness on different carbon sources, different sporulation efficiencies and ability to flocculate. The integration of the same cassette at the two target *loci* can be processed in different ways generating strains different in karyotype and consequently in phenotype and physiology. These data suggest that the scrambling of gene regulation throughout the genome triggered by the integration of a linear DNA fragment through recombination is a great force for evolution. Indeed, among the broad panorama of mutants generated by a translocation, few of them, or perhaps only one of them, will be favored in survival and life span, adapting better to new

environmental conditions or oxidative stress. That is exactly what happens to the mammalian cells after neoplastic transformation, when genomic defects are translated to phenotypic aberrations and it represents the great plasticity and diversity of cancer cells.

GENOME-WIDE EFFECTS ON REGULATION OF GENE EXPRESSION

It was demonstrated that the BIT system causes an increased expression of the genes around the breakpoints up to five times (cis effect), coinciding with an increased level of the RNA polymerase II binding to their promoters, and with the pattern of histone acetylation (Nikitin et al., 2008). Furthermore, many other genes not involved in the specific translocation events are deregulated (trans effect). Extensive transcriptome and fluorescence-activated cell sorting (FACS) analysis of the translocant pointed out that the acentric chromosome fragments are duplicated or integrated through micro-homology in the genome and that many cells are blocked in G2/M phase. These results indicate that the translocant cells have adapted to the checkpoint response after the initial DNA damage induced by BIT. More recently, an implementation of the BIT system was created in order to bridge together two homologous chromosomes in a diploid cell (Tosato et al., 2009). In this case, the experiments demonstrated that BIT happens with low frequencies producing LOH and regions of hemizygosity by deletion. The frequency of targeted BIT between homologous chromosomes is lower or the same than between heterologs, supporting the idea that a checkpoint system might actively prevent mitotic LOH in eukaryotic diploid cells. The phenotypic and transcriptional aberrations of the translocant between homologous chromosomes are negligible if compared to those of non-reciprocal translocants between heterologs. Moreover, the quantitative analysis of the expression of several genes around the breakpoints indicated the over-expression of the multi-drug resistance gene VMR1. Remarkably, VMR1 is the budding yeast homolog to the human MRP4, which is highly expressed in LOH-associated types of cancer such as primary neuroblastoma (Norris et al., 2005).

ANEUPLOIDY

Recently, it was demonstrated that the HRS and the BIR pathway are both responsible for the formation of the initial non-reciprocal translocation and that the proximity of the targeted *loci* with specific genomic elements, such as autonomously replicating sequences (ARS) or repeated DNA regions, may influence not only the efficiency of the event, but also the frequency of secondary rearrangements and aneuploidies (Tosato and Rossi, personal communication).

Aneuploidies are a landmark for cancer, but it is still not completely clear if they are an innocent by-product due to checkpoint gene alterations or a driver of evolutionary processes leading to neoplastic transformation. Further investigations of the molecular players hidden behind the BIT system and responsible for the primary and secondary rearrangements, will shed light to this complex question.

FUTURE PERSPECTIVES AND CONCLUSIONS

In this short review we analyze some analogies between yeast and cancer cells by the metabolic and genomic point of view. Typical traits of a neoplastic transformation are loss of growth control, the consequential continuous energetic demand and aneuploid conditions due to genome instability. We found that in some translocants, where clear phenotypic defects are visible, there are also important metabolism impairments such as a reduced fitness to grow on glucose-deprived media. Effectively, after a wide proteomic and transcriptomic analysis (Nikitin et al., 2008; Nikitin and Bruschi, personal communication), we found that the trans effect of BIT does not mainly concern, as expected, recombination-related genes, but on the contrary, metabolic genes. From a purely logic point of view, to adapt to a different environmental condition and to evolve (a malignant status is also an evolution) the cells at first must change their own metabolism. In this way, the cellular fitness will be improved and the new mutants will be suddenly ready to overgrow the normal, low life span population. To understand how to stop this amazing ability to adapt and immortalize, we have to use simple single-cell models able to retain an induced aneuploid status and chromosomal alterations like telomere–telomere fusions, typical of many cancers. The ideal organism is *S. cerevisiae* because it has a good amount of chromosomes to play with, the best annotated genome, an ability to survive and grow in haploid and diploid state, a simple switch between fermentation and respiration and a great tolerance to ploidy variations. The phenotypic and metabolic changes observed in *Saccharomyces* after a translocation resemble some of the peculiarities observed in tumorigenesis. Studying metabolism, experiments conducted in yeast are less biased compared to mammalian cell culture, as culture conditions and genetic background have strong influence on the status of the metabolic network. Most routes of central metabolism are strongly conserved between yeast and human, and it appears that the same is true for basic control mechanisms. We have reviewed the regulatory function of yeast and human PK on metabolism, and conclude that this enzyme presents a central and conserved coordinator between energy production, ROS clearance, and amino acid metabolism. Elaborating the principles of the metabolism of rapidly proliferating cells (ROS quantitation, respiratory proficiencies) and extensively studying the altered genetic expression in a collection of different BIT translocants will help finding the effectors to revert, if not the altered karyotype, at least some abnormal phenotype of aneuploid cancerogenic cells. In addition, this straightforward technology could be extrapolated to higher organisms to implement a molecular modeling of spontaneous genome rearrangements leading to speciation in lower eukaryotes or LOH in mammalian cells.

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Hypothesis: is yeast a clock model to study the onset of humans aging phenotypes?

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In this paper we report the growth and aging of yeast colonies derived from single cells isolated by micromanipulation and seeded one by one on separated plates to avoid growth interference by surrounding colonies. We named this procedure clonal life span, and it could represent a third way of studying aging together with the replicative life span and chronological life span. In this study we observed over time the formation of cell mass similar to the human "senile warts" (seborrheic keratoses), the skin lesions that often appear after 30 years of life and increase in number and size over the years. We observed that similar signs of aging appear in yeast colonies after about 27 days of growth and increase during aging. In this respect we hypothesize to use yeast as a clock to study the onset of human aging phenotypes.

Keywords: aging, apoptosis, cancer, colony, dedifferentiation, keratoses, yeast

Nowadays, *Saccharomyces cerevisiae* has been widely accepted as a model for the study of aging of multicellular eukaryotes (Kaeberlein, 2010). In fact, in this organism, we can measure the number of mitotic events an individual mother cell can undergo before senescence (Mortimer and Johnston, 1959), referred as replicative life span (RLS). In addition, we can measure the time a non-dividing cell population can remain viable and this is called chronological life span (CLS; Fabrizio and Longo, 2007). RLS has been suggested to be a model for the aging of mitotic tissues, whereas CLS has been likened to the aging of post-mitotic tissues (MacLean et al., 2001) and both can induce apoptosis (Buttner et al., 2006).

Different evolutionary studies are using yeast as a model to investigate on the initial emergence of multicellularity. The formation of multicellular aggregates in liquid cultures can be the result of incomplete cell separation or following the selection of clusters of cells whether by post-division adhesion or by aggregation (Koschwanez et al., 2011; Ratcliff et al., 2012). On solid medium yeast forms colonies after repeated cell divisions.

Here we report the development and aging over 50 days of yeast colonies derived from individual cells of the wild type strain CML39-11A (Mazzoni et al., 2005) isolated by micromanipulation and placed each in one synthetic dextrose (SD) plate. We named this experimental approach clonal life span (ClLS) in that it allows to study the development of clonal cell avoiding the growth interference mediated by the acidic-base pulse generated by surrounding colonies (Váchová and Palková, 2011).

The organization of yeast colonies is ensured by signals transmitted and received by dividing cells within a colony and by chemical alkaline/acid pulse resulting from metabolic activity of colonies growing nearby.

Ammonia signaling is the first non-directed alkaline pulse produced by neighboring colonies and it is followed by a second step leading to acidification of the medium. A second ammonia pulse of higher intensity then occurs and is oriented toward the neighbor colonies. Ammonia signaling results in growth inhibition of the facing parts of near developing colonies (Palková et al., 1997).

The acid phase induces the production of reactive oxygen species (ROS) and other harmful products by all the cells forming the colony and induces apoptosis. The subsequent ammonia signal triggers metabolic changes that allow cells to lower their ROS production (Palková and Vachova, 2006). There is hence a selection of cells within the colony, determined also by the surrounding cells, which limit colony size and development. Cells located at the colony border grow slowly and are healthier compared to the ones located in the center of colonies, which predominantly undergo death.

In the absence of negative growth control exerted by neighboring colonies border cells of an individual colony can divide more and more times, probably without neutralizing ROS. Consequently, colonies might show larger size, peculiar phenotypes, and increased frequency of mutation during aging.

As shown in **Figure 1**, the size of the individually plated colonies increased within 50 days up to 12–13 mm. Interestingly, after 27 days we observed the appearance of some wart-shaped formations. The nature of these excrescences is still unknown, but one can hypothesize that during aging DNA damage and mutation frequency increase (Fabrizio et al., 2005) leading to cells escaping growth control. Although not demonstrated for warts, a similar phenomenon occurs during yeast CLS where the regrowth of few cells, probably adapted mutants generated within aging populations, has been observed (Fabrizio and Longo, 2008). The latter authors suggested that such mutants are reminiscent of cancer cells, which become resistant to apoptosis and duplicate under conditions that are normally not permissive for growth.

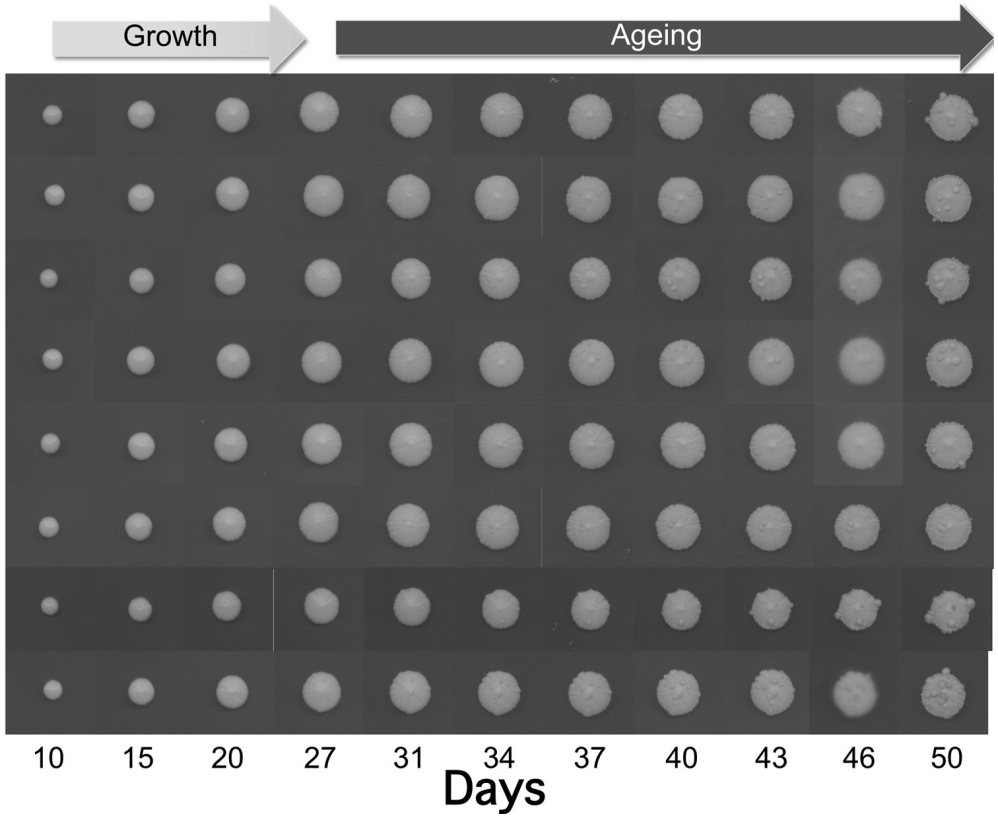

FIGURE 1 | Growth and aging of colonies derived from of eight yeast cells of the wild type strain CML39-11A (Mat a, ade1-101, his3-Δ1, leu2, ura3, trp1-289) isolated by micromanipulation and placed each in one SD (0.67% yeast nitrogen base without amino acids), 2% glucose, and 20 μg/ml of the appropriate nutritional requirements according to the genotype of the strain) plate. Plates were incubated at 28°C and recorded at the indicated days.

It has been demonstrated that these mutant cells arise from altruistic death program partially mediated by superoxide (Fabrizio et al., 2004). The frequency of this cancer-like regrowth phenotype in yeast liquid cultures is greatly reduced under calorie restriction (CR) and in the presence of mutations in the Tor/Sch9 and Ras/AC/PKA (rat sarcoma/adenylate cyclase/protein kinase A) pathways. For these reasons yeast regrowth has been proposed as a useful phenomenon to study the age-dependent effect of mutations associated with cancer (Madia et al., 2007).

Initially the yeast warts mainly appear in the middle of the colony, where older cells are located, and then they enlarge and propagate along the edge of the whole colony. Anyway, the number of such warts significantly differs from colony to colony suggesting the stochastic nature of these events.

To look closer at the nature of warts, we repeated the experiments extending the aging time up to around 100 days. We compared cell viability and mutation frequency of cells from warts (w) and from the smooth layer (L), picked from the same or different colonies. As shown in **Figure 2A**, w cells showed mainly higher viability compared to L cells. At the same time, we also determined the occurrence of mutations in both w and L cells by measuring the reversion frequency to the prototrophic phenotype of the auxotrophic mutation trp1-289 carried by the CML39-11A strain.

As shown in **Figure 2C**, the number of warts, compared to 50 days aged colonies, increased significantly, indicating that this phenomenon can occur for long time.

In **Figure 2B** is reported the number of revertants to Trp^+ phenotype (capability to grow in synthetic medium without tryptophan) normalized to 10^6 viable cells. As can be seen, the reversion frequency of w cells although heterogeneous, was higher than in L cells, reaching in some cases very high levels (i.e., w29, w31, and w32 values are out of scale).

In cancer cells, nuclear morphology is often altered and nuclei appear bigger and irregular in their contours (Zink et al., 2004). 4′,6-Diamidino-2-phenylindole (DAPI) staining of DNA revealed the presence of round nuclei in cells coming from the smooth surface (**Figure 3A**) as well as in a fraction of w cells (not shown). In addition, part of w cells population showed abnormal nuclei morphology, including bigger dimension, fragmentation, and irregular contours (**Figures 3B–D**). Finally, the presence of many cells showing diffused DNA not organized in defined nuclei, together with multinucleated cells, indicates possible defects in cell division.

We like to speculate that these warts could represent the signs of age, just like senile warts [seborrheic keratoses (SKs)], the skin lesions that appear in humans around the age of 30 years and increase in number during aging (Parish and Witkowski, 2005).

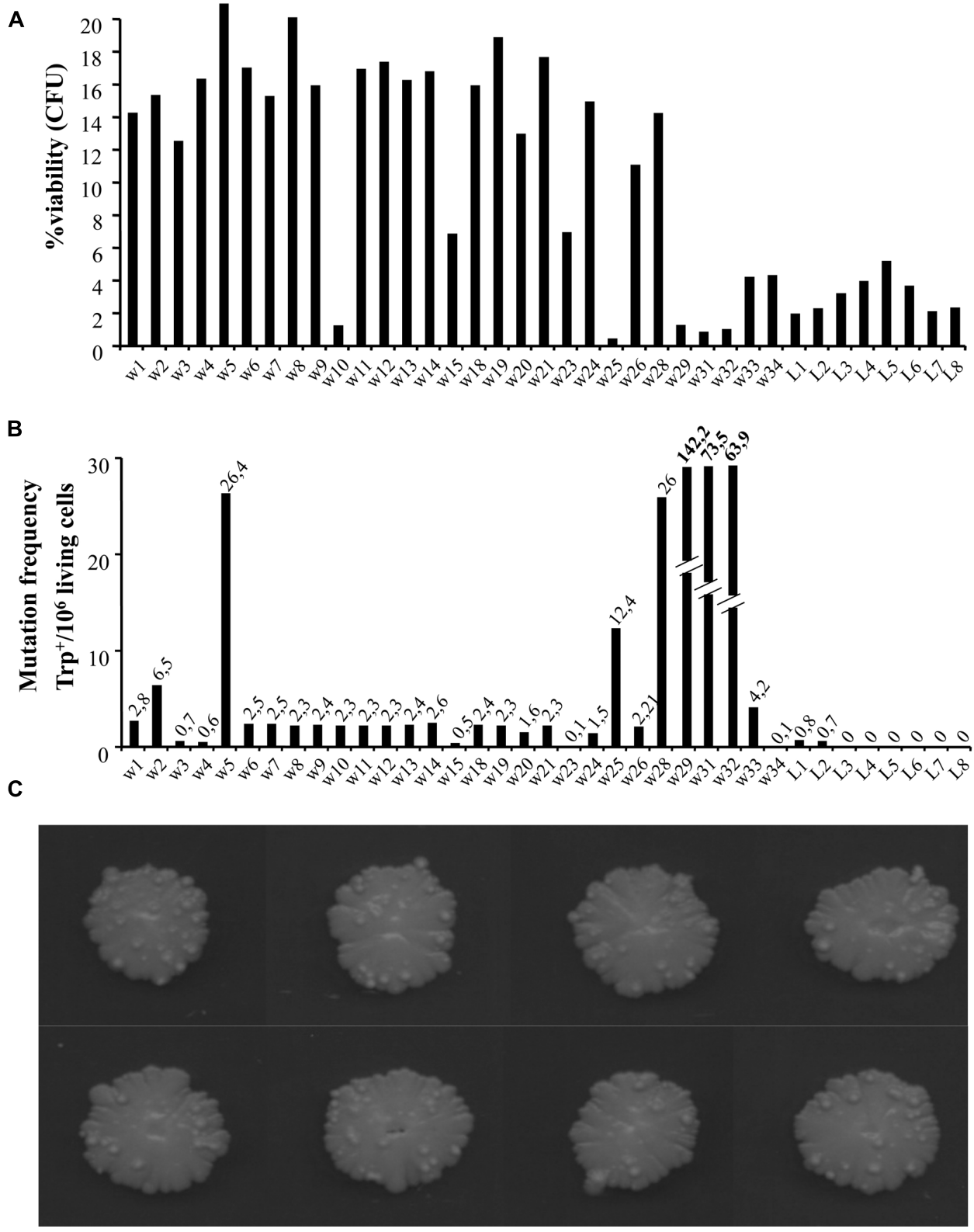

FIGURE 2 | (A) Percentage viability of cells picked from single warts (w) or from the smooth layer (L) of colonies after 100 days of growth [shown in **(C)**]. About 1000 cells for each sample were analyzed for their capability to form microcolonies (Palermo et al., 2007). The viability scale was fixed to 20% to better show the lower values. **(B)** Mutation frequency of the same cells measured as Trp^+ revertants normalized to 10^6 viable cells.

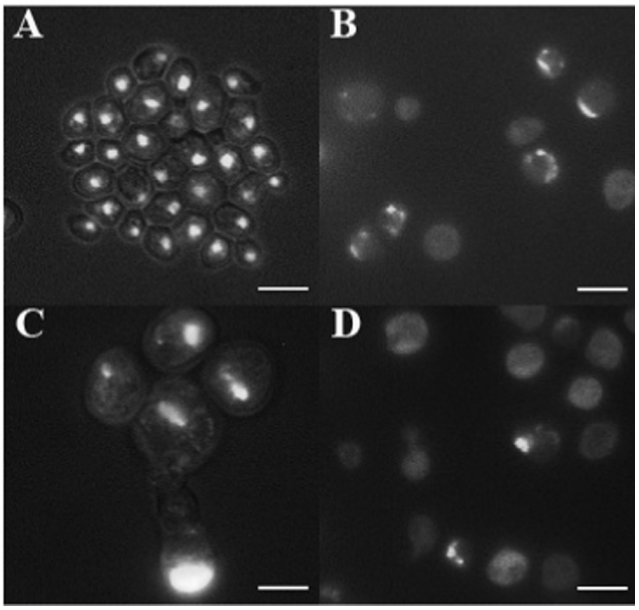

FIGURE 3 | DAPI staining of cells picked after 100 days from the smooth layer of colonies (A) and from three warts (B–D). Cells were fixed with 70% ethanol and stained with DAPI at the concentration of 1 μg/ml, and observed by fluorescence microscopy. Cells are shown at the same magnification. Bar, 10 μm.

Seborrheic keratoses are the most common benign tumors in older individuals and they develop from the proliferation of epidermal cells. Although no specific etiologic factors have been identified, it is known that SK occurs more frequently in sunlight-exposed areas of the body such as the face and the neck (Yeatman et al., 1997). SKs are benign but secondary tumors, and Bowen disease (squamous cell carcinoma *in situ*) or malignant melanoma may occasionally arise within these lesions (Terada, 2010; Böer-Auer et al., 2012).

A similar situation seems to occur in yeast warts, as they can show both normal and cancer-like phenotypes.

Up to now, the patho-mechanisms of SK are not fully understood. Several studies showed that mutations in the fibroblast growth factor receptor 3 (FGFR3) and in the 110 kDa catalytic subunit of the phosphoinositide-3-kinase (PIK3CA) are present in human and mice benign skin tumors (Logie et al., 2005). More recently, some novel insight into the molecular basis of these benign skin lesions came from gene expression analysis study by DNA microarray that identified several upregulated genes, including the oncogenic form ΔNp63 of the transcriptional regulator p63 (Seo et al., 2012).

Recently, a yeast gene related to p63, NDT80, has been identified in controlling the aging process. In fact, this gene is involved in rejuvenilation of yeast cells during sporulation and yeast cells over-expressing this gene are able to double their lifespan (Unal et al., 2011).

We propose the ClLS approach as an additional method to study the onset of cells escaping growth control that, in turn, can be isolated from colonies for further studies, including genome wide analysis.

The treatment of SK human lesions varies from topical application of 5-fluorouracil (5-FU), cryotherapy, electrodessication, curettage to excisional surgery (Park, 2005; Sand et al., 2008).

Yeast has already been successfully used to screen new compound and/or to assess their mechanisms of action (Cassidy-Stone et al., 2008; La Regina et al., 2009; Palermo et al., 2010, 2011, 2012). In this respect, ClLS can provide a useful tool for the screening of molecules that are able to delay and/or reduce the onset of warts in yeast colonies.

In conclusions, we propose that yeast, by means of the regrowth phenotype during CLS and the appearance of warts during ClLS, can be a valuable model to study the formation of cell mass escaping the control of growth and it will be very interesting to study the nature of genes involved in this phenomenon. Moreover, yeast might represent a nice clock to measure the onset of aging phenotypes in humans.

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Acetic acid treatment in *S. cerevisiae* creates significant energy deficiency and nutrient starvation that is dependent on the activity of the mitochondrial transcriptional complex Hap2-3-4-5

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Metabolic pathways play an indispensable role in supplying cellular systems with energy and molecular building blocks for growth, maintenance and repair and are tightly linked with lifespan and systems stability of cells. For optimal growth and survival cells rapidly adopt to environmental changes. Accumulation of acetic acid in stationary phase budding yeast cultures is considered to be a primary mechanism of chronological aging and induction of apoptosis in yeast, which has prompted us to investigate the dependence of acetic acid toxicity on extracellular conditions in a systematic manner. Using an automated computer controlled assay system, we investigated and model the dynamic interconnection of biomass yield- and growth rate-dependence on extracellular glucose concentration, pH conditions and acetic acid concentration. Our results show that toxic concentrations of acetic acid inhibit glucose consumption and reduce ethanol production. In absence of carbohydrates uptake, cells initiate synthesis of storage carbohydrates, trehalose and glycogen, and upregulate gluconeogenesis. Accumulation of trehalose and glycogen, and induction of gluconeogenesis depends on mitochondrial activity, investigated by depletion of the Hap2-3-4-5 complex. Analyzing the activity of glycolytic enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pyruvate kinase (PYK), and glucose-6-phosphate dehydrogenase (G6PDH) we found that while high acetic acid concentration increased their activity, lower acetic acids concentrations significantly inhibited these enzymes. With this study we determined growth and functional adjustment of metabolism to acetic acid accumulation in a complex range of extracellular conditions. Our results show that substantial acidification of the intracellular environment, resulting from accumulation of dissociated acetic acid in the cytosol, is required for acetic acid toxicity, which creates a state of energy deficiency and nutrient starvation.

Keywords: growth dynamic, response surface modeling, automated assay, acetic acid, Hap4p, intracellular pH, metabolic control

INTRODUCTION

Basic metabolic pathways provide energy and molecular building blocks required for growth, maintenance and repair. Changes in the metabolome correlate with declining functions with age. Network analysis of metabolism can help to determine how failures in metabolic control, required to maintain stability and homeostasis within living systems, can lead to senescence and aging.

Acetic acid is a normal end product of alcoholic fermentation in *S. cerevisiae* that cannot be metabolized by glucose-repressed yeast cells. In undissociated form acetic acid is freely membrane permeable and enters the cell by simple diffusion. At higher extracellular pH acetic acid will dissociate to the acetate anion, a form that is relatively membrane-impairment. Acetic acid was also shown to induce apoptosis in yeast cells involving release of cytochrom c in a mitochondria dependent apoptotic pathway (Ludovico et al., 2002; Pereira et al., 2007). Almeida et al. (2009) presented that induction of apoptosis triggered by acetic acid is accompanied by severe amino acids starvation and activation of the TOR signaling pathway. Acetic acid was also identified as a cell-extrinsic mediator of cell death during chronological aging in *S. cerevisiae* (Burtner et al., 2009).

These observations suggest that the cellular response to acetic acid and the induction of apoptosis could strongly depend on specific combinations of extracellular conditions, like medium pH or glucose availability. To investigate how various combinations of extracellular factors and metabolic activity modulate the biological response of yeast cells to acetic acid treatment,

we performed a large scale study using a computer-controlled robot-system together with a mathematical algorithm for experimental planning to appropriately cover the full space of multiple culture/medium parameters by automated variation of experimental conditions (Bonowski et al., 2010). Experiments on the robot systems are controlled by R scripts using object-oriented descriptions of experimental parameters. Thus, providing only a small set of defined stock solutions, yeast growth is automatically analyzed in a multidimensional parameter space. By covering the whole range of all possible combinations, we try to avoid a bias in experimental conditions that could influence the experimental results (Kovárová-Kovar and Egli, 1998; Narendranath and Power, 2005). The resulting datasets of growth kinetics depending on the variable parameters are visualized using multidimensional regression methods and response surface modeling that facilitate the determination of the optimum values for the factors under investigation. Using this automated procedure we provide a detailed model for the dynamic dependence of biomass yield and growth rate on extracellular glucose concentration, pH conditions and acetic acid concentration. Further investigation revealed that accumulation of acetic acid in the cytosol results in inhibition of the respiratory chain and ceased uptake of carbohydrates creating significant energy deficiency and nutrient starvation. Acetic acid also directly influenced the activity of key metabolic enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pyruvate kinase (PYK), and glucose-6-phosphate dehydrogenase (G6PDH).

MATERIALS AND METHODS

STRAINS AND CULTURING CONDITIONS

The yeast strains used in this study are: FF 18984 (*MATa leu2-3,112 ura3-52, lys2-1, his7-1*) and Δ*hap4* (*MATa leu2-3,112 ura3-52, lys2-1, his7-1;hap4::KanMX4)*. Yeast cells were grown in rich (YPD) medium containing 10 g l^{-1} Yeast Extract, 20 g l^{-1} Bacto peptone, and different glucose concentrations (as indicated; 20, 10, 5 g, or 2.5 g l^{-1}). Cell were grown over night at 30° with agitation, collected by centrifugation at 3500 rpm for 5 min, suspended in water and used as a cell stock for the assays.

GROWTH DYNAMIC EXPERIMENTAL SETUP

Experiments were carried out by a Tecan Genesis RSP 150 robot and a Tecan Ultra II plate reader. The system is controlled by a set of R packages developed in our lab that provide a general experimental framework for fluid mixture based experiments. The program packages enable automated variation of experimental conditions by generating tables of concentrations of each assay component. Pipetting volumes are calculated from these concentrations and all pipetting and measurement steps are executed automatically. The robot pipettes all components into 96 U-shaped well plates and growth is automatically analyzed in a plate reader controlled by a modified version of the XFluor Excel macros. The protocols allow fully automated configuration, execution and export of measurement data without user-interactions [for more details see Bonowski et al. (2010)].

Medium stock solutions were defined as fixed components (4xYP-yeast extract/peptone/dextrose medium (YPD) and variable components (glucose, pH, and acetic acid). The parameter space for the variable medium components was specified in terms of concentrations and pH values and a *space-filling design* was used to cover it with measurement points, 48 conditions for each strain. The pH was controlled by combination of two buffers adjusted to different pH values (50 mM citrate/phosphate buffer). A spline fit method was applied to convert between buffer fractions and pH values. Medium components were pipetted first by multi-pipetting mode. Yeast cells in water were added to a final concentration of 0.2 OD_{600} and growth kinetics were measured at 620 nm for 114 cycles at an interval of 10 min. Reader thermostat was adjusted to 30°C.

QUANTITATIVE ASSESSMENT OF GLUCOSE, GLYCOGEN, AND TREHALOSE CONTENT

In order to quantify glucose consumption and glycogen and trehalose accumulation, culture supernatant and cell pellet were collected at indicated time points. The procedure was performed as described previously (Parrou and Francois, 1997). Briefly, the cell pellet (collected from 20 OD_{600} units of culture) was suspended in 250 µl 0.25 M Na_2CO_3 and heated at 95°C for 4 h with occasional stirring. The suspension was adjusted to pH 5.2 with 150 µl 1 M acetic acid and 600 µl 0.2 M sodium acetate buffer, pH 5.2. Half of this mixture was incubated overnight at 57°C with continuous shaking on a rotary shaker in the presence of 100 µg of α-amyloglucosidase from *Aspergillus niger* (Sigma). The second half of the mixture was incubated overnight at 37°C in the presence of 3 mU trehalase (Sigma). The glucose released from glycogen and trehalose digestion as well the glucose content in the medium were determined with the glucose oxidase/peroxidase method (Cramp, 1967).

MONITORING OF OXYGEN CONSUMPTION

Oxygen consumption was monitored in OxoPlate® (PreSens; Germany) covered with a breathable membrane (Diversified Biotech, USA) in 150 µl volumes containing the indicated medium conditions and 0.1 OD_{600} units of cells. The plates were prepared using our computer-controlled automated experimental design. The signal of the oxygen fluorescence sensor and the optical density of the culture at 600 nm were measured continuously during indicated times with intervals of 10 min. The calibration of the fluorescence reader was performed using a two-point calibration curve with oxygen-free water (80 mM Na_2SO_3) and air-saturated water. Partial pressure of oxygen was calculated from the calibration curve.

PREPARATION OF CELL-FREE EXTRACTS AND ENZYME ASSAYS

All procedures were carried out at 0–4°C. Crude extracts were prepared from 20 OD_{600} units of cells with 1 g glass beads (0.4–0.5 mm diameter) in 0.5 ml 20 mM Hepes, pH 7.1, 100 mM KCl, 5 mM MgCl2, 1 mM EDTA, and 1 mM DTT. Samples were vortexed (3 × 5 min with cooling on ice in between) in Mixer Mill MM 300 (Retsch). After centrifugation at 16,000 g for 15 min at 4°C, the supernatants were immediately used for enzymatic assays. Protein content was determined by the method of Bradford (1976). All chemicals and enzymes for enzymatic assays were purchased from Sigma.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12), pyruvate kinase (PYK; EC 2.7.1.40), and

glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activity were measured at 30°C by measuring NADH consumption or NADPH production, using a spectrophotometric assays as described earlier (Kitanovic et al., 2009).

Fructose 1,6 bisphosphatase (FBP1; EC 3.1.3.11) activity was measured at 30°C by coupling reactions of FBP1, phosphoglucoisomerase and G6PDH and measuring NADPH production, using a spectrophotometric assay. The reaction was made in 0.15 ml containing 100 mM Imidazole, pH 7.1, 100 mM KCl, 5 mM $MgSO_4$, 5 mM EDTA, 0.7 mM $NADP^+$, 3.4 mM glucose-6-phosphate, and 10 units of phosphoglucoisomerase and G6PDH (EC 5.3.1.9 and EC 1.1.1.49, respectively). The reaction was initiated by the addition of crude extract and increase of absorbance at 340 nm was monitored.

Malate dehydrogenase (MDH; EC 1.1.1.37) activity was measured at 30°C by monitoring NADH consumption, using a spectrophotometric assay. The reaction was made in 0.15 ml containing 50 mM Tris/MES, pH 7.4, 200 mM KCl, 10 mM $MgCl_2$, 0.625 mM NADH, and 0.5 mM oxaloacetate. The reaction was initiated by the addition of crude extract and decrease of absorbance at 340 nm was monitored.

Isocitrate dehydrogenase (IDH; EC 1.1.1.41) activity was measured at 30°C by monitoring NADH production, using a spectrophotometric assay. The reaction was made in 0.15 ml containing 50 mM Tris/MES, pH 7.5, 100 mM KCl, 12 mM $MgCl_2$, 0.625 mM NADH, and 8 mM isocitrate. The reaction was initiated by the addition of crude extract and decrease of absorbance at 340 nm was monitored.

MONITORING OF INTRACELLULAR pH

For cytosolic expression of ratiometric pHluorin we used pHluorin in an expression plasmid with a strong constitutive ADH1 promoter, kindly provided by Tobias Dick (Heidelberg) (Braun et al., 2010). For estimating the calibration curve, the cells were resuspended in a series of 50 mM citrate-phosphate calibration buffers of defined pH, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 (supplements). Cells containing pHluorin reporter plasmid were grown in 260 µl low fluorescence minimal F1 medium (Kitanovic and Wölfl, 2006) at 30°C in Tecan Ultra microplate reader (Tecan). Kinetic parameters were measured at 390/510 and 480/510 nm Ex/Em. According to the calibration curve, we then calculated the internal pH values (pHin) from fluorescence ratios measured in the experimental part. Samples were measured in triplicates; the data shown represents one of three independent experiments.

DATA ANALYSIS

Multivariate response surface modeling

The large multidimensional datasets generated by our experimental framework require new approaches of explorative data-analysis. To visualize the response of yeast to changes in medium composition, we use 2D slices of multivariate response surface models (RSMs).

Classical RSMs based on low order polynomials have a long and successful history as a tool for optimizing biotechnological processes (Popa et al., 2007; Fereidouni et al., 2009; Singh et al., 2009). The main advantages of these models are that they are mathematically simple, easy to optimize and provide a method to improve processes without having to carry out a large number of measurements. Unfortunately, fitting a fixed functional form to a dataset introduces a massive bias and is unlikely to yield an accurate description of a complex non-linear system (Jones, 2001), making them unsuitable as an explorative tool.

Non-parametric and semi-parametric regression techniques like Gaussian Random Process Regression (GRPR) can help to avoid these shortcomings (Cressie, 1993). The assumptions behind GRPR are much more general than those behind classical RSMs, giving them much more flexibility to fit the data in a less biased fashion. In this work, we show how the combination of automated experimenting and visualization of datasets using GRPR can help to get an intuitive understanding of the combined quantitative influence of multiple factors on the growth dynamic of yeast cultures.

Yeast growth in a liquid batch culture was expressed as exponential rate constant $\mu(t)$ where:

$$\mathrm{dOD(t)/dt} = \mu(t) \cdot \mathrm{OD(t)} \tag{1}$$

In order to define growth kinetic over time a piecewise linear regression of log (OD) was determined:

$$\begin{aligned} \mathrm{OD(t)} &= \mathrm{OD(t_0)} \cdot e^{\mu(t) * t} \\ &\Rightarrow \ln(\mathrm{OD(t)}) = \ln(\mathrm{OD(t_0)}) + t \cdot \mu(t) \end{aligned} \tag{2}$$

We developed an extension to the GRPR implemented in the *fields* R package for visualizing slices of multidimensional datasets and obtaining non-parametric surrogate models of experimental systems. The *fields* implementation uses generalized cross validation to obtain an estimation of the noise-level of the data and find optimal smoothing parameter (Marcotte, 1995). Our implementation also performs an optimization of the length-scale parameter of the covariance function in all dimensions of the model using the Nelder-Mead method as implemented in the R function *optim* with cross-validation error of *fields* as the objective function, and allows scaling of the axes with arbitrary functions that reflect *a-priori* assumptions about the sensitivity of the system to parameter changes in different regions of the parameter space. Certain axes are switched to logarithmic scaling, which is useful for many biochemical systems that show a large variability at low concentrations (Bonowski et al., 2010).

GRPR has been used extensively in the field of geostatistics, where it is commonly called Kriging (Cressie, 1993). A Gaussian process is a collection of random variables, any finite number of which have a joint Gaussian distribution (Rasmussen and Williams, 2006). In our setting, each observation at a point x_i in the parameter space corresponds to one random variable of the Gaussian process. In GRPR, one assumes that the covariance of different observations is a function $k(x_i, x_j)$ of their locations in the parameter space. The joint distribution $f(X)$ of observations at a set X of parameter combinations is given by

$$f(X) \sim N\left(\mu, \sum = K(X)\right) \tag{3}$$

where N is a multivariate normal distribution and the entries $K_{ij} = k(x_i, x_j)$ of the covariance matrix are given by the covariance function.

Predictions $f(X^*)$ for unknown parameter combinations X^* can be derived from the joint distribution with observations from measurements at locations X as described in Cressie (1993). The choice of a covariance function $k(x_i, x_j)$ provides a way to influence on the properties of the resulting RSM.

Our models are based on a stationary covariance function of the "Gaussian" type that is one of the standard choices for a smooth covariance function (Cressie, 1993):

$$k(x_i, x_j) = \sigma_f^2 \exp\left(-\frac{1}{2l^2}(x_i - x_j)^2\right) + \sigma_n^2 \delta_{ij} \quad (4)$$

In this formula, σ_f describes the strength of correlation between measurements with similar parameters, l describes the range of the correlation, σ_n describes the measurement noise, and δ_{ij} is 1 if $i = j$ and 0 otherwise.

Quantifying of survival and growth conditions influence

To investigate the influence of complex combination of extracellular conditions on yeast growth kinetic we used the numeric calculation of the OD integral and the O2 integral. The integral was defined as the area under the growth curve or oxygen saturation curve:

$$\text{OD integral}(\text{OD}, \text{t}) = \sum_{i=1}^{n}\left(\frac{1}{2} * (\text{OD}_i + \text{OD}_{i-1}) * (\text{t}_i - \text{t}_{i-1})\right)$$

where n is the total number of discrete time points in the measurement, and in the case of O2 integral the oxygen saturation of medium was used instead the OD. By using non-parametric GRPR we defined multidimensional model of the OD integral and O2 integral dependence on quantitative influence of multiple extracellular factors. From the integral dependence on acetic acid concentration we defined the EC50 for the growth or mitochondria inhibition as the amount of acetic acid needed for 50% of growth or mitochondria respiratory inhibition:

$$EC_{50}(\text{OD integral}) := \text{c(acetic acid)}$$

with

$$\text{OD integral}(\text{c(acetic acid)}) = \frac{\max[\text{OD integral}] - \min[\text{OD integral}]}{2}$$

where in the case of EC50 for mitochondria inhibition the O2 integral was used.

To estimate relative growth or mitochondria viability we calculated the area below curves that represent OD or O2 integral (calculated from GRPR) dependence on acetic acid concentration and defined these areas as OD toxicity or O2 toxicity integrals. These integrals were further normalized to the highest values for each investigated strain and the resulted normalized parameters were defined as relative growth viability or relative mitochondria viability:

$$\text{viability}(\text{OD integral}, \text{c(acetic acid)}) = \sum_{i=1}^{n}\left(\frac{1}{2} * (\text{OD integral}_i + \text{OD integral}_{i-1}) * (\text{c(acetic acid)}_i - \text{c(acetic acid)}_{i-1})\right)$$

where n is the total number of grid points in the multidimensional interpolation space, and in the case of O2 integral the oxygen saturation of medium was used instead the OD.

RESULTS

DEPENDENCE OF ACETIC ACID TOXICITY ON THE EXTRACELLULAR pH CONDITIONS

To study dependence of acetic acid toxicity on the extracellular pH and glucose concentration we performed an automated multifactorial experiment in which the multidimensional experimental space is generated by an algorithm that sequentially adds parameter combinations maximizing the difference to data points already in the experimental design set. The resulting space filling experimental design covers the entire feasible parameter space evenly. By using a log-scaled axis for glucose concentration, the data points in our experimental design provide a dense coverage of the lower concentration range. The calculation of growth kinetic is performed by piece-wise linear regression of log (OD) and the maximal growth rate and maximal biomass yield are determined for each measured set of conditions. The obtained results are used to develop a RSM of growth dependence on extracellular glucose concentration and pH condition by GRPR (see Materials and Methods).

RSMs of cell growth dynamics confirmed a strong shift of optimal pH conditions from low pH values toward higher ones in presence of increasing acetic acid concentrations (**Figure1**). Lack of Hap4p resulted in a slightly reduced growth rate (**Figure 1A**) and significantly reduced biomass yield (data not shown). Although, the growth of yeast cells under high glucose concentration does not require mitochondrial activity, our results indicate that functional mitochondria and the transcriptional complex Hap 2-3-4-5 are indispensable for optimal growth rates and biomass production. Upon treatment with acetic acid survival is facilitated in conditions that favor the dissociation of acetic acid, e.g., higher pH environment. However, dissociation to acetate anion and hydrogen ion leads to acidification of the extracellular milieu. If the buffering capacity of the medium (50 mM citrate/phosphate buffer) is exceeded by high acetic acid concentrations a considerable amount of acetic acid will be present in undissociated form and able to enter the cell. Therefore, the treatment with very high concentrations of acetic acid will cause significant growth reduction even in high extracellular pH conditions (**Figure 1**).

The response of Δ*hap4* mutant to acetic acid treatment was significantly different from the response of wild-type cells. Surprisingly, Δ*hap4* mutant cells could survive in much lower

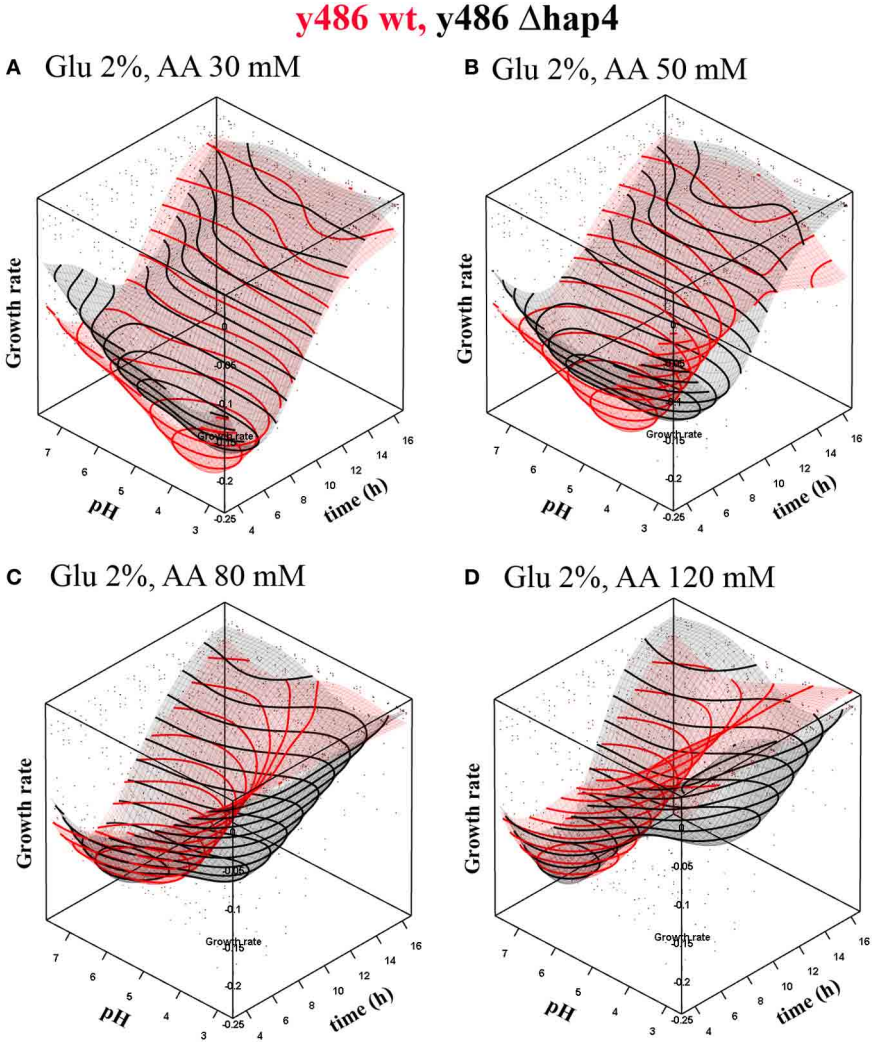

FIGURE 1 | Dependence of growth rate on pH conditions in YPD medium upon treatment with (A) 30 mM or (B) 50 mM, (C) 80 mM and (D) 120 mM acetic acid and constant 2% glucose concentration. The graphs represent interpolated response surface models of growth rates (μ) over time in dependence on pH conditions for wild-type (red surface) and hap4 mutant cells (black surface). Note: inverted growth scale; the best growth is in the valley. Individual data point in the graph represent single measurements that were base for the interpolation. As the interpolation surface presented in the graph was based on the standard glucose concentration, data points for other glucose concentration conditions lay outside the interpolated surface.

extracellular pH conditions (**Figure 1**). The maximal growth rate of the Δ*hap4* mutant is reached at a later time point of cultivation if cells are grown at lower pH. In contrast, wild-type yeast showed a complete growth inhibition under these conditions. At higher acetic acid concentration (120 mM; **Figure 1D**) growth rate was also reduced in Δ*hap4* cells.

Presence of acetic acid in growth medium appeared to be toxic for the mitochondria respiratory activity as well (**Figure 2**). Based on the measurements of oxygen consumption of cells grown in various combinations of extracellular conditions (see Materials and Methods) we developed a RSM of respiratory activity in dependence of acetic acid, glucose concentration, and extracellular pH. The O_2 integral was defined as the area under the oxygen saturation curve over time during the experiment. These values were used for computing the RSM of the acetic acid inhibition of respiratory activity. The inhibition of respiration was clearly concentration and pH dependent (**Figure 2**).

The ability of acetic acid to inhibit cell growth or mitochondria activity was characterized as "relative growth viability" or "relative mitochondria viability" by using non-parametric GRPR we defined multidimensional RSM of the OD-integral and O_2-integral dependence on multiple extracellular factors (see Materials and Methods). From these models, we calculated the area below curves representing OD- or O_2-integral dependence on acetic acid concentration calculated from RSM for various pH and glucose concentrations in the medium as OD toxicity or O_2 toxicity integrals. These integrals were further normalized to the highest values of each strain investigated to obtain normalized values that we defined as "relative growth viability" or "relative mitochondria viability." With this computational processing we

obtained numerical values describing the cellular sensitivity to the complex combination of environmental factors that can be presented in 2D graphs with one variable. Our results clearly show the highest toxicity of acetic acid (growth and respiration) in low pH conditions and lower sensitivity of the Δ*hap4* mutant (**Figure 3**). At higher pH conditions the Δ*hap4* mutant exhibited reduced mitochondrial respiratory activity. Interestingly, inhibition of respiration by acetic acid was comparable between wild-type and Δ*hap4* mutant suggesting that further metabolic alterations, differently regulated in those strains, are responsible for the increased resistance of the mutant strain to acetic acid (**Figure 3B**).

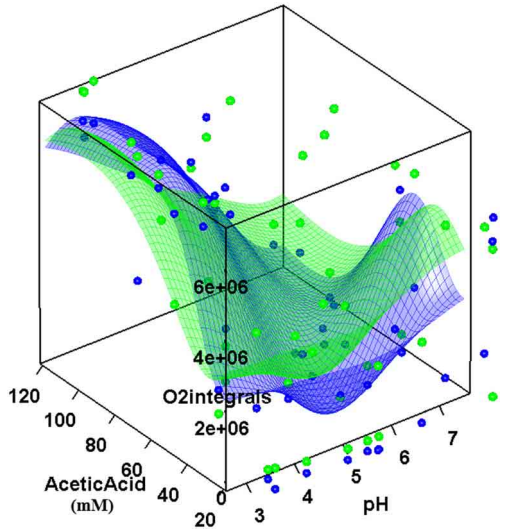

FIGURE 2 | Respiratory activity dependence on acetic acid concentration and extracellular pH. The graphs represent interpolated response surface models (RSM) of O_2 integrals, defined as the area under the oxygen saturation curve, in dependence on pH and acetic acid concentrations for wild-type (blue surface) and hap4 mutant cells (green surface) at a standard glucose concentration in the medium (2%). Note: high integral represents low oxygen consumption and respiratory inhibition; the best respiratory activity is in the valley. Individual data points in the graph represent single measurements that were the basis for the interpolation. The interpolation surface presented in the graph is based on one glucose concentration, and the data points for other glucose concentrations lay outside the interpolated surface.

INCREASED GLUCOSE CONCENTRATION PROTECTS CELLS AGAINST ACETIC ACID TOXICITY

Growth rate of yeast cells at pH 3.0, in the presence of very low, non-toxic concentrations (30 mM) of acetic acid, progressively increased over time, reaching a maximum after about 5 h of cultivation (**Figure 4A**). After this time point growth rate was decreasing as nutrient supply was depleted and metabolic by-products accumulated. Increasing glucose concentration (up to 2%) had a stimulating effect on the growth rate. The optimum glucose concentration under these conditions was between 2% and 4%. Both, wild-type and Δ*hap4* mutant, showed a similar growth dependence on glucose in the medium. Interestingly, when acetic acid concentration was increased to 50 mM (**Figure 4B**), the optimum glucose concentration also increased, with the optimum being above 3.5%. In Δ*hap4* cells, however, acetic acid did not trigger a shift in optimal glucose levels in the medium, although, the toxic influence of acetic acid, observed as decreased growth rate and delayed time when maximal growth rate is reached, was similar in both strains.

To quantify the protective effect of increased extracellular glucose concentrations on the cellular sensitivity to acetic acid we used the interpolated RSM of the OD integral and O_2 integral to visualize the influence of multiple extracellular factors. From the integral of the dependence on acetic acid concentration we defined the specific EC50 as the amount of acetic acid needed for 50% of growth or mitochondria respiratory inhibition. As mentioned before, we also calculated the area below curves that represent OD or O_2 integral dependence on acetic

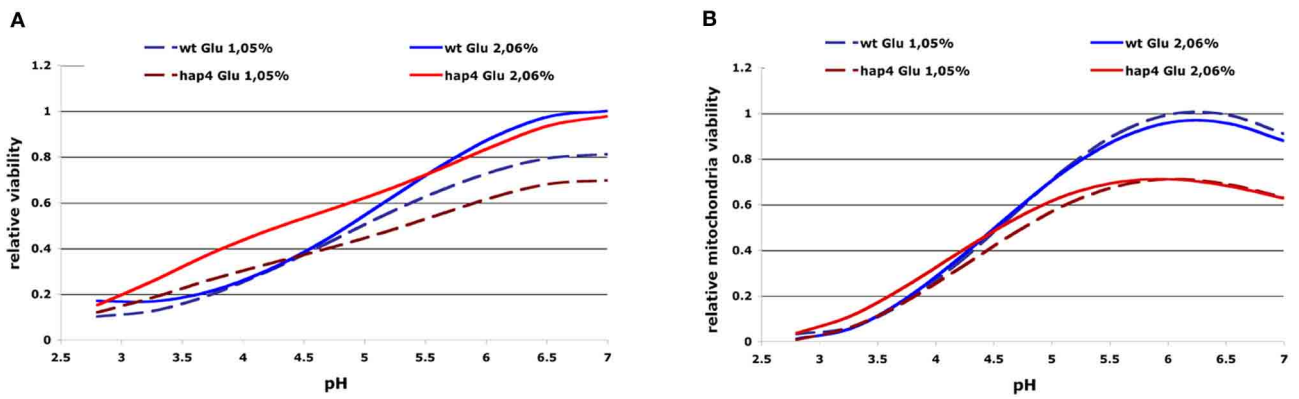

FIGURE 3 | 2D presentation of calculated "relative growth viability" (A) and "relative mitochondrial viability" (B) upon acetic acid treatments in dependence on extracellular pH and glucose concentration. The curves represent the OD/O_2-integral dependence on acetic acid concentration calculated from RSM for the whole range of pH values (x-axis) and were normalized to the highest values for each investigated strain. Results for selected glucose concentrations and strains are presented as indicated. The resulting normalized values were defined as "relative growth viability" and "relative mitochondria viability" (see Materials and Methods). Sensitivity to acetic acid is clearly reduced in the hap4 mutant at lower pH, while impaired mitochondrial activity of the Δ*hap4* mutant can be seen at higher pH.

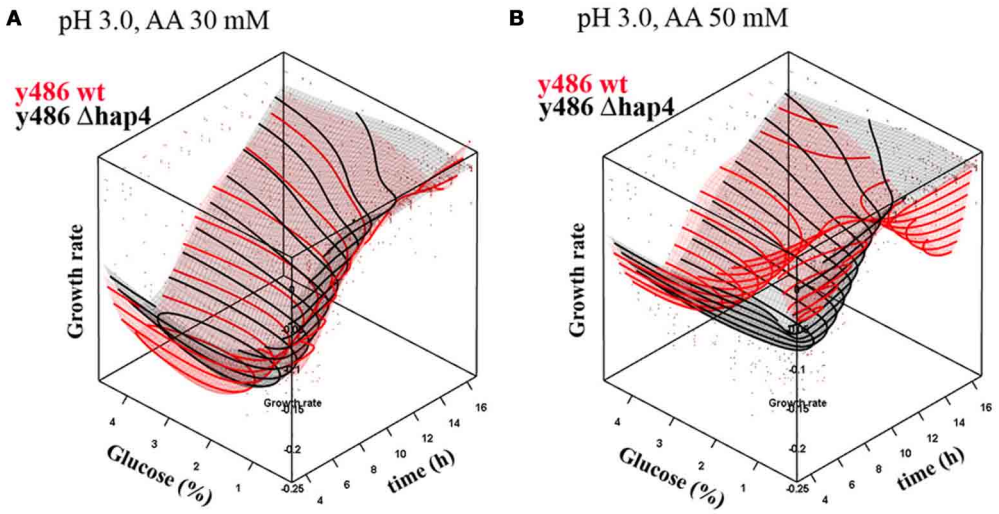

FIGURE 4 | Dependence of growth rate on glucose concentration in YPD medium upon treatment with (A) 30 mM or (B) 50 mM acetic acid (AA) at pH 3.0. The graphs represent response surface models of growth rates (μ) over time in dependence on glucose concentration for wild-type (red surface) and hap4 mutant cells (black surface). Note: inverted growth scale; the best growth is in the valley. Individual data points in the graph represent single measurements that were used for the interpolation. The interpolation surface presented in the graph is based on one pH (pH 3.0), and data points for the other pH conditions lay outside the interpolated surface.

acid concentration calculated from RSM for various pH and glucose concentrations in the medium and defined these areas as OD toxicity or O_2 toxicity integrals. As before, results were normalized to the highest values for each investigated strain and the resulting normalized parameters were again defined as "relative growth viability" or "relative mitochondria viability." The results show that increasing extracellular glucose concentrations resulted in an increased EC50 for growth inhibition and inhibition of mitochondrial respiration in both investigated strains (**Figures 5A,C**). The data also indicate a steady increase of EC 50 values for both, growth and mitochondria inhibition, with increased extracellular pH (**Figures 5A,C**). These results were also reflected in an increase of "relative growth viability" at high extracellular glucose or pH conditions (**Figure 5B**). However, "relative mitochondria viability" was decreased at elevated glucose concentrations in the medium as a result of decreased aerobic and increased fermentative metabolism (**Figure 5D**).

EFFECT OF ACETIC ACID ON GLUCOSE UPTAKE AND CARBOHYDRATE STORAGE

Glucose-repressed cells are impermeable to the anion (Cássio et al., 1987) and only the undissociated form of acetic acid is able to enter the cells by simple diffusion. Acetic acid affects the transport of glucose by acting on the transport proteins as uncoupler dissipating ΔpH and membrane potential (Sousa et al., 1995). Our results show that acetic acid significantly inhibited mitochondria respiration (**Figure 2**). The inhibitory effect on mitochondria can be explained as a result of respiratory chain uncoupling and an impinging effect on the $\Delta\Psi$.

In order to test whether glucose consumption directly depends on the concentration of acetic acid we performed time-course measurements of glucose concentration in presence of increasing concentrations of acetic acid. The results confirmed an acetic acid concentration-dependent inhibition of glucose uptake (**Figure 6A**). Surprisingly, the inhibitory effect of acetic acid observed was much lower in the Δhap4 mutant. Although reduced, glucose consumption was still detected in Δhap4 mutant treated with 50 mM acetic acid. In contrast, wild-type cells treated with the same acetic acid concentration completely ceased glucose consumption already after 2 h of treatment (**Figure 6A**). Moreover, the inhibition of growth by acetic acid was reciprocally proportional to the inhibition of glucose consumption (**Figure 6B**). Thus, sustained glucose uptake in Δhap4 mutant supported cell growth even in the presence of 50 mM acetic acid.

Respiratory deficient cells exhibit high level of glycogen and trehalose accumulation (Enjalbert et al., 2000; Kitanovic et al., 2009). In general, various types of stress, like nutrient starvation, heat-shock, or oxidative stress, induce futile cycling of storage carbohydrates, trehalose and glycogen, meaning that both biosynthesis and biodegradation pathways are activated almost to the same extend (Parrou and Francois, 1997). Glycogen and preferentially trehalose, serve as a fuel reserve that enable yeast cells to survive starvation. Upon return to favorable conditions or upon defeating stress conditions these storage carbohydrates can be quickly mobilized to help fuel growth (Shi et al., 2010). However, in conditions where glucose uptake is inhibited by acetic acid, increased synthesis of glycogen and trehalose would create significant energy deficiency and dissipation of important metabolic intermediates that could be used in catabolic reactions to sustain sufficient levels of ATP within cells. To test this hypothesis, we measured glycogen and trehalose accumulation upon treatment of wild-type and Δhap4 mutant with increasing acetic acid concentrations. The experiments showed that acetic acid treatment caused significant accumulation of both, glycogen and trehalose only in wild-type cells (**Figures 6C,D**). In the Δhap4 mutant

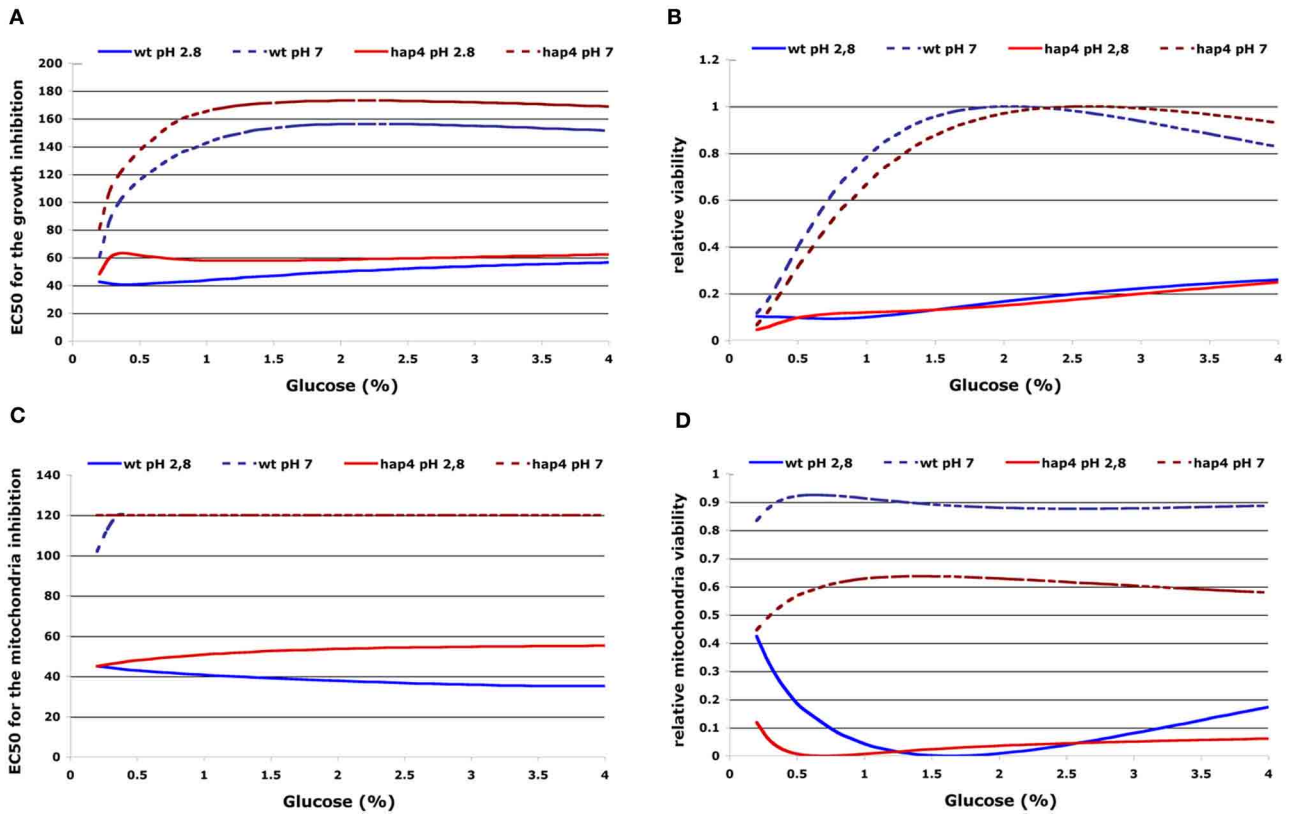

FIGURE 5 | 2D presentation of calculated EC50 for growth inhibition (A) or inhibition of respiration (B) and "relative growth viability" (C) or "relative mitochondria viability" (D) upon acetic acid treatment in dependence on glucose concentration and extracellular pH. The EC50 for growth or mitochondria inhibition was defined by the integral dependence on acetic acid concentration as the amount of acetic acid needed for 50% of growth or mitochondria respiratory inhibition. "Relative growth viability" or "relative mitochondria viability" were defined as the normalized values that represent OD/O_2-integral dependence on acetic acid concentration (see Materials and Methods). Results for selected pH values and strains are presented as indicated.

we detected increased levels of these storage carbohydrates only at the onset of glucose exhaustion, the conditions which normally facilitate their accumulation. A short, transient increase of trehalose and glycogen levels followed by their fast mobilization was observed in Δhap4 mutant only after treatment with very high acetic acid concentrations (80 mM; **Figures 6C,D**).

INTRACELLULAR ACIDIFICATION AND GLYCOLYTIC FLUX UPON ACETIC ACID TREATMENT

Yeast cells are unable to maintain a stable pH gradient across the plasma membrane on starvation (Dechant et al., 2010). It was shown earlier that reduced glycolytic flux upon starvation directly results in significant acidification of the cytosol (Dechant et al., 2010) as a consequence of ATP depletion. For ATPase to accomplish its function of regulating internal pH (pHin), metabolic activity and sustainable level of ATP is required. Therefore, it is to be expected that increased glucose concentration in the medium could help to keep sufficient ATP level for ATPase function and prevent intracellular acidification.

To investigate alterations of intracellular pH upon acetic acid treatment we performed *in vivo* monitoring of cytosolic pH using ratiometric measurements with pHluorin that exhibits pH-dependent dual excitation peaks at 395 and 475 nm (Braun et al., 2010). Cells containing reporter plasmid were grown in minimal SD medium with different glucose concentrations in presence or absence of 40 mM acetic acid. In control culture grown in extracellular pH 3.0 and 2% glucose a fast drop of intracellular pH was observed in the late stationary phase (**Figure 7**). Acetic acid treatment under these conditions resulted in much earlier cytosolic acidification already after 20 h of treatment probably as a consequence of ATP depletion following inhibition of glucose uptake (as shown in **Figure 6A**). Cultivation of cells in 4% glucose medium could prevent intracellular acidification in both control and acetic acid treated cells. In contrast, decrease of glucose concentration in medium resulted in rapid cytosolic acidification at a much earlier time point of cultivation independently of acetic acid. These results confirmed that maintenance of intracellular pH depends on glucose signaling. In conditions of glucose exhaustion in the medium or inhibition of glucose uptake by acetic acid, the intracellular ATP pool becomes limited and—as a consequence—disturbs the pH balance, significantly reducing cytosolic pH.

Dechant et al. (2010) showed that changes in the ATP level directly impinge on cytosolic pH, which acts as a second messenger in response to glucose. A similar mechanism of glucose sensing was described in pancreatic β-cells, in which increasing

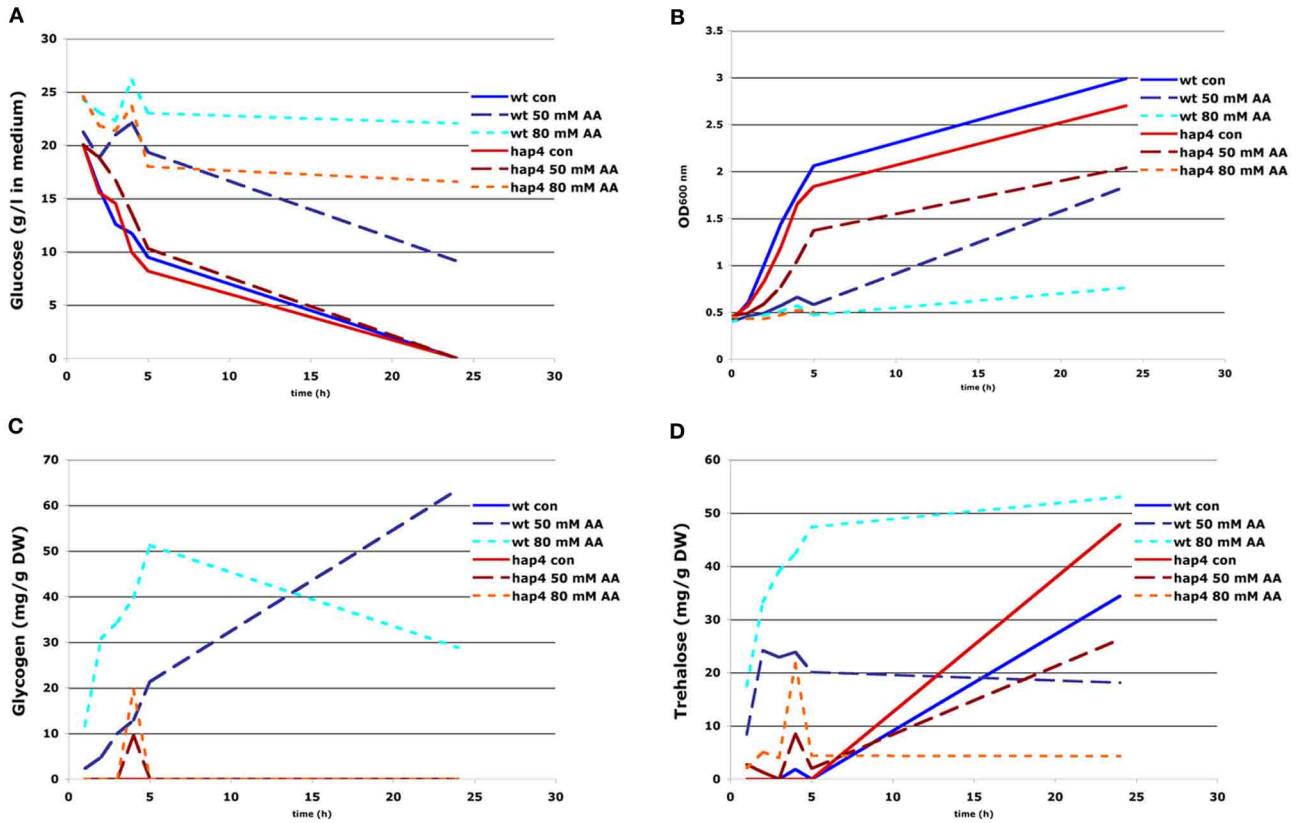

FIGURE 6 | Effect of acetic acid treatment on cell growth, glucose consumption, and accumulation of glycogen and trehalose. FF18984 wild-type and hap4 mutant cells were cultured to mid-log phase (OD_{600} =0.6–1.0) in full (YPD) medium either mock- (control) or acetic acid-treated (AA) with concentration of 30, 50, and 80 mM of AA. Glucose content in the medium **(A)**, optical density of the culture (OD_{600}) **(B)**, and kinetics of glycogen **(C)** and trehalose **(D)** accumulation were monitored during 26 h of cultivation.

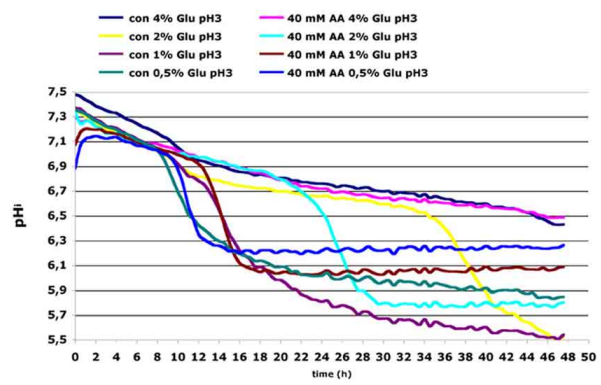

FIGURE 7 | Wild-type (wt) yeast cells transformed with pHluorin containing reporter plasmid were incubated in a low fluorescent F1 medium at pH 3 containing increasing concentrations of glucose (0.5, 1, 2, and 4% Glu). Equal amounts of cells were plated in each well of 96-well microtiter plate with round bottom. Cells were either mock-treated (con) or treated with 40 mM acetic acid. The kinetic of the fluorescence signal (Ex/Em pairs 390/510 and 480/510) was monitored in a plate reader. The intracellular pH was calculated based on a calibration curve that was made with the same cells incubated in phosphate/citrate buffer adjusted to pH between 5 and 8 in presence of 0.16 % digitonin.

ATP concentrations mediate glucose sensing through inactivation of ATP-dependent K^+ channels (MacDonald and Wheeler, 2003). Our results show that trehalose and glycogen were synthesized in acetic acid treated cells even in absence of glucose uptake. Together with the observation of Almeida et al. (2009) that amino acids pools are significantly reduced as a consequence of acetic acid treatment, it can be concluded that acetic acid treatments should lead to gluconeogenesis and consumption of glycolytic and tricarboxilic acid pathway (TCA) intermediates in favor of storage carbohydrate synthesis.

To test this hypothesis we analyzed the activity of glycolytic and TCA pathway enzymes upon treatment with 50 mM acetic acid in wild-type and the Δhap4 mutant. The results obtained (**Figures 8A–C**) show a significant increase in the activity of the glycolytic enzymes PYK, GAPDH, and G6PDH activity in both wild-type and Δhap4 mutant cells. Higher concentration of acetic acid (from 80 mM) significantly inhibited the activity of all three enzymes (data not shown). The activity of the TCA pathway enzymes MDH, and IDH, and the key enzyme in gluconeogenesis, FBP1, however, was elevated only in wild-type cells. The Δhap4 mutant showed significantly reduced activity of those enzymes in both control and acetic acid treated cells.

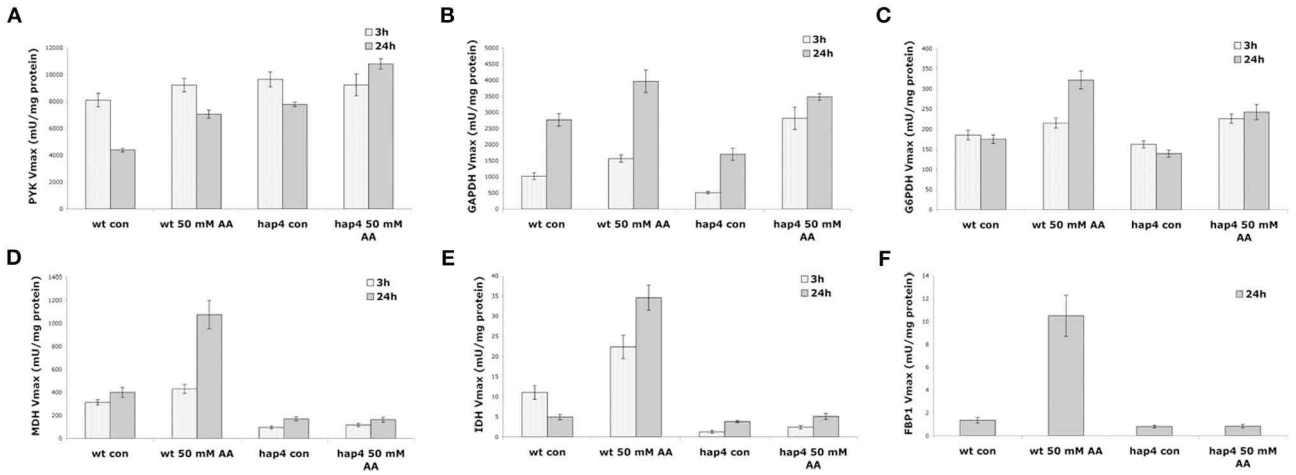

FIGURE 8 | Effect of acetic acid treatment on the activity of metabolic enzymes. Cultures of yeast wild-type (wt) and Δhap4 mutant cells growing exponentially in full (YPD) medium at pH 3 were divided into two batches, one of which was mock-treated (con), the other challenged with 50 mM acetic acid (AA). At the indicated times of incubation aliquots were withdrawn and **(A)** pyruvate kinase (PYK), **(B)** glyceraldehyde-3-phosphate dehydrogenase (GAPDH), **(C)** glucose-6-phosphate dehydrogenase (G6PDH), **(D)** malate dehydrogenase (MDH), **(E)** isocitrate dehydrogenase (IDH), and **(F)** fructose 1,6 bisphosphatase (FBP1) activities were measured from the cellular protein extract. The values are given in mU/mg protein ± SE (U = μmol/min).

The yeast *Saccharomyces cerevisiae* adapts to glucose exhaustion through induced transcription of genes involved in various cellular processes, including gluconeogenesis, the glyoxylate cycle, the tricarboxylic acid (TCA) cycle, respiration, β-oxidation, and utilization or transport of alternative sugars. Enzymes of gluconeogenesis and the glyoxylate cycle are indispensable for growth on non-fermentable carbon sources, such as, ethanol, lactate, or glycerol (Schüller, 2003). Hap4p is a subunit of the Hap2/3/4/5 transcriptional complex, which is involved in the transcriptional regulation of TCA cycle genes, glyoxylate cycle genes and stress response genes (Raghevendran et al., 2006). Hap4p overexpression results in increased growth rates and biomass formation and prolonged life span (Lin et al., 2002). Therefore, decreased toxicity of acetic acid in Δhap4 mutant could be explained by lower inhibition of glucose uptake and, in parallel, decreased activity of gluconeogenesis, TCA cycle, and synthesis of storage carbohydrates. This would result is increased ATP levels that could support the function of membrane ATPases in maintaining intracellular pH homeostasis and prevent amino acids starvation.

DISCUSSION

The accumulation of acetic acid in stationary phase budding yeast cultures is considered to be the primary mechanism of chronological aging in yeast and recent results suggest that the mechanism of acetic acid toxicity in yeast could be related to the induction of growth signaling pathways and oxidative stress (Burhans and Weinberger, 2009; Burtner et al., 2009). Recent publications showed that buffering medium could inhibit the age-dependent accumulation of reactive oxygen species preferentially superoxide anions that is produced by dysfunctional mitochondria (Burhans and Weinberger, 2009; Pan et al., 2011). The accumulation of acetic acid in stationary phase induces oxidative stress, a factor previously implicated in chronological aging of yeast and aging in other organisms as well. Interestingly, the accumulation of acetic acid in stationary phase cultures inhibits growth arrest of cells in G1 and is preferentially toxic to cells that fail to undergo a G1 arrest (Burhans and Weinberger, 2009).

We used a multifactorial experimental design to investigate the impact of acetic acid on cellular growth kinetics in dependence on glucose concentration as well as extracellular pH, covering the whole range of combinations in experimental conditions. Raising the extracellular pH clearly reduced the toxic influence of acetic acid. The accumulation of undissociated acids within the cell is a function of ΔpH and glucose concentration in the medium (Thomas et al., 2002). By raising the pH to a value higher than the pKa of the acid, the concentration of undissociated acid is reduced for a given amount of total acid, placing less stress on cells. Raising the extracellular glucose concentration results in increased intracellular ATP supporting the activity of ATPases (Thomas et al., 2002). Both conditions result in a lower waste of energy for maintenance of the pHin in the range optimal for growth. The outcome is a decreased inhibitory effect of acetic acid on yeast growth and metabolism.

The protecting effect of high glucose concentrations observed in our experiments could be explained by several mechanisms. Acetic acid can enter the cells only in its undissociated, uncharged form. The charged acetate anion is generally considered as non-toxic (Piper et al., 2001). A higher pH on the cytosolic side of the membrane can cause a substantial fraction of this acid to dissociate to the anion, a form which is relatively membrane-impermeable and that therefore will accumulate inside the cell resulting in intracellular acidification (Piper et al., 2001).

In budding yeast and many other fungi, intracellular acidification activates highly conserved Ras2 and cAMP-dependent signaling pathways that respond to glucose (Thevelein and de Winde, 1999). Thus, despite the oxidative stress, inhibition of

glycolysis, induction of gluconeogenesis and synthesis of storage carbohydrates, acetic acid treated cells are continuously subjected to growth signals that promote entry into S phase. Constitutive activation of the Ras-cAMP-PKA pathway would also result in a PKA-dependent loss of mitochondrial function (Gourlay and Ayscough, 2006), which requires *HAP4* transcriptional regulation and accumulation of damaged, high ROS producing mitochondria (Leadsham and Gourlay, 2010). The result is a conflicting situation. On one side, inhibition of glucose uptake and mitochondria function by acetic acids will result in significant ATP depletion and disturbed pH homeostasis. Available nutrients are then redirected toward synthesis of storage carbohydrates causing insufficient synthesis of dNTPs and inefficient DNA replication. On the other side, Ras-cAMP-PKA activation will stimulate growth. Together, intracellular acid accumulation seems to trigger an inappropriate growth signal and replication stress, which leads to cell death. Feeding with high glucose concentrations can, therefore, prevent the energetic collapse in mitochondria impaired cells where the glycolytic flux is reduced because of low pH. This also fits with the observation that neutralizing buffering of yeast media could extend chronological life span in yeast cells, implicating that other mechanism than just simple acidification of the environment are involved in acetic acid-induced metabolic alterations and apoptosis induction (Pan et al., 2011).

Reduced sensitivity of the Δhap4 mutant to acetic acid may be explained by impaired expressions of gluconeogenic, glyoxylate, and TCA cycle enzymes, which are regulated by the Hap 2-3-4-5 complex (**Figure 8**). The low gluconeogenic activity in the Δhap4 mutant will prevent the synthesis of storage carbohydrates, trehalose and glycogen, in conditions where glucose uptake is inhibited by acetic acid. Lin et al. (2002) showed that aged cells respond to glucose-deprivation by shifting the metabolism away from glycolysis toward gluconeogenesis and energy storage. In acetic acid induced aging, increased gluconeogenesis and trehalose/glycogen synthesis pathways will compete with amino acids synthesis pathways for the same glycolytic and TCA intermediates. In consequence, this will result in a condition of amino acids starvation and ATP depletion. In contrast, in the Δhap4 mutant the limited available glucose in the cells can be fully used for sustaining the ATP pool and pHin homeostasis and significantly decrease acetic acid toxicity.

Our results clearly show the interdependence between an important metabolic by-product of yeast fermentation, acetic acid, and the efficiency of the cellular metabolism as well as aging of cells. In addition to providing some new insight into the role of extracellular conditions and availability of nutrients on glucose metabolism, respiration, cellular proliferation and aging, which play a central role in different diseases, our results also suggest controlled stress conditions as a means to increase fermentation efficiency. Given the urgent need to optimize the production of fuel ethanol from cellulosic biomass as a more environmental-friendly fossil fuel alternative, our results suggest that investigating the role of metabolic by products and other stress conditions on fermentation could still lead to further optimization.

AUTHOR CONTRIBUTIONS

Ana Kitanovic and Stefan Wölfl planed and designed the project. Felix Bonowski conceived and designed the computational framework, Ana Kitanovic, Florian Heigwer, Igor Kitanovic, and Christin Ungewiss designed and performed experiments, Ana Kitanovic, Florian Heigwer, Peter Ruoff, and Stefan Wölfl modeled experimental results, Ana Kitanovic, Igor Kitanovic, and Stefan Wölfl wrote the manuscript.

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Lack of Ach1 CoA-transferase triggers apoptosis and decreases chronological lifespan in yeast

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ACH1 encodes a mitochondrial enzyme of *Saccharomyces cerevisiae* endowed with CoA-transferase activity. It catalyzes the CoASH transfer from succinyl-CoA to acetate generating acetyl-CoA. It is known that *ACH1* inactivation results in growth defects on media containing acetate as a sole carbon and energy source which are particularly severe at low pH. Here, we show that chronological aging *ach1*Δ cells which accumulate a high amount of extracellular acetic acid display a reduced chronological lifespan. The faster drop of cell survival is completely abrogated by alleviating the acid stress either by a calorie restricted regimen that prevents acetic acid production or by transferring chronologically aging mutant cells to water. Moreover, the short-lived phenotype of *ach1*Δ cells is accompanied by reactive oxygen species accumulation, severe mitochondrial damage, and an early insurgence of apoptosis. A similar pattern of endogenous severe oxidative stress is observed when *ach1*Δ cells are cultured using acetic acid as a carbon source under acidic conditions. On the whole, our data provide further evidence of the role of acetic acid as cell-extrinsic mediator of cell death during chronological aging and highlight a primary role of Ach1 enzymatic activity in acetic acid detoxification which is important for mitochondrial functionality.

Keywords: ***Saccharomyces cerevisiae*, Ach1, acetic acid, mitochondria, chronological aging, apoptosis**

INTRODUCTION

In the single-celled yeast *Saccharomyces cerevisiae*, the replicative and chronological aging paradigms have been described. In the latter, chronological lifespan (CLS) is the mean and maximum survival period of a population of non-dividing cells in postmitotic stationary phase. Viability over time is defined as the ability to resume mitotic growth upon return to rich fresh medium (Fabrizio and Longo, 2003). This growth arrest simulates the postmitotic quiescent state of multicellular organisms.

Yeast cells respond to nutrient scarcity by inducing a series of metabolic, physiological, and morphological changes which mainly increase stress resistance in order to survive starvation (Smets et al., 2010). Moreover, in this context, unfit cells can undergo apoptosis for the benefit of the whole population (Longo et al., 2005; Fabrizio and Longo, 2008). Apoptosis is a highly regulated cellular "suicide" program whose activation can rely on different exogenous or endogenous stimuli (Carmona-Gutierrez et al., 2010). Chronological aging is an example of an endogenous, physiological trigger (Herker et al., 2004), while treatment of yeast cells with a harsh environmental stress, such as acetic acid, is an example of an exogenous one (Ludovico et al., 2001). Acetic acid which is also a by-product of the yeast metabolism and in some settings has been reported to restrict CLS (Burtner et al., 2009; Murakami et al., 2010). In both chronological aging and acetic acid-induced apoptosis, mitochondria play an active and fundamental role (Ludovico et al., 2002; Bonawitz et al., 2006; Aerts et al., 2009; Pan, 2011). In addition, among the different mitochondrial proteins involved in the execution of the acetic acid-induced apoptotic program, Dnm1 has been shown to be also implicated in chronological aging. This protein is required for mitochondrial fission and its lack of function impairs not only mitochondrial apoptotic fragmentation but also increases CLS (Scheckhuber et al., 2007), underlying a connection among mitochondrial dynamics, apoptosis, and aging.

Mitochondria are also important organelles for yeast carbon metabolism and become essential for growth on non-fermentable substrates such as acetate. Acetate metabolism requires acetate activation to acetyl-CoA by acetyl-CoA synthetase isoenzymes, the mitochondrial Acs1, and cytosolic Acs2 which are known as the gluconeogenic and glycolytic isoforms, respectively (Verduyn et al., 1992; van den Berg et al., 1996). Once generated, acetyl-CoA can be used to fuel the glyoxylate and TCA cycles and also for the synthesis of macromolecules which requires active gluconeogenesis (dos Santos et al., 2003). The concentration of cellular acetyl-CoA is primarily controlled by the balance between its synthesis and utilization in the different metabolic pathways.

The mitochondrial enzyme Ach1 was initially assumed to act as acetyl-CoA hydrolase, probably involved in reducing mitochondrial accumulation of acetyl-CoA during growth on acetate to avoid toxic effects (Lee et al., 1990; Buu et al., 2003). However, hydrolysis of a high energy thioester bond has no apparent metabolic advantage since would result in losing two ATP molecules which have been consumed for acetate activation in the ester. Similarly, the ortholog Acu-8 from *Neurospora crassa* had been classified as

an acetyl-CoA hydrolase (Connerton et al., 1992), giving no further possible explanation for the physiological role of this "energy wasting" process. Thus, the existence of such an enzyme has been denoted as a biochemical conundrum (Buu et al., 2003). Successively, the mitochondrial enzyme CoaT from *Aspergillus nidulans*, which is involved in propionyl-CoA detoxification in the presence of acetate, was characterized (Fleck and Brock, 2009). This protein shows a high aminoacidic identity to Ach1 and Acu-8, but displays a CoA-transferase activity being able to transfer the CoASH moiety from propionil-CoA to acetate (Fleck and Brock, 2009). A re-characterization of Ach1 has been performed indicating that this enzyme acts as a CoA-transferase by catalyzing the transfer of the CoASH moiety from succinyl-CoA to acetate. Thus, it could detoxify mitochondria from acetate by an enzymatic reaction which would save one ATP (Fleck and Brock, 2009).

In this work we provide evidence that *ACH1* inactivation severely impairs mitochondrial functions. This influences the acetate metabolism, the CLS which is restricted, and the occurrence of apoptosis.

MATERIALS AND METHODS

YEAST STRAINS AND GROWTH CONDITIONS

All haploid strains with null mutations were generated by PCR-based methods in a W303-1A background (*MAT*a *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*): *ach1*Δ (*ach1*Δ::*KlLEU2*), *yca1*Δ (*yca1*Δ::*URA3*; Bettiga et al., 2004) and *ach1*Δ *yca1*Δ (*ach1*Δ::*KlLEU2 yca1*Δ::*URA3*). The accuracy of gene replacement was verified by PCR with flanking and internal primers. Standard methods were used for DNA manipulation and yeast transformation. Yeast cells were grown in batches at 30˚C in minimal medium (Difco Yeast Nitrogen Base without amino acids, 6.7 g/l), supplemented with 2% w/v or 0.05% w/v (Calorie Restriction, CR) glucose. Auxotrophies were compensated for with a fourfold excess of supplements (Fabrizio et al., 2005). For shift experiments in acetate-containing medium, cells were grown in minimal medium 2% glucose up to exponential phase (10^7 cells/ml), centrifuged, and resuspended in fresh acetate medium. For acetate medium, pre-calculated amounts of 0.2 M acetic acid and 0.2 M sodium acetate solutions were mixed and added to minimal medium to obtain the required pH and molarity. All strains were inoculated at the same cellular density (culture volume no more than 20% of the flask volume) and growth was monitored by determining cell number using a Coulter Counter-Particle Count and Size Analyser, as described (Vanoni et al., 1983). Duplication times (Td) were obtained by linear regression of the cell number increase over time on a semi-logarithmic plot.

For growth assays on agar plates, exponentially growing cells were dropped (5 μl from a concentrated solution of 10^8 cells/ml and from serial 10-fold dilutions) onto rich medium (YEP, 1% w/v yeast extract, 2% w/v bacto peptone) agar plates supplemented with acetic acid (YEPA) at the indicated pH and molarity. Plates were incubated at 30˚C for 3–5 days.

METABOLITE MEASUREMENTS

At designated time-points, aliquots of the yeast cultures were centrifuged and supernatants were frozen at −20˚C until used. Glucose, ethanol, and acetate concentrations in the growth medium were determined using enzymatic assays (K-HKGLU, K-ETOH, and K-ACET kits from Megazyme). Values represent the average of three independent experiments.

CLS DETERMINATION

Survival experiments in expired medium were performed on cells grown in minimal medium (with a fourfold excess of supplements) containing 2% glucose as described by (Fabrizio and Longo, 2003; Fabrizio et al., 2005). During growth, cell number and extracellular glucose, ethanol, and acetic acid were measured in order to define the growth profile (exponential phase, diauxic shift, post-diauxic phase, and stationary phase) of the culture. Cell survival was monitored by harvesting aliquots of cells starting 72 h (Day 3, first age-point) after the diauxic shift, when cells stopped dividing and cell density reached a plateau value. Subsequent age-points were taken every 2–3 days. Cells were plated onto rich medium/2% glucose (YEPD) plates and viability was scored by counting colony-forming units (CFUs). The number of CFUs at Day 3 was considered the initial survival (100%). For survival experiments in water, post-diauxic cells (at Day 1) were harvested, washed with sterile distilled water, and resuspended in a volume of water equal to the initial culture volume. Every 48 h, cells were washed with water and resuspended in fresh water to remove nutrients released by dead cells (Fabrizio and Longo, 2003). Survival experiments in water containing acetic acid were performed essentially as described by (Fabrizio et al., 2005) for survival in water/ethanol. In our setting, acetic acid (10 mM) substituted for ethanol and the pH of the water was adjusted to 2.8 for both the control and acetic acid add-back cultures (Burtner et al., 2009). Viability was measured as described above. Survival experiments in expired medium were also performed on cells grown in minimal medium (with a fourfold excess of supplements) containing 0.05% glucose.

Index of respiratory competence (IRC) was also measured according to Parrella and Longo (2008) by plating identical samples on YEPD plates and on rich medium 3% glycerol (YEPG) plates. IRC was calculated as colonies on YEPG divided by colonies on YEPD times 100%.

TESTS FOR OXIDATIVE STRESS AND CELL DEATH MARKERS

Reactive oxygen species (ROS) were detected with dihydrorhodamine 123 (DHR123, Sigma) and dihydroethidium (DHE, Sigma; Madeo et al., 1999). TdT-mediated dUTP nick end labeling (TUNEL, Roche) and Annexin V (ApoAlert Annexin V Apoptosis Kit, Clontech) assay for apoptotic markers as well as propidium iodide (PI, Sigma) staining for necrotic ones were performed as described (Orlandi et al., 2004). Caspase activity was measured with 50 μM FITC-VAD-fmk (CaspACE, Promega) as described (Bettiga et al., 2004). The mitochondrial membrane potential was assessed by staining with rhodamine 123 (RH123) and 3,3″-dihexyloxacarbocyanine iodide ($DiOC_6$; both from Molecular Probes, Invitrogen), according to (Koning et al., 1993; Du et al., 2008).

A Nikon Eclipse E600 fluorescence microscope equipped with a Leica DC 350 F ccd camera was used. Digital images were acquired using FW4000 software (Leica).

STATISTICAL ANALYSIS

All values are presented as the mean ± standard error of the means (SEM). Differences in measurements were assessed by Student's *t*-test. The level of statistical significance was set at a *P* value of ≤ 0.001.

RESULTS

LOSS OF *ach*1 RESTRICTS CLS AND INCREASES APOPTOSIS

Since acetic acid is a factor whose presence in the growth medium promotes chronological aging and Ach1 is a mitochondrial CoA-transferase which has been proposed to be involved in acetic acid detoxification (Fleck and Brock, 2009), we decided to analyze the effects of *ACH1* inactivation on CLS. In the context of a standard CLS experiment (Fabrizio and Longo, 2003), after the diauxic shift, when cells switched from fermentation- to a respiration-based metabolism, we also measured extracellular ethanol and acetic acid concentrations (see Material and Methods). As a by-product of glucose fermentation, the wild type (wt) strain and *ach1*Δ mutant produced a similar maximal amount of ethanol which, during the post-diauxic phase, decreased at a similar rate in both culture media (**Figure 1A**). Acetic acid concentration, in the wt culture, initially increased and then rapidly decreased because it is utilized by the cells for the respiratory metabolism during the post-diauxic phase (**Figure 1B**). On the contrary, *ach1*Δ mutant showed a prolonged accumulation of this C2 compound. In fact, high levels of acetic acid were still present 6 days following the entry in post-diauxic phase (**Figure 1B**) indicating a severe impairment in its utilization in line with a previous study (Fleck and Brock, 2009). Moreover, *ACH1* inactivation significantly reduced CLS (**Figure 1C**).

Yeast cells undergo apoptosis during chronological aging as well as upon exposure to acetic acid (Ludovico et al., 2001; Herker et al., 2004; Fabrizio and Longo, 2008; Rockenfeller and Madeo, 2008). Thus, we assessed different apoptotic features in the short-lived *ach1*Δ cells. DNA fragmentation was detected by TUNEL assay, exposure of phosphatidylserine at the outer leaflet of the plasma membrane and membrane integrity were evaluated by combined Annexin V/PI staining which allows for the identification of early apoptotic events (Annexin V^+) and necrotic cell death (PI^+; Madeo et al., 1997). At Day 6, when survival of *ach1*Δ cells began to decrease (**Figure 1C**), in these cells DNA strand breakage occurred and the percentage of $TUNEL^+$ was fourfold higher (24 ± 1%) as compared to wt ones (6 ± 1%; **Figure 2A**). At the same time-point, Annexin V staining, under conditions where plasma membrane was not compromised, as indicated by exclusion of PI co-staining (data not shown), detected about 18.4 ± 3.1% of Annexin V^+ *ach1*Δ cells in comparison to 7.3 ± 1.9% in the wt (**Figure 2B**). All these data indicate that the *ach1*Δ mutant is subject to a much faster chronological aging process accompanied by an early insurgence of apoptosis.

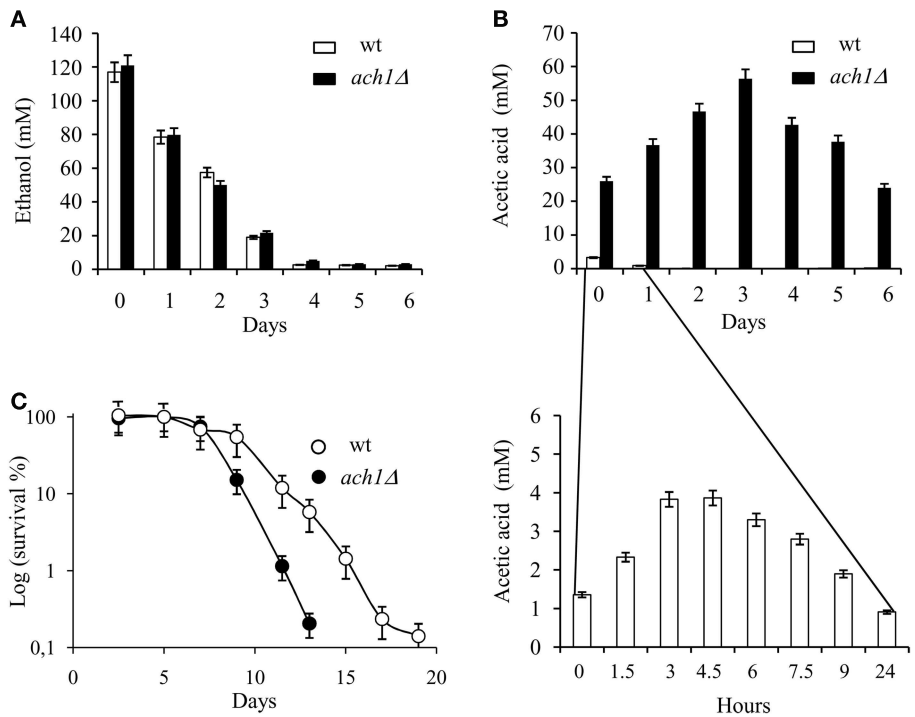

FIGURE 1 | *ACH1* inactivation shortens CLS in concert with increased extracellular acetic acid. Wild type (wt) and *ach1*Δ mutant cells were grown in minimal medium (with a fourfold excess of supplements) containing 2% glucose and followed up to stationary phase. Bar charts of ethanol **(A)** and acetic acid **(B)** concentrations measured in the medium of both cultures at the indicated time-points. Day 0, diauxic shift. Inset: time-scale blow-up. Error bars are the standard deviation of three replicates. **(C)** CLS of wt and *ach1*Δ mutant cells. At every time-point, viability was determined by counting CFUs on YEPD plates. 72 h after the diauxic shift (Day 3), was considered the first age-point (see Materials and Methods). Error bars are the standard deviation of three replicates.

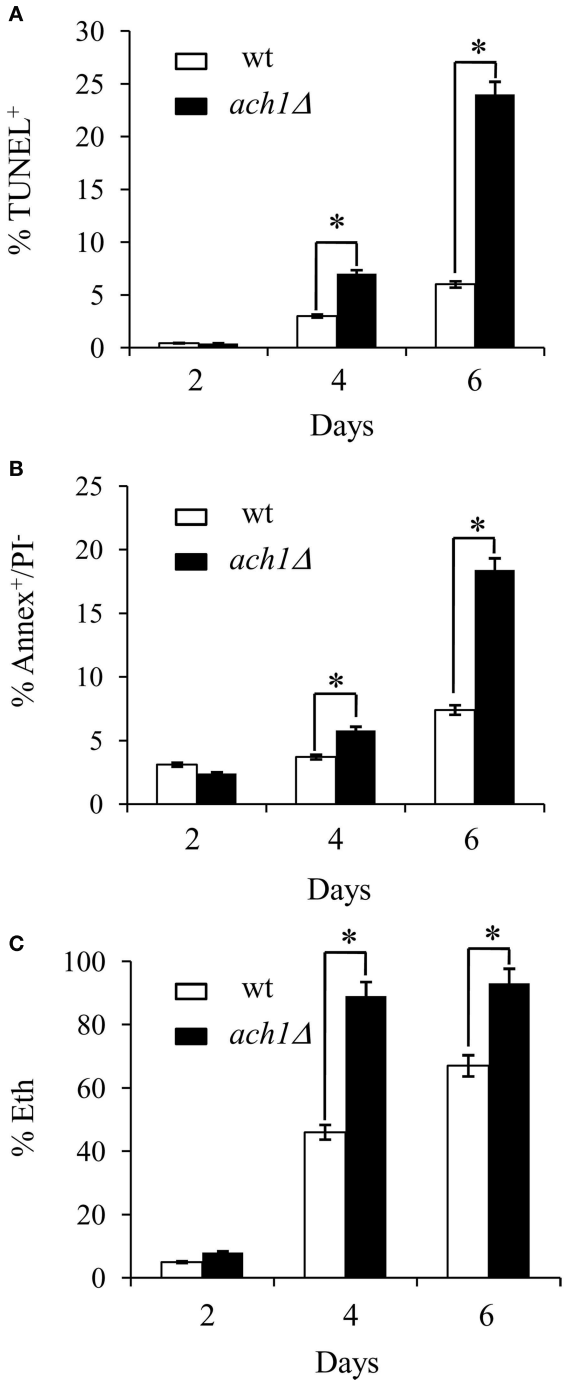

FIGURE 2 | Chronological aging *ach1*Δ cells display an early insurgence of apoptosis. Chronological aging cultures of **Figure 1** at the indicated time-points were assessed for DNA fragmentation by TUNEL assay **(A)**, phosphatidylserine externalization and membrane integrity by Annexin V/propidium iodide (PI) co-staining (Annex V^+/PI^-, **(B)** and intracellular superoxide by dihydroethidium conversion into fluorescent ethidium (Eth), **(C)**. Summary graphs of the percentage of positive cells for each test are indicated. Evaluation of about 1000 cells for each sample in three independent experiments was performed. Standard deviations are indicated. Statistical significance between the strains is indicated ($*P \leq 0.001$, Student's *t*-test).

IN THE *ach1*Δ MUTANT MITOCHONDRIAL FUNCTIONALITY IS IMPAIRED

ROS accumulation is an important endogenous trigger which has been associated with apoptosis during chronological aging and acetic acid treatment (see Carmona-Gutierrez et al., 2010, for a review). ROS content, measured as the superoxide-driven conversion of non-fluorescent DHE into fluorescent ethidium (Eth), was already significantly enhanced in the *ach1*Δ mutant ($89 \pm 3\%$) as compared to the wt strain ($46 \pm 4\%$) at Day 4 (**Figure 2C**) revealing an endogenous situation of higher oxidative stress in the mutant.

Mitochondria are key organelles in superoxide generation. This radical can directly induce oxidative damage or can be converted to other ROS which, in turn, induce aging-associated damage (Pan, 2011). In addition, a direct correlation between reduced CLS and dysfunctional mitochondria has been reported (Bonawitz et al., 2006; Fabrizio and Longo, 2007). Consequently, we decided to analyze whether *ACH1* inactivation could affect mitochondrial functionality. *S. cerevisiae* can grow by either fermentation on glucose as carbon source or by respiration by using different non-fermentable substrates such as glycerol. The growth on the latter can take place only when mitochondria are functional. This feature is exploited to evaluate whether mitochondria are extensively damaged at a point when the cell is still viable. The percentage of viable cells which are competent to respire defines the IRC (Parrella and Longo, 2008). At Day 1, both the wt and *ach1*Δ chronologically aging cells had an IRC of about 100% (**Figure 3A**) indicating that all the cells are respiration-competent. During the following days, this value never dropped below about 80% for the wt, while it decreased quickly for the *ach1*Δ mutant reaching about 15–20% by Day 6 which is indicative of a time-dependent loss of mitochondrial functionality. In parallel, mitochondrial morphology was examined by using $DiOC_6$ dye. In fact, at low concentrations (20–100 ng/ml), this dye accumulates specifically at mitochondrial membranes and can be observed by fluorescence microscopy. However, cells with low mitochondrial membrane potential will fail to accumulate $DiOC_6$ (Koning et al., 1993). Balanced fusion and fission of mitochondria results in tubular mitochondrial morphology, as was the case for wt cells, whereas for *ach1*Δ cells punctiform formations were observed at Day 2 (**Figure 3B**). The conversion of mitochondrial morphology from tubular structures to punctuate ones is also referred to as mitochondrial fragmentation. It is presumed to occur by excessive mitochondrial fission (Fabrizio and Longo, 2008) and has been observed in yeast apoptosis induced by different stimuli including acetic acid treatment (Fabrizio and Longo, 2008). Moreover, at Day 4, $DiOC_6$ staining was greatly reduced in *ach1*Δ cells (**Figure 3B**) revealing a reduction in mitochondrial membrane potential. Taken together these data indicate that the early insurgence of apoptosis in chronological aging *ach1*Δ cells is preceded by a severe damage of mitochondria.

ACETIC ACID IS RESPONSIBLE FOR THE REDUCED CLS OF *ach1*Δ MUTANT

In order to determine whether the reduced CLS of *ach1*Δ mutant was linked to the excess of acetic acid in the extracellular environment, we first examined the effects of lowering glucose concentration from 2 to 0.05% in the initial culture medium. This

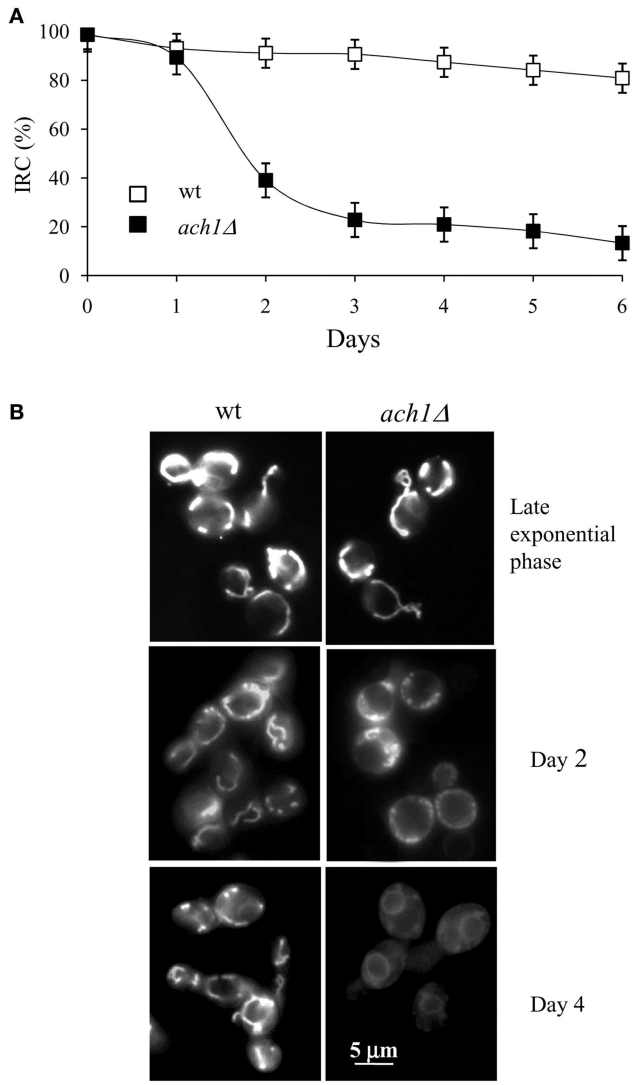

FIGURE 3 | Chronological aging *ach1*Δ cells display compromised mitochondrial activity. Chronological aging cultures of **Figure 1** at the indicated time-points were serially diluted, plated onto YEPD and YEPG plates and the index of respiratory competence (IRC) was determined **(A)**. Standard deviations of three independent experiments are indicated. **(B)** Same cells stained with $DiOC_6$ to visualize mitochondrial membranes. Morphologies of the mitochondria of cells in late exponential phase are also reported. Representative images are shown. Bar: 5 μm.

is a growth condition of CR which reduces acetic acid production in the chronological aging culture and extends CLS (Burtner et al., 2009). As expected, almost undetectable extracellular acetic acid was present in wt cultures grown in CR (**Figure 4A**) which displayed an enhanced survival relative to wt cultures grown on 2% glucose (**Figure 4B** and **Figure 1C**). Growth in a CR regimen for the *ach1*Δ mutant produced undetectable extracellular acetic acid as well (**Figure 4A**). Additionally, it was also sufficient to avoid completely the deleterious effect on cell viability associated with growth on 2% glucose. In fact, CR *ach1*Δ cells had a CLS comparable to CR wt cells (**Figures 4B** and **1C**).

We next monitored survival of cells switched from the expired medium to water. Incubation in water is an extreme form of CR which is known to dramatically extend CLS (Fabrizio et al., 2005). Wt and *ach1*Δ mutant cells were grown on 2% glucose and after the diauxic shift switched to water (see Materials and Methods). As shown in **Figure 4B**, both wt and *ach1*Δ cells, incubated in water, increased CLS to the same extent. Thus, the short-lived phenotype of the *ach1*Δ mutant seems to be mainly due to the toxicity of acetic acid which is accumulated in the environment of chronologically aging *ach1*Δ cells.

Starting from the aforementioned results, we wondered whether the addition of acetic acid could influence the chronological survival of *ach1*Δ cells. It has been previously reported that the addition of acetic acid (10 mM) to low pH (2.8) water can prevent CLS extension of chronologically aging cells associated with their transfer to water (Burtner et al., 2009). Wt and *ach1*Δ cultures grown in 2% glucose were transferred to low pH water supplemented with acetic acid after the diauxic shift and cell viability monitored. In line with acetic acid pro-aging role, acetic acid add-back cultures had a reduced CLS compared to cells incubated in water alone (**Figure 4C**). Notably, acetic acid affected chronological survival of *ach1*Δ cells to a higher extent relative to wt cells. In fact, acetic acid add-back wt cultures displayed a CLS similar to that of wt cells aged in their exhausted medium in agreement with (Burtner et al., 2009), while for the acetic acid add-back mutant cultures the CLS was much more reduced (**Figure 4C**). Together all these data are fully consistent with the notion that the extracellular acetic acid is responsible for the reduced CLS of the *ach1*Δ mutant and also suggest that the lack of Ach1 makes cells more sensitive to acetic acid.

THE *ach1*Δ MUTANT DISPLAYS AN APOPTOTIC CASPASE-DEPENDENT PHENOTYPE

Since *ACH1* deletion results in growth defects on media containing acetic acid as a sole carbon and energy source which are particularly severe under acidic conditions (Fleck and Brock, 2009), to further refine our investigation, we examined whether such a growth impairment could be ascribed to mitochondrial damages. For this purpose, wt and *ach1*Δ cells exponentially growing on 2% glucose were harvested and transferred to 50 mM acetic acid medium, pH 4.5. In this way, cells were released from glucose repression and were able to express all the genes involved in acetate catabolism and assimilation (Paiva et al., 2004), including *ACH1* (Lee et al., 1996). Moreover, the combination 50 mM acetic acid/pH 4.5 is still a permissive growth condition for wt cells but not for the mutant (Fleck and Brock, 2009). Analyses were performed 4 and 16 h after the metabolic shift corresponding to time-windows during which gene expression changes required for acetate metabolism (Paiva et al., 2004) and a significant CoA-transferase activity (Fleck and Brock, 2009) were respectively detected. At these time-points, wt and *ach1*Δ cells were incubated with RH123 in order to visualize active mitochondria, and with the ROS-sensing dye, DHR123. After 4 h, in both strains similar mitochondrial patterns were observed with RH123 associated with DHR123-negative staining indicating that mitochondria retain their membrane potential and no ROS accumulation took place (**Figure 5A**). This was still the case for wt cells *at 16 h*,

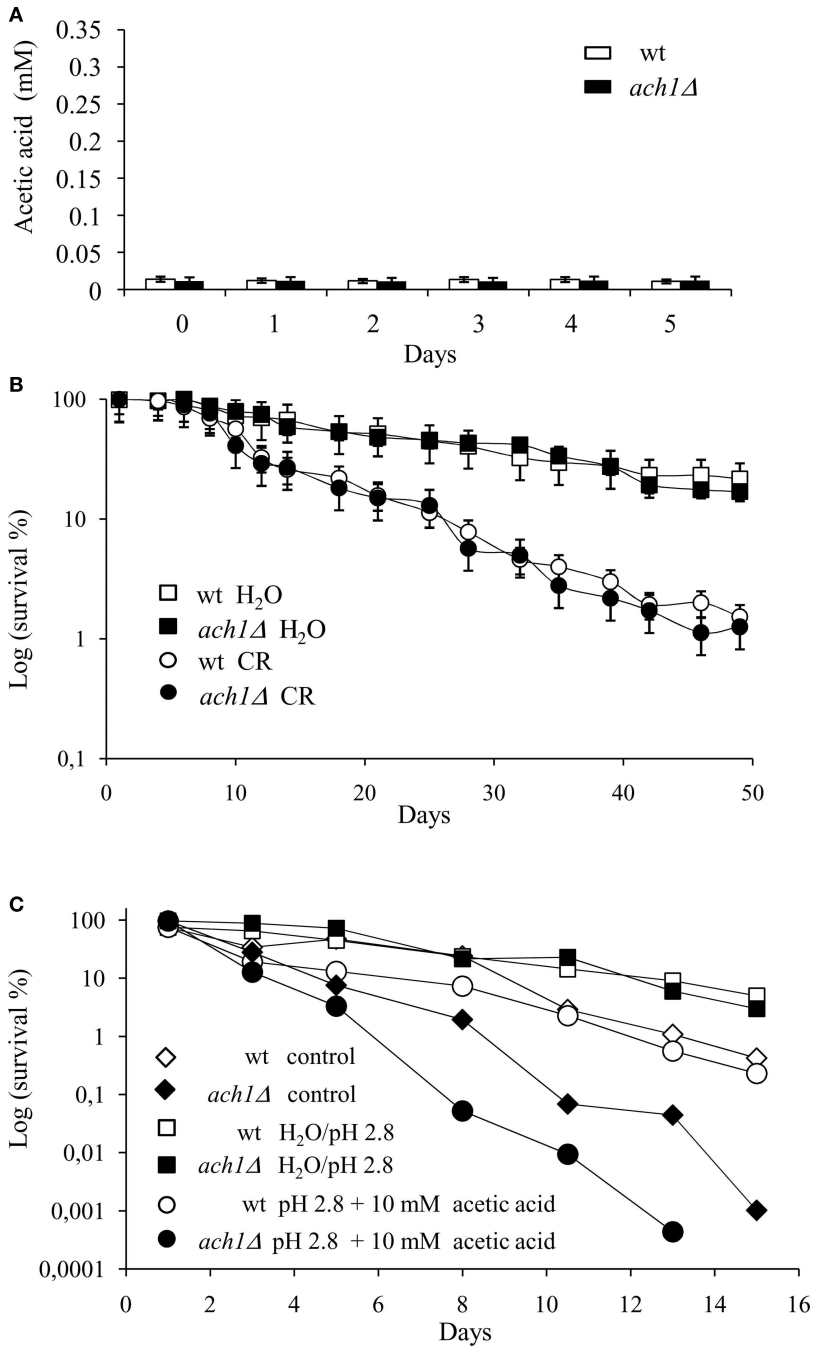

FIGURE 4 | CR suppresses the CLS-shortening effect of *ACH1* inactivation. Wild type and *ach1*Δ cells were grown on 0.05% glucose (CR) and extracellular acetic acid concentration was measured for both cultures at the indicated time-points. Day 0, diauxic shift **(A).** In parallel, cell survival in the exhausted medium was determined by counting CFUs on YEPD plates **(B)**. Wild type and *ach1*Δ chronological aging cells grown on 2% glucose were switched to water (extreme CR) at Day 1. Every 48 h, cultures were resuspended in fresh water and at every time-point, viability was measured by counting CFUs on YEPD plates **(B)**. Survival of cells in their exhausted medium was also monitored as control (reported in **Figures 1C** and **4C**). Error bars are the standard deviation of three replicates. (**C**) Wild type and *ach1*Δ chronological aging cells grown on 2% glucose were switched to water adjusted to pH 2.8 and water/pH 2.8 containing 10 mM acetic acid at Day 1. Every 48 h, cultures were resuspended in fresh water/pH 2.8 and each time 10 mM acetic acid was added. At every time-point, viability was measured as in **(B).** Survival of cells in their exhausted medium was also monitored (control). One representative experiment is shown.

while in the mutant a severe reduction in mitochondrial membrane potential and increase in ROS-accumulating cells (about 80%) were observed (**Figures 5A,B**). These features were also accompanied by the appearance of Annexin V^+ (about 29%) and

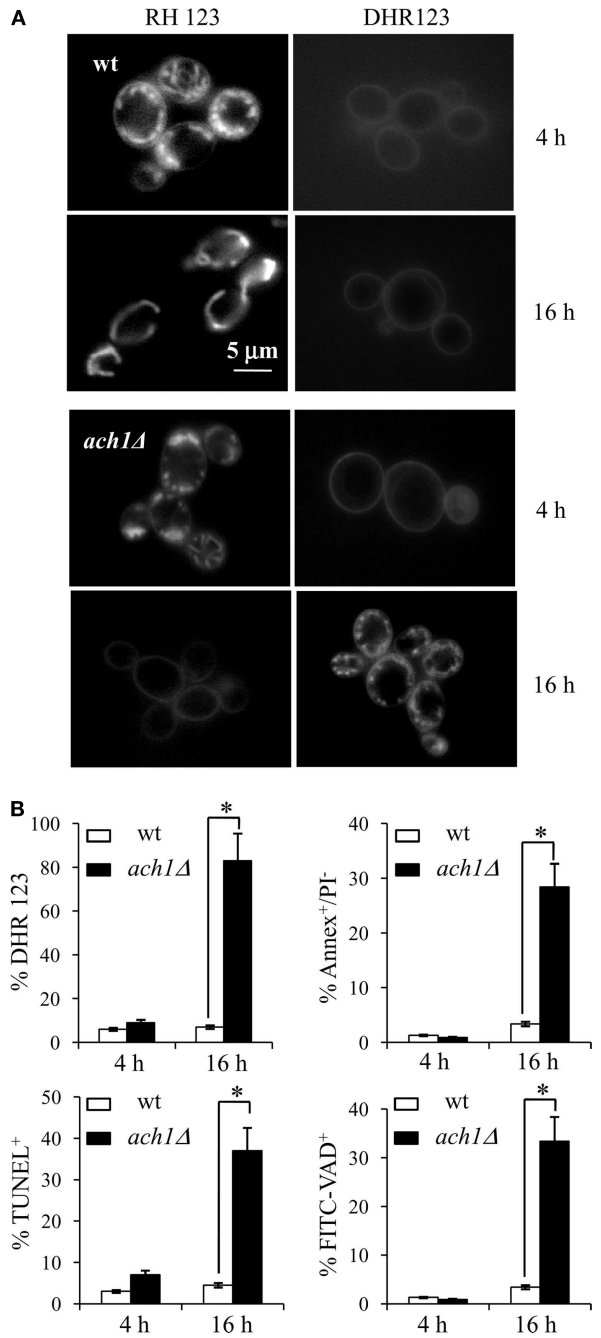

FIGURE 5 | The presence of acetic acid induces apoptosis in *ach1*Δ cells. Wild type and *ach1*Δ cells exponentially growing on 2% glucose were shifted to acetate (50 mM) medium, pH 4.5 as sole carbon source. At the indicated time-points after the shift, cells were stained with Rhodamine 123 (RH123) to visualize functional mitochondria and with DHR123 for the presence of intracellular ROS **(A)**. Representative images are shown. Bar: 5µm. The same cultures were analyzed for apoptotic markers by Annexin V/PI co-staining and TUNEL assay, and for caspase activity by FITC-VAD-fmk. Summary graphs of the percentage of ROS-accumulating cells, of Annexin V^+/PI^-, of $TUNEL^+$ and $FITC\text{-}VAD^+$ are reported **(B)**. Evaluation of about 500 cells for each sample in three independent experiments was performed. Standard deviations are indicated. Statistical significance between the strains is indicated ($*P \leq 0.001$, Student's *t*-test).

$TUNEL^+$ (about 37%) cells (**Figure 5B**) suggesting the onset of apoptosis.

Both caspase-dependent and caspase-independent cell death pathways have been described in yeast (Madeo et al., 2009). The Yca1 metacaspase is a yeast functional ortholog of mammalian caspases which mediates the apoptotic process triggered by several intrinsic and extrinsic inducers including acetic acid (Guaragnella et al., 2006; Madeo et al., 2009). To evaluate whether *ach1*Δ cells shifted to the acetate medium showed an endogenous metacaspase activity, the FITC-labeled caspase inhibitor VAD-fmk, which binds to activated caspase, was used. 16 h after the shift, about 32 ± 2.3% of *ach1*Δ cells were FITC-positive (**Figure 5B**) suggesting that apoptosis in *ach1*Δ cells occurs through a caspase-dependent cascade. Consequently, we analyzed the effects of *YCA1* inactivation in the *ach1*Δ background. Initially, we assessed cellular growth on 50 mM acetic acid media whose pH had been adjusted to 5.8 or 4.5: a permissive and a restrictive growth condition for the *ach1*Δ mutant, respectively (Fleck and Brock, 2009). As depicted in **Figure 6A**, both wt and *yca1*Δ cells grew on all the acetic acid-containing media while acidification of the medium affected severely the *ach1*Δ mutant viability. Notably, this effect was almost completely prevented by deleting *YCA1* (**Figure 6A**). Changes in the pH of glucose-containing media did not influence the growth of all strains (data not shown). Moreover, on 50 mM acetic acid medium, pH 4.5, the measurement of apoptotic markers (Annexin V^+/PI^-) and ROS showed that *YCA1* inactivation partly rescued the *ach1*Δ mutant from apoptosis, as well as from ROS accumulation (**Figure 6B**). Decreases in Annexin V^+/PI^- cells and ROS were also observed in the *ach1*Δ *yca1*Δ mutant compared to the *ach1*Δ mutant in 35 mM acetic acid medium, pH 4.5 (**Figure 6B**). This was accompanied by an improvement of cellular growth (**Figure 6C**) confirming the results obtained for 50 mM acetic acid medium. Together all these data point to an involvement of Yca1 in the caspase-dependent apoptosis of the *ach1*Δ mutant promoted by acetic acid.

DISCUSSION

Acetic acid, a well known by-product of yeast glucose fermentation, has been identified as a cell-extrinsic mediator of cell death during chronological aging (Burtner et al., 2009). Data reported in this paper provide more experimental evidence of a role for acetic acid toxicity as a determinant of CLS and of the positive feed-forward connection between mitochondrial damage and apoptosis. In fact, we show that inactivation of *ACH1* encoding a mitochondrial CoASH transferase which catalyzes the transfer of the CoASH moiety from succinyl-CoA to acetate (Fleck and Brock, 2009), reduces CLS. The short-lived phenotype relies on an excess of extracellular acetic acid which is accumulated in the medium of chronologically aging *ach1*Δ cells. In fact, the faster chronological aging process can be completely abrogated either by transferring chronologically aging *ach1*Δ cells from the expired medium to water, thus alleviating the acid stress experienced by the cells, or by growing them under a CR regimen (0.05% glucose) and consequently avoiding acetic acid production (Burtner et al., 2009; this work). Since 0.05% is a glucose concentration which is well below what is normally needed to relieve glucose repression (Yin et al., 2003), yeast metabolism switches from fermentation

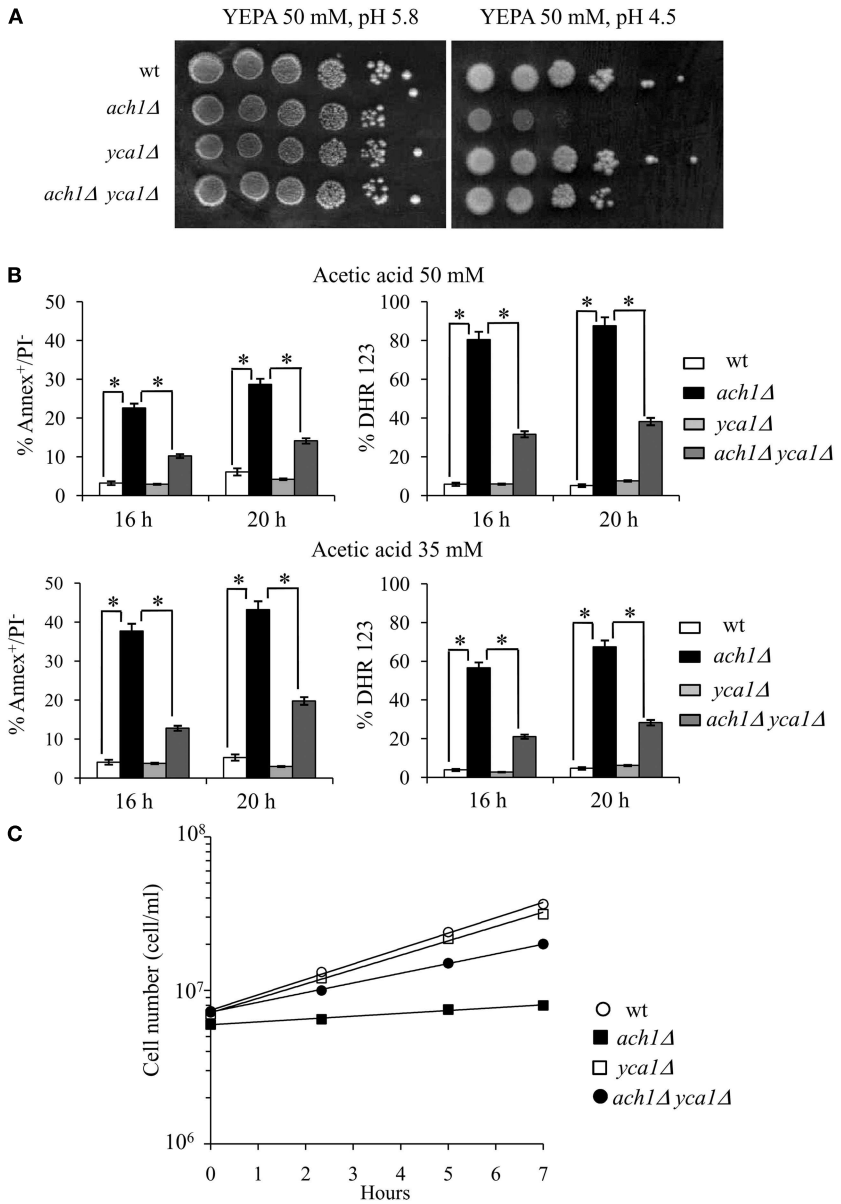

FIGURE 6 | Effects of *YCA1* inactivation on *ach1*Δ cells. (A) The indicated strains exponentially growing on 2% glucose were spotted (10-fold serial dilutions) onto plates containing 50 mM acetic acid (YEPA) as a carbon source at pH 5.8 and pH 4.5. Plates were incubated at 30˚C for 4 days. One significant experiment out of 3 is shown. **(B)** The indicated strains exponentially growing on 2% glucose were shifted to 35 mM and 50 mM acetic acid-containing media, pH 4.5. At the indicated time-points after the shift, cells were analyzed for apoptotic markers by Annexin V/PI co-staining and stained with DHR123 for the presence of intracellular ROS. Summary graphs of the percentage of Annexin V^+/PI^- and of ROS-accumulating cells are reported. Evaluation of about 500 cells for each sample in three independent experiments was performed. Standard deviations are indicated. Statistical significance between the strains is indicated ($*P \leq 0.001$, Student's *t*-test). **(C)** Growth curves of the indicated strains grown in 35 mM acetic acid-containing medium, pH 4.5. Growth was measured as increase in cell number over time. One representative experiment is shown.

to respiration. Acetyl-CoA is synthesized directly from pyruvate by oxidative decarboxylation, catalyzed by the mitochondrial pyruvate dehydrogenase complex, and in such a way glycolysis is coupled to the TCA cycle (Pronk et al., 1996).

In parallel with the faster drop of cell survival, chronologically aging *ach1*Δ cells undergo an early insurgence of apoptosis. In addition, *ach1*Δ cells also undergo apoptosis when they are inoculated in 35 mM or 50 mM acetate medium at pH 4.5; a growth condition which has a detrimental effect on the viability of this mutant. It is well known that acetic acid represents a compound commonly used to induce yeast apoptosis when applied in the presence of glucose at pH 3 (Ludovico et al., 2001, 2002; Giannattasio et al., 2005). Here, we show that acetic acid alone triggers apoptosis in glucose-derepressed *ach1*Δ cells. Uptake of acetate

is linked to a proton symport mechanism (subjected to glucose repression) accompanied by passive/facilitated diffusion of the uncharged/undissociated acid through the Fps1 aquaglyceroporin channel (see Casal et al., 2008, for a review). Acetic acid displays toxicity at low extracellular pH primarily due to the undissociated acid diffusion. In fact, the acetic/acetate couple forms a buffer system; at pH values below the pKa of the acid (4.75) the undissociated form prevails and diffuses through the plasma membrane. Once inside the cell (pH close to neutral), the acid dissociates causing anion accumulation and intracellular acidification that, in turn, is thought to have negative effects on yeast metabolic activity. Additionally, free radical production is also affected leading to severe oxidative stress (Piper et al., 2001). In line with this, *ach1*Δ cells on acetate display ROS accumulation and a strong reduction in mitochondrial membrane potential similar to that elicited by acetic acid in glucose-repressed wild type cells (Ludovico et al., 2002) supporting the notion of a role for Ach1 as a mitochondrial detoxifying enzyme (Fleck and Brock, 2009). Moreover, as in the case of the acetic acid-induced apoptosis of glucose-repressed cells, occurrence of apoptotic markers in the *ach1*Δ mutant is accompanied by caspase activation. So far, four proteases have been described in the yeast apoptotic scenarios: the separase Esp1 (Yang et al., 2008), the nuclear HtrA-like protein Nma111 (Fahrenkrog et al., 2004; Wissing et al., 2004), Kex1 (Hauptmann et al., 2006), and the metacaspase Yca1 (Madeo et al., 2002). Here, evidence is provided that Yca1 is involved in the caspase-dependent apoptosis of the *ach1*Δ mutant promoted by acetic acid since *YCA1* deletion decreases apoptotic markers, as well as ROS accumulation in the *ach1*Δ mutant and, conversely, improves its cellular growth on acetic acid-containing media at low pH. Such an involvement is fully consistent with the requirement of this metacaspase for the ROS-dependent acetic acid-induced apoptosis (Guaragnella et al., 2010a,b).

A pattern of endogenous severe oxidative stress is also observed in chronologically aging *ach1*Δ cells in concert with elevated levels of extracellular acetic acid. Here, a precocious increase of mitochondrial superoxide which is well known to target primarily several mitochondrial enzymes (Longo et al., 1999; Fabrizio et al., 2001) and to play a major role in chronological aging (Fabrizio and Longo, 2003; Mesquita et al., 2010; Pan, 2011), is associated with loss of respiratory competence which precedes apoptotic death suggesting that Ach1 is required to protect mitochondrial function during chronological aging. Fleck and Brock (2009) proposed that under extracellular acidic conditions, the diffusional entry of the undissociated acid through the plasma membrane into the cell might also lead to an influx of acetic acid over the mitochondrial membrane. This would result in acetate accumulation and mitochondrial acidification affecting negatively mitochondrial functionality. Ach1 enzymatic activity would prevent this acetate accumulation by a CoASH transfer from succinyl-CoA (produced by the TCA cycle) to acetate generating acetyl-CoA. Ach1 has a low Km for both succinyl-CoA and acetate (Fleck and Brock, 2009). In addition, the CoA-transferase reaction saving ATP, compared with the acetate activation to acetyl-CoA by acetyl-CoA synthetase, would be favored in a condition where in order to counteract intracellular acidification induced by acetic acid, cells are already consuming energy (Piper et al., 2001). Taken together our data are fully consistent with this hypothesis since they indicate that mitochondria are the main target of acetic acid toxic effects in *ach1*Δ cells in extracellular acidic conditions such as acetate medium at pH 4.5 and during chronological aging (pH about 3; Burtner et al., 2009). This implies that Ach1 can function as a mitochondrial detoxifying enzyme. Moreover, the mitochondrial damage resulting from Ach1 loss of function can account for the growth impairment on acetate and the CLS decrease.

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Oxidative stress and programmed cell death in yeast

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Yeasts, such as *Saccharomyces cerevisiae*, have long served as useful models for the study of oxidative stress, an event associated with cell death and severe human pathologies. This review will discuss oxidative stress in yeast, in terms of sources of reactive oxygen species (ROS), their molecular targets, and the metabolic responses elicited by cellular ROS accumulation. Responses of yeast to accumulated ROS include upregulation of antioxidants mediated by complex transcriptional changes, activation of pro-survival pathways such as mitophagy, and programmed cell death (PCD) which, apart from apoptosis, includes pathways such as autophagy and necrosis, a form of cell death long considered accidental and uncoordinated. The role of ROS in yeast aging will also be discussed.

Keywords: oxidative stress, yeast, apoptosis, necrosis, mitophagy, autophagy, aging

INTRODUCTION

It is a well known fact that the majority of living organisms depend on oxygen for survival. However, organisms also had to evolve a multitude of antioxidant defenses to protect their cells from oxygen's toxic properties (Bilinski, 1991; Halliwell, 1999), which stem mainly from its propensity to produce reactive oxygen species (ROS) such as the superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\bullet}$). These ROS typically arise because of electron leakage from the electron transport chain onto dioxygen (O_2) during aerobic respiration (Boveris et al., 1972; Turrens and Boveris, 1980; Turrens, 1997; Halliwell and Gutteridge, 2007). Exposure to heavy metals (Liang and Zhou, 2007; Xu et al., 2011), ultraviolet (UV) irradiation, herbicides, air pollutants, xenobiotics, and other exogenous factors can also induce significant generation of ROS (Halliwell and Cross, 1994; Gille and Sigler, 1995). Failure of cell antioxidant defenses to impede ROS accumulation inevitably results in oxidative stress, a condition broadly defined as an imbalance between prooxidants and antioxidants, in favor of the former. This potentially leads to a situation where important cell biomolecules suffer severe oxidative damage, thus compromising the viability of cells (Sies, 1991; Halliwell and Cross, 1994; Halliwell and Gutteridge, 2007).

In fact, accumulated ROS have been shown to inflict oxidative damage upon essential biomolecules such as nucleic acids (Yakes and Van Houten, 1997), proteins (Cabiscol et al., 2000), and lipids (Bilinski et al., 1989). Furthermore, ROS accumulation has long been found to play an important role in mediating programmed cell death (PCD) such as apoptosis, or even – at very high concentrations – necrosis, in various cell types (Pierce et al., 1991; Kasahara et al., 1997; Madeo et al., 1999; Chandra et al., 2000; Simon et al., 2000; Ludovico et al., 2001; Jeon et al., 2002; Avery, 2011). More significantly, oxidative damage and cell death induced by ROS have been linked to many serious human pathologies including diabetes (Giugliano et al., 1996; Baynes and Thorpe, 1999; Yokozawa et al., 2011) and neurodegenerative diseases such as Parkinson's disease (Hirsch, 1993; Jenner and Olanow, 1996; Jenner, 2003; Facecchia et al., 2011), Alzheimer's disease (Behl, 1999; Nunomura et al., 2001; Reddy et al., 2009), and amyotrophic lateral sclerosis (ALS; Andrus et al., 1998; Barber et al., 2006; Barber and Shaw, 2010). ROS have also been implicated in the aging process (Harman, 1956; Orr and Sohal, 1994; Barja, 2004; Fabrizio et al., 2004; Herker et al., 2004) and are known to play a pivotal role in the development of cancer (Ames et al., 1993, 1995; Loft and Poulson, 1996; Naka et al., 2008; Khandrika et al., 2009; Acharya et al., 2010).

Yeast cells have steadily evolved into one of the most preferred experimental models for the study of oxidative stress and its effects, in the context of PCD and aging. Yeast species such as the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*, enjoy several advantages over other experimental models. For instance, they have a short life-cycle and are easy to manipulate genetically – much like bacteria, whilst still retaining the core cellular processes that are characteristic of eukaryotes (Carmona-Gutierrez et al., 2010a). Most importantly, past studies have shown that, amongst these cellular processes, core elements of PCD, such as apoptosis, are also conserved in yeast (Madeo et al., 1997, 1999, 2002; Fröhlich and Madeo, 2001; Fahrenkrog et al., 2004; Wissing et al., 2004; Büttner et al., 2007) and that accumulation of ROS plays a key role in these pathways (Madeo et al., 1999; Jeon et al., 2002). Hence, yeast can make a significant contribution to our understanding of oxidative stress, and its consequences such as PCD and the misregulation of PCD in the context of pathologies such as cancer, neurodegenerative diseases, and aging (Carmona-Gutierrez et al., 2010a).

This review aims to present a broad overview of current knowledge of oxidative stress and its role in yeast PCD, with emphasis on *S. cerevisiae*. The role of oxidative stress and ROS in yeast aging will also be discussed.

OXYGEN TOXICITY AND OXIDATIVE STRESS IN YEAST

The accumulation of ROS, in yeast, generally stems from internal metabolic processes associated with respiration, but can also be triggered by environmental stress stimuli (Jamieson, 1998; Perrone et al., 2008).

REACTIVE OXYGEN SPECIES GENERATION IN YEAST

Mitochondria have long been established as a major source of ROS (Longo et al., 1996) such as the superoxide radical $O_2^{\bullet-}$, which is generated from O_2 by electron leakage originating in the mitochondrial transport chain, during respiration. (Fridovich, 1998; Cadenas and Davies, 2000). In yeast species such as *S. cerevisiae*, the specific sources of $O_2^{\bullet-}$ in the mitochondrial chain include the external NADH dehydrogenases Nde1p and Nde2p (the active sites of which face the mitochondrial intermembrane space) and complex III (Fang and Beattie, 2003).

Although superoxide can directly inactivate certain proteins such as catalases (Kono and Fridovich, 1983; Fridovich, 1989) and dehydratases (Murakami and Yoshino, 1997), it is a relatively selective and unreactive ROS (Fridovich, 1998; Halliwell and Gutteridge, 2007). Nevertheless, the prompt and efficient removal of $O_2^{\bullet-}$ from living cells is of great biological importance, given its tendency to generate H_2O_2 and subsequent, more harmful ROS (Fridovich, 1989; Gille and Sigler, 1995). In fact, studies have shown that $O_2^{\bullet-}$ detoxification mechanisms such as the superoxide dismutase enzymes (SODs), are essential for survival of *S. cerevisiae* against hyperoxia (Outten et al., 2005). Recently $O_2^{\bullet-}$ has also been implicated as a signaling molecule in yeast cell processes such as chronological aging (Weinberger et al., 2010; Lewinska et al., 2011).

Superoxide radical dismutation, which mainly occurs via enzymatic SOD activity, is a major source of H_2O_2 *in vivo* (McCord and Fridovich, 1969). In yeast, H_2O_2 is also generated by D-amino acid oxidases, peroxisomal acyl-coenzyme A oxidases (Halliwell and Cross, 1994; Herrero et al., 2008), and protein folding events in the endoplasmic reticulum (ER; Gross et al., 2006). Like $O_2^{\bullet-}$, H_2O_2 is relatively unreactive (Gille and Sigler, 1995; Halliwell and Gutteridge, 2007) but can also travel long distances and penetrate biological membranes, allowing it to exert oxidative damage in locations far from its point of origin (Saran and Bors, 1991). It is also a signaling molecule in mammals (Sundaresan et al., 1995), plants (Vergara et al., 2012), and yeast (Bienert et al., 2006; Bartosz, 2009). In fact, H_2O_2 signaling activates transcription factors that regulate antioxidant gene expression in yeast (Wemmie et al., 1997; Delaunay et al., 2000, 2002; Kuge et al., 2001). This is believed to take place primarily via the direct oxidation and concomitant functional alteration of redox-sensitive thiol peroxidases (Fomenko et al., 2011). Furthermore, pre-treatment with 150 μM H_2O_2 enhances the sensitivity of budding yeast cells to heat stress, suggesting that ROS (presumably H_2O_2) relay signals induced by heat stress to yeast heat shock transcription factors that initiate the heat shock response (Moraitis and Curran, 2004).

Both $O_2^{\bullet-}$ and H_2O_2 can combine to form the hydroxyl radical ($OH^{\bullet}$), via the Fenton and Haber–Weiss reactions catalyzed by free metal cations such as those of iron (Fe; Haber and Weiss, 1934). Ferrous Fe^{2+} ions are oxidized to ferric Fe^{3+} ions by H_2O_2 to produce $OH^{\bullet}$. These Fe^{3+} ions can be reduced again by $O_2^{\bullet-}$, resulting in a reaction cycle capable of generating an infinite supply of $OH^{\bullet}$.

The $OH^{\bullet}$ radical is an exceedingly powerful oxidant which indiscriminately oxidizes cell biomolecules at a diffusion-limited rate (Fridovich, 1989, 1998). Biomolecules oxidized by $OH^{\bullet}$ can become radicals themselves, which propagate even further non-specific cell oxidative damage (Evans et al., 1998). In fact, most oxidative damage in cells is mediated by $OH^{\bullet}$, which is far more toxic than its precursors $O_2^{\bullet-}$ and H_2O_2, the impact of which mainly lies in their propensity to form $OH^{\bullet}$ (Halliwell and Cross, 1994).

The nitric oxide radical ($NO^{\bullet}$) is another important free radical species associated with oxidative damage in organisms. Upon reacting with $O_2^{\bullet-}$, it produces peroxynitrite ($ONOO^-$), a strongly oxidizing reactive nitrogen species (RNS) which can generate further radicals such as $OH^{\bullet}$ (Beckman et al., 1990) and induce oxidation of proteins and nucleic acids (Radi, 2004; Poyton et al., 2009). The $NO^{\bullet}$ radical has also been linked to increased ROS generation and cell death in *S. cerevisiae*, where endogenous $NO^{\bullet}$, generated by $NO^{\bullet}$ synthase-like activity (Osório et al., 2007), was found to induce ROS accumulation and apoptosis in yeast cells treated with H_2O_2 (Almeida et al., 2007). Importantly, this was also accompanied by S-nitrosation of the glycolytic glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Almeida et al., 2007). Given that S-nitrosation, defined as the covalent attachment of $NO^{\bullet}$ to the cysteine thiol groups of target proteins, is a mechanism by which $NO^{\bullet}$ regulates varied cellular processes in organisms (Hess et al., 2005), these observations also exemplify the role of $NO^{\bullet}$ radicals as signaling molecules (Almeida et al., 2007).

Under normoxic conditions, cells can very efficiently prevent ROS accumulation and attenuate oxidative damage, using various defensive strategies and antioxidants. However, environmental conditions can undergo frequent changes, such as progressive depletion of nutrients, increases in ambient temperature, or sudden xenobiotic contamination. Regardless of their nature, such environmental changes invariably cause an element of stress to organisms, to which they must effectively adapt in order to survive. This stress is frequently associated with ROS (Avery, 2011), which either deplete functional antioxidants or induce further ROS accumulation, or both (Costa and Moradas-Ferreira, 2001).

For instance, UVA radiation (Kozmin et al., 2005) and cadmium (Brennan and Schiestl, 1996) cause oxidative damage or outright deletion of genes in yeast cells. Deletion or impairment of genes associated with antioxidant enzymes such as manganese superoxide dismutase (MnSOD) and copper-zinc superoxide dismutase (CuZnSOD) causes increased mitochondrial protein oxidation (O'Brien et al., 2004) and loss of viability of respiring stationary phase cells (Longo et al., 1996). Also, prooxidant xenobiotics, including aminotriazole (AMT) and diethyldithiocarbamate (DDC), directly inactivate catalase (Bayliak et al., 2008) and CuZnSOD (Lushchak et al., 2005), respectively, sensitizing yeast to oxidative stress (Lushchak, 2010). Redox-cycling xenobiotics, such as paraquat and menadione, stimulate superoxide production in yeast by accepting electrons from cellular reducers and transferring them to oxygen (Lushchak, 2010). Other environmental stimuli such as heat stress (Davidson et al., 1996), ethanol-induced diauxic shift (Drakulic et al., 2005), H_2O_2 (Madeo et al., 1999), acetic acid (Ludovico et al., 2001), cadmium (Brennan and Schiestl, 1996), and arsenic (Du et al., 2007) amongst several other agents, can all induce ROS accumulation in yeast.

MOLECULAR TARGETS OF ROS IN YEAST

The accumulation of cellular ROS inevitably results in oxidative damage to important cell biomolecules such as proteins, DNA,

and lipids. Failure to curb such damage is ultimately associated with cell death (Costa and Moradas-Ferreira, 2001). The following sections will briefly outline some of the most prominent molecular targets of ROS in yeast.

Oxidative damage of proteins

Oxidative damage of proteins adversely influences cell homeostatic functions and rapidly compromises cell viability. In fact, accumulation of oxidized proteins has been associated with aging and several ROS-related diseases such as ALS and Alzheimer's disease. All ROS, including lipid peroxides, can oxidize proteins via several different pathways, the net result of which is usually the formation of protein carbonyls. This is also accelerated by free metal cations as a result of Fenton chemistry (Berlett and Stadtman, 1997).

Protein carbonyls are irreversible products and thus susceptible to proteolytic degradation (Wolff and Dean, 1986). Prompt removal of protein carbonyls is important, given their tendency to form large protein aggregates which cannot then be degraded by cells via normal proteolytic pathways. Failure to effectively degrade such aggregates will only enhance further carbonyl accumulation and eventually disrupt cell homeostasis altogether (Cecarini et al., 2007).

Thiol (–SH) groups of cysteine, methionine, and aromatic phenylalanine amino acid residues are particularly sensitive to oxidation by all ROS (Berlett and Stadtman, 1997). Thiol groups can be transiently oxidized to disulfide groups (S–S) and sulfenic acid groups (–SOH), or irreversibly oxidized to sulfinic ($–SO_2H$) or sulfonic ($–SO_3H$) acid groups (Klatt and Lamas, 2000).

Additionally, ROS can cause dysfunctional protein synthesis, such as by mistranslation of mRNA. For instance, in *S. cerevisiae*, oxygen-dependent chromate ion Cr (VI)-induced mistranslation of mRNA was found to cause accumulation of insoluble and toxic protein carbonyl aggregates composed of improperly synthesized, inactive proteins. This is considered the primary means of Cr (VI) toxicity in yeast (Holland et al., 2007).

Otherwise, major yeast protein targets include mitochondrial citric acid cycle proteins, such as α-ketoglutarate dehydrogenase, pyruvate dehydrogenase, and the iron-sulfur [4Fe-4S] cluster enzymes aconitase and succinate dehydrogenase, all of which are readily inactivated by H_2O_2 and by menadione-derived superoxide radicals (Cabiscol et al., 2000; Cecarini et al., 2007). The superoxide-mediated oxidation of labile [4Fe-4S] clusters in dehydratase enzymes, such as aconitase, also releases free Fe^{2+} ions that participate in Fenton chemistry, promoting further protein oxidation (Fridovich, 1998). Aconitase in yeast is also inactivated by $NO^{\bullet}$-induced oxidative stress (Lushchak and Lushchak, 2008). Important enzymes such as GAPDH (Cabiscol et al., 2000) and CuZnSOD (Costa et al., 2002) are also easily inactivated via H_2O_2-induced carbonylation, which is largely mediated by metal ion-catalyzed formation of $OH^{\bullet}$ (Costa and Moradas-Ferreira, 2001).

The yeast actin cytoskeleton is another important target of ROS. The recessive loss-of-function mutation of an *ASC1* sequence in yeast induces hyper-assembly of actin filaments and consequent cytoskeletal morphological defects, whilst further increasing actin's sensitivity to oxidative stress (Haarer and Amberg, 2010). The physiological consequences of actin oxidation in yeast include accelerated aging and apoptotic cell death (Farah and Amberg, 2007). Similarly, the decline of actin dynamics and subsequent cytoskeletal stabilization in yeast, induced by the disruption of actin regulatory proteins Sla1p and End3p, leads to ROS accumulation and cell death, mediated by hyperactivation of the Ras/cAMP kinase pathway (Gourlay and Ayscough, 2006).

Oxidative damage of DNA

Nucleic acids can suffer various forms of mutational damage caused by ROS. These include single or double strand breaks, base modification, abasic sites, and protein-DNA cross-linkage (Henle and Linn, 1997; Finn et al., 2011). Oxidatively induced mutations such as these, which compromise DNA integrity and functionality, have long been implicated in the development of pathologies such as cancer (Ames et al., 1995) and in aging (Burhans and Weinberger, 2012). Oxidized DNA also interferes with the normal response to environmental oxidative stress in yeast (Salmon et al., 2004), causing ROS accumulation and PCD in both *S. cerevisiae* and *S. pombe* (Burhans et al., 2003). Oxidative DNA damage and chromosome fragmentation are in fact frequently detected in ROS-mediated killing of yeast exposed to acetic acid (Ludovico et al., 2002), H_2O_2 (Madeo et al., 1999), and hyperosmotic stresses (Ribeiro et al., 2006).

The ROS which plays a dominant role in oxidizing DNA is $OH^{\bullet}$. Singlet oxygen ($^1O_2$) also oxidizes guanine bases (Halliwell and Cross, 1994) into 7,8-dihydro-8-oxoguanine, a well-characterized marker of oxidative DNA damage (Jenner, 1994; Daroui et al., 2004). Other prooxidants or ROS can only oxidize DNA via these two species (Halliwell and Aruoma, 1991). This is true for stresses such as ionizing radiation (Frankenberg et al., 1993), UVA radiation (Kozmin et al., 2005), heavy metals such as cadmium (IV) (Jin et al., 2003) and prooxidants that produce H_2O_2 such as mitomycin and paraquat (Brennan et al., 1994) or $O_2^{\bullet-}$ such as menadione (Lee and Park, 1998) – these induce damages such as strand breakages, oxidation of bases, and intrachromosomal recombination in the DNA of *S. cerevisiae* cells, primarily via formation of $OH^{\bullet}$.

Oxidative damage of lipids

Oxidative damage of lipid molecules generally involves lipid peroxidation, an autocatalytic process initiated by the oxidation of polyunsaturated fatty acids (PUFAs) into labile lipid hydroperoxides, by $OH^{\bullet}$ radicals. Lipid hydroperoxides propagate the synthesis of further hydroperoxides and other reactive derivatives, all of which can inflict extensive oxidative damage to cell biomolecules (Halliwell and Gutteridge, 2007).

Yeast cells are incapable of synthesizing PUFAs, but will readily incorporate them into their membrane structures if cultured in PUFA-enriched growth medium, thus raising the risk of lipid peroxidation on exposure to oxidative stress (Bilinski et al., 1989). In fact, yeast cells with PUFA-enriched yeast membranes, showed hypersensitivity to cadmium toxicity, which was mediated by lipid peroxidation (Howlett and Avery, 1997). Expression of human Bax in yeast cells containing PUFA-enriched mitochondrial membranes also caused pronounced lipid peroxidation (Priault et al., 2002). Lipid peroxidation was also reported in yeast cells undergoing rapamycin-induced autophagy (Kissová et al., 2006), and

oxidative stress induced by H_2O_2 (Reekmans et al., 2005) and menadione (Kim et al., 2011).

PRO-SURVIVAL CELLULAR RESPONSES TO OXIDATIVE STRESS IN YEAST

Yeast cells express limited pools of antioxidants which sufficiently protect against ROS. However, these constitutive defenses cannot protect the cells from sudden oxidative insults. Therefore, yeast has had to evolve the ability to sense aggressive stress stimuli and to generate rapid adaptive responses to increased ROS accumulation. The onset of oxidative stress in yeast cells generally induces an early response, where pre-existing antioxidant defenses provide immediate protection against the initial sub-lethal accumulation of ROS. There is also early transmission of stress signals and consequent activation of transcription factors which promote the synthesis of further antioxidant defenses (Gasch et al., 2000; Temple et al., 2005). This leads to the late response involving synthesis and activation of new antioxidant defenses that scavenge ROS, repair oxidized biomolecules, and restore cellular redox balance (Costa and Moradas-Ferreira, 2001).

YEAST TRANSCRIPTION FACTORS AND THEIR ROLE IN YEAST OXIDATIVE STRESS RESPONSES

The adaptive response mechanisms to oxidative stress in *S. cerevisiae* are regulated at the transcriptional level (Collinson and Dawes, 1992; Jamieson et al., 1994) mainly by the transcription factors Yap1p, Skn7p, Msn2p, and Msn4p (Morgan et al., 1997; Takeuchi et al., 1997; Lee et al., 1999; Gasch et al., 2000). These transcription factors collectively coordinate appropriate responses to different oxidative stressors, by repressing or upregulating the transcription of specific genes, many of which are associated with antioxidant defenses (Gasch et al., 2000; Temple et al., 2005).

Transcription factor Yap1p

The yeast activator protein 1 (AP-1), Yap1p, is a basic leucine zipper domain (b/ZIP) type transcription factor that bears great similarity to mammalian AP-1 type transcription factors such as c-Jun, in terms of sequence structure and DNA-binding affinity. It generally binds to a consensus Yap1p recognition element (YRE; 5′-TT/GAC/GTC/AA-3′) contained in the promoter sequences of several genes (Fernandes et al., 1997) and plays a key role in the cellular response of *S. cerevisiae* yeast cells to oxidative stress and xenobiotic insults, particularly drugs and heavy metals (Wemmie et al., 1994; Kolaczkowska and Goffeau, 1999; Gasch et al., 2000). In fact, Yap1p-deficient (Δ*yap1*) mutants exhibit hypersensitivity and an impaired adaptive response to oxidants such as H_2O_2 (Stephen et al., 1995), which is accompanied by reduced activities of antioxidants such as γ-L-glutamyl-L-cysteinylglycine or glutathione (GSH), SODs, and GSH reductase (Schnell et al., 1992).

Activation of Yap1p in response to oxidants, particularly H_2O_2, has been well studied (Wemmie et al., 1997; Delaunay et al., 2000, 2002; Kuge et al., 2001). Under normoxic conditions, most of the cell's Yap1p molecules are retained in the cytosol by the nuclear export activity of chromosome regional maintenance protein 1 (Crm1p), which is bound to a nuclear export signal (NES) in the C-terminal cysteine-rich domain (CRD) of Yap1p (Kuge et al., 1998). These redox-sensitive domains are highly conserved among Yap1p and its homolog proteins, and are essential for Crm1p-mediated nuclear export and binding (Kuge et al., 1998; Kudo et al., 1999). Because of their redox-sensitive nature, the CRDs easily respond to ROS-induced oxidative signaling. However, Yap1p is not directly oxidized by ROS (Delaunay et al., 2002).

In H_2O_2-induced oxidative stress, Yap1p is activated by a yeast GSH peroxidase (GPX)-like protein called oxidant receptor peroxidase 1 (Gpx3p/Orp1p). The cysteine residues of this peroxidase are themselves oxidized to cysteine-sulfenic acid (–SOH) groups by H_2O_2. The oxidized cysteine residues of Orp1p then react with the CRDs of Yap1p, inducing the formation of an intra-molecular disulfide bond between the cysteine residues of the Yap1p C- and N-terminal CRDs (Delaunay et al., 2002). This bond masks the NES contained in the C-terminal CRD and thus inhibits Yap1p-to-Crm1p bond formation, allowing the newly oxidized Yap1p transcription factor to migrate back into the nucleus (Delaunay et al., 2000). Once it approaches nuclear DNA, Yap1p initiates the transcription of specific genes by binding to the YREs contained in their promoter sequences (Lushchak, 2010). This Orp1p-mediated activation of Yap1p is further facilitated by Yap1p-binding protein (Ybp1p), which forms an H_2O_2-induced complex with Yap1p (Veal et al., 2003).

In response to H_2O_2, Yap1p promotes the transcription of several genes encoding antioxidants or antioxidant-associated molecules, such as those involved in the thioredoxin (TRX) and GSH systems. Upregulated sequences include *GSH1* (encoding γ-glutamylcysteine synthetase), *GSH2* (encoding GSH synthetase), *TRX2* (encoding TRX), *SOD1* (encoding CuZnSOD), *SOD2* (encoding MnSOD), and several others (Schnell et al., 1992; Stephen et al., 1995; Gasch et al., 2000). Activated Yap1p further upregulates expression of Ret tyrosine kinase inhibitor *RPI1* in yeast cells (Dumond et al., 2000). This represses the cAMP-dependent Ras/protein kinase A (Ras/PKA) signaling pathway which, under non-stress conditions, actively represses the activation of Yap1p and of other stress response transcription factors (Fernandes et al., 1997). The Ras/PKA pathway is then itself repressed by *RPI1* expression when cells are at stationary phase or exposed to oxidative stress (Dumond et al., 2000).

Oxidized Yap1p can be reduced and rendered H_2O_2-sensitive again by TRX-mediated reduction of the disulfide bond (Kuge and Jones, 1994; Delaunay et al., 2000; Kuge et al., 2001). TRX is in turn reduced by TRX reductase at the expense of reduced nicotinamide adenine dinucleotide (NADPH; Holmgren, 1989). Therefore, Yap1p's functionality is intimately associated with the redox status of the cell and with cellular NADPH content in particular (Lushchak, 2010). By promoting the synthesis of antioxidant molecules, Yap1p essentially sustains its own transcription activity until sufficiently inhibited by TRX, at which point the increase in antioxidants is likely sufficient for the cells to adapt to oxidant-induced stress (Delaunay et al., 2000; Temple et al., 2005).

Yap1p can also be activated via alternate pathways, as in the Orp1p- and Ybp1p-independent response to diamide, where the N- and C-terminal CRD cysteine residues of Yap1p do not form a disulfide between themselves, as they do in H_2O_2-induced activation (Wemmie et al., 1997; Delaunay et al., 2000; Kuge et al., 2001). Besides its sensitivity to ROS, Yap1p also regulates the yeast cell response to cadmium ion toxicity by upregulating transcription of

yeast cadmium factor *YCF1*. This sequence encodes a membrane transport protein involved in conferring cadmium tolerance to cells (Wemmie et al., 1994).

At least seven other Yap proteins – Yap2p to Yap8p – have been identified in *S. cerevisiae*. It has been suggested that these proteins each have different, albeit slightly overlapping, physiological roles in the regulation of cellular responses to oxidative stresses and xenobiotic insults, along with Yap1p (Fernandes et al., 1997).

Similarly to budding yeast, fission yeast *S. pombe* (Toone et al., 1998), facultative yeast pathogen *Candida albicans* (Zhang et al., 2000) and lactose-metabolizing yeast species *Kluyveromyces lactis* (Billard et al., 1997) each possess their own Yap1p homologs, which mediate responses to oxidative stress (Toone and Jones, 1999).

Transcription factor Skn7

The Skn7 protein is a kinase-regulated transcription factor that also modulates the response to oxidative insults in budding yeast. It possesses a C-terminus receiver domain which is essential for its role in the regulation of cell wall biosynthesis, the cell cycle, and the response to osmotic shock and to oxidative stress (Brown et al., 1994; Maeda et al., 1994; Morgan et al., 1995, 1997; Krems et al., 1996). The Skn7p transcription factor also shows homology to heat shock factor protein (Hsf1p). In fact, both Skn7p and Hsf1p upregulate heat shock proteins by binding to heat shock elements (HSEs) in H_2O_2-treated *S. cerevisiae* cells exposed to heat shock (Raitt et al., 2000).

Skn7p also acts as an auxiliary transcription factor to Yap1p, in response to oxidants such as H_2O_2 (Lee et al., 1999) where it upregulates proteins such as TRX reductase (encoded by *TRR1*) and TRX (Morgan et al., 1997). Both Yap1p and Skn7p upregulate the transcription of genes encoding proteins such as SODs, catalases, TRXs, and heat shock proteins (Lee et al., 1999). Other yeast species have their own respective homolog of Skn7p, such as Prr1p in *S. pombe* (Ohmiya et al., 1999).

Transcription factors Msn2p and Msn4p

Another two yeast transcription factors involved in the response of *S. cerevisiae* cells to oxidative stress are Msn2p and Msn4p. These are proteins with zinc-finger type DNA-binding domains. In response to a variety of general stresses such as osmotic shock, hypersalinity, heat stress, oxidants, and also diauxic transition, Msn2p and Msn4p are reversibly translocated to the nucleus, where they bind to the stress response element (STRE) 5′-CCCCT-3′contained in certain DNA promoter sequences (Martínez-Pastor et al., 1996; Schmitt and McEntee, 1996; Boy-Marcotte et al., 1998). They play a considerably important role in the response to oxidative stress in yeast (Gasch et al., 2000), as shown by the increased sensitivity of Δ*msn2*Δ*msn4* budding yeast mutants to high concentrations of H_2O_2 and their limited ability to adapt to low H_2O_2 concentrations (Hasan et al., 2002). Two antioxidant-encoding sequences are upregulated by Msn2p and Msn4p: *CTT1* (encoding cytosolic catalase) and *GRX1* (encoding glutaredoxin). Otherwise, they largely upregulate genes encoding proteases, heat shock proteins, metabolic enzymes, and other molecules implicated in stress recovery, restoration of metabolic homeostasis, and the repair or removal of damaged biomolecules (Hasan et al., 2002). Activation of general Msn2p/4p-mediated stress responses is negatively regulated by kinase enzyme signaling systems such as cAMP-dependent Ras/PKA activity (Hasan et al., 2002).

YEAST ANTIOXIDANTS AND THEIR ROLE IN YEAST OXIDATIVE STRESS RESPONSES

Following their upregulation by transcription factors and their subsequent synthesis, yeast cell antioxidant defenses remove excess ROS and restore redox balance. Prominent among these are mitochondrial MnSOD and cytosolic CuZnSOD, which scavenge $O_2^{\bullet-}$ and convert it to H_2O_2 (McCord and Fridovich, 1969; Weisiger and Fridovich, 1973a,b). The SOD-derived H_2O_2 can in turn be degraded to water and oxygen by the redox-sensitive heme groups of cytosolic catalase T (Ctt1p; Hartig and Ruis, 1986) and peroxisomal catalase A (Cta1p) enzymes (Cohen et al., 1988). Other enzymatic antioxidants include GPXs and TRX peroxidases/peroxiredoxins (PRX), which use electron-donating cysteine thiol groups as active sites to catalyze the reduction of hydroperoxides (Jamieson, 1998). In fact, GPXs protect against lipid peroxidation (Avery and Avery, 2001) and heavy metal toxicity (Avery et al., 2004) and help activate Yap1p transcription factors in response to H_2O_2 signaling (Delaunay et al., 2000; Gasch et al., 2000; Fomenko et al., 2011). PRX also respond to heavy metal toxicity (Nguyên-Nhu and Knoops, 2002) and zinc-metal deficiency (Wu et al., 2007), and reduce ROS, such as H_2O_2 and alkylhydroperoxides (Chae et al., 1994). PRX also reduce peroxynitrites (Wood et al., 2003).

Yeast cells also possess thiol oxidoreductase enzymes such as glutaredoxins and TRXs, which both catalyze the reduction of disulfides to thiols, using thiolated cysteine residues as an active site (Herrero et al., 2008). TRXs, the functionality of which is maintained by NADPH and the catalytic action of TRX reductase, restore functionality to oxidized PRX, maintain redox balance (Holmgren, 1989), and confer protection against hydroperoxides in yeast (Garrido and Grant, 2002). Similarly, glutaredoxins protect yeast against superoxide radicals (Luikenhuis et al., 1998) and hydroperoxides (Pujol-Carrion et al., 2006), reduce GSH-protein mixed disulfides and protein disulfides, and also play an important role in iron homeostasis (Yamaguchi-Iwai et al., 2002; Ojeda et al., 2006).

Glutathione, an abundant non-enzymatic antioxidant in yeast (Izawa et al., 1995; Zechmann et al., 2011), is required for the glutaredoxin-catalyzed reduction of disulfides and also reduces oxidized GPXs (Holmgren, 1989). Oxidized GSH (GSSG) is reduced back to GSH by GSH reductase, in a reaction that requires electrons from NADPH, the main source of which is the pentose phosphate pathway (Izawa et al., 1995; Juhnke et al., 1996). Besides being a crucial source of electrons for many antioxidants, GSH can also protect protein thiol groups from oxidation, by S-thiolation (Klatt and Lamas, 2000). Other prominent non-enzymatic yeast antioxidants include $O_2^{\bullet-}$-scavenging manganese ion complexes regulated by nutrient-sensing kinase pathways (Reddi et al., 2009; Reddi and Culotta, 2011), erythroascorbates, and vitamin E (Herrero et al., 2008).

INTRACELLULAR DEGRADATION OF OXIDIZED BIOMOLECULES

Despite the rapid upregulation and high efficiency of yeast antioxidant defenses, cell biomolecules can still sustain severe oxidative

damage that cannot be repaired. Irreparably damaged cell components such as carbonylated proteins and oxidatively damaged mitochondria must be efficiently removed before they are allowed to form large aggregate masses that lead to cell death. Yeast has two stress response mechanisms that facilitate the removal of irreparably oxidized biomolecules: the ubiquitin-dependent proteasome system (UPS) and autophagy.

The ubiquitin-dependent proteasome system in yeast

The UPS is a highly conserved mechanism of targeted protein degradation, which facilitates the removal of small oxidized proteins from cells. The precise mechanism by which polyubiquitinated substrates are delivered to the proteasome is presently unclear, despite the identification of key UBL/UBA ubiquitin receptor proteins such as Rad23 (Schauber et al., 1998; Chen and Madura, 2002; Elsasser et al., 2004), Dsk2 (Wilkinson et al., 2001), and Ddi1 (Bertolaet et al., 2001) in yeast. A recent study by Li et al. (2010) has shed some light on this aspect, demonstrating that Rad4, a regulatory protein previously associated only with nuclear excision repair (NER) of DNA, together with Rad23, regulate a post-ubiquitination event, presumably the delivery of specific ubiquitinated substrates to the proteasome. In fact, Rad4 shares specific substrates with Rad23 and possesses a binding domain which is crucial for Rad23 to exert its degradative function. The authors suggested that Rad4 plays an accessory role in proteolysis by keeping the Rad23 protein in the required active conformation to associate with ubiquitinated substrates. On the other hand, Rad23 is believed to play an accessory role in NER, by protecting Rad4 from degradation by proteins such as the yeast deubiquitinase Ubp3 (Mao and Smerdon, 2010).

Because the UPS mainly operates in the cytosol, oxidized proteins located within cell compartments segregated by membranes, such as the ER and the mitochondria, cannot be degraded by this mechanism unless they are exported into the cytosol (Taylor and Rutter, 2011). In fact, an ER-associated degradation (ERAD) pathway facilitates the translocation of oxidized or misfolded ER proteins to the cytosol for UPS-mediated proteasomal degradation (Ye et al., 2001). An ERAD-like mechanism, called the mitochondria-associated degradation (MAD) pathway, has also been proposed whereby oxidized mitochondrial proteins are extruded through the outer mitochondrial membrane into the cytosol for UPS-mediated degradation (Chatenay-Lapointe and Shadel, 2010; Heo et al., 2010).

Autophagic pathways in yeast

Autophagy involves the intracellular degradation and recycling of long-lived biomolecules such as macromolecular proteins and organelles, by lysosomes (in mammalian cells) or vacuoles (in yeast cells; Klionsky et al., 2010). These pathways are activated by the expression of autophagy-related genes (*ATG*), of which there are at least 33 in yeast, along with many other *ATG* homologs in mammals (Reggiori and Klionsky, 2002; Goldman et al., 2010). Autophagy is indeed a highly conserved process among eukaryotes. It is involved in metazoan cell differentiation and plays a crucial pro-survival role in response to stresses such as nutrient starvation and ROS accumulation in eukaryotes such as yeast (Kissová et al., 2004, 2006, 2007; Bhatia-Kissová and Camougrand, 2010). In fact, deficiency of autophagy in yeast causes ROS accumulation after stresses such as nitrogen starvation (Suzuki et al., 2011). The protective role of autophagy against oxidative stress is also very evident in ROS-associated pathologies such as aging (Donati et al., 2001) and neurodegenerative diseases (Rubinsztein, 2006), where autophagic pathways are largely disrupted and cells suffer extensive intracellular aggregation of oxidized proteins (Terman and Brunk, 1998; Rubinsztein, 2006; Scherz-Shouval and Elazar, 2007; Rubinsztein et al., 2011).

The principal autophagic pathway in yeast and in other eukaryotes is macroautophagy. This involves the non-selective sequestration of intracellular macromolecules and portions of cytosol by a double-membraned vesicle called the autophagosome, which then delivers its enclosed material to a vacuole (or lysosome in higher eukaryotes) replete with hydrolytic enzymes that facilitate biomolecular degradation (Klionsky et al., 2010). Another non-selective autophagic pathway, called microautophagy, involves the direct sequestration and degradation of organelles and portions of cytoplasm by vacuoles/lysosomes themselves, excluding the involvement of autophagosomes. Autophagy predominantly proceeds via these two non-selective pathways, both of which take place in yeast depending on factors such as the metabolic state of the cells (Kissová et al., 2007).

Selective autophagic degradation of specific biomolecular targets can also take place, often in conjunction with non-selective autophagy (Nair and Klionsky, 2005; Kissová et al., 2007; Kraft et al., 2009). This includes selective autophagic degradation of the Golgi apparatus (crinophagy; Glaumann, 1989), ER (reticulophagy; Hamasaki et al., 2005), ribosomes (ribophagy; Kraft et al., 2008), peroxisomes (pexophagy; Sakai et al., 1998), and mitochondria (mitophagy; Lemasters et al., 1998; Lemasters, 2005; Kissová et al., 2007).

In terms of its physiological role in yeast, it is believed that selective autophagy is required for (i) selective removal of defective organelles under normal physiological conditions to maintain homeostatic cell structure integrity, and (ii) specific removal of excess or damaged organelles in response to stresses such as nutrient starvation and ROS accumulation (Goldman et al., 2010). During oxidative stress, autophagic pathways involved in the specific removal of mitochondria assume particular importance (Bhatia-Kissová and Camougrand, 2010). Mitochondria, as previously described, constitute a major production site and target of ROS, therefore the risk of accumulation of defective mitochondria in cells increases during oxidative stress. If left unabated, such accumulation will only lead to further cell oxidative damage and eventual disruption of cell homeostasis. Therefore the rapid removal of these dysfunctional organelles, a function facilitated by mitophagy, is of paramount importance (Lemasters, 2005; Scherz-Shouval and Elazar, 2007; Bhatia-Kissová and Camougrand, 2010).

Mitophagy in yeast as a response to oxidative stress

Mitochondrial degradation by autophagy in yeast was first reported by Takeshige et al. (1992), who observed sequestered mitochondria in the autophagic bodies of yeast cells subjected to nitrogen starvation, after growth on glucose (Takeshige et al., 1992). Similarly, Ashford and Porter (1962) had earlier reported an abundance of mitochondria in rat hepatocyte autophagosomes.

These observations suggested that autophagy is a primary removal mechanism of mitochondria in cells. In fact, subsequent studies showed that selective autophagic degradation of mitochondria, defined by Lemasters (2005) as mitophagy, can indeed take place in yeast in response to adverse stimuli involving mitochondrial damage and ROS accumulation (Goldman et al., 2010).

Campbell and Thorsness (1998) were among the first to indicate a link between mitochondrial damage and the upregulation of specific mitochondrial degradation in yeast. *S. cerevisiae* Δ*yme1* mutants, the mitochondria of which were impaired by the absence of yeast mitochondrial ATP-dependent metalloprotease enzyme Yme1p, exhibited increased migration of mtDNA from their dysfunctional mitochondria, when grown on ethanol/glycerol medium. This was accompanied by the increased degradation and proximity of dysfunctional mitochondria to the surfaces of degradative vacuoles, although this did not conclusively demonstrate that the mitochondria were degraded by autophagy.

Priault et al. (2005) then unequivocally demonstrated the preferential removal of dysfunctional mitochondria by autophagy in yeast. It was shown that Δ*fmc1* yeast mutants, the mitochondria of which had an impaired mitochondrial membrane potential due to aggregation of mitochondrial ATP synthase, exhibited preferential degradation of their dysfunctional mitochondria by mitophagy under anaerobic conditions. The possibility that autophagy was induced by ATP depletion (due to ATP synthase aggregation) as opposed to mitochondrial damage was excluded, given that Δ*fmc1* yeast cells had similar cellular ATP levels to the wildtype cells. Further studies showed that osmotic swelling of mitochondria due to impairment of K^+/H^+ exchange proteins (Nowikovsky et al., 2007) and impairment of the mitochondrial membrane potential due to malfunctioning mtDNA replication machinery (Zhang et al., 2007), also initiate mitophagy.

Kissová et al. (2006) further showed that upregulation of mitophagy in wildtype yeast cells, in response to the target of rapamycin (TOR) kinase inhibitor, rapamycin, is accompanied by early ROS accumulation and mitochondrial membrane lipid peroxidation. The addition of resveratrol to rapamycin-treated cells inhibited these oxidative effects and largely impaired mitophagy, clearly suggesting that ROS-induced mitochondrial damage also plays a crucial role in activating this pathway. This is further corroborated by the fact that, unlike macroautophagy, selective mitophagy is specifically suppressed by the antioxidant *N*-acetylcysteine (NAC), which replenishes the cellular GSH pool (Deffieu et al., 2009; Kissová and Camougrand, 2009). This showed that mitophagy is sensitive to cellular redox imbalances induced by the depletion of GSH and that it is a discretely, independently regulated pathway.

Key genetic evidence that mitophagy is a discrete process, regulated independently of non-selective macroautophagy in yeast, was provided by Kissová et al. (2007). Nitrogen-starved wildtype yeast cells, grown under aerobic conditions, exhibited early selective mitochondrial autophagy followed by non-selective autophagic removal of mitochondria after prolonged stress. The early selective mitophagic process is dependent on an outer mitochondrial membrane protein, Uth1p. Removal of this protein, facilitated by the deletion of *UTH1*, resulted in the activation of only the non-selective (Uth1p-independent) autophagic degradation of mitochondria. Subsequent genetic screening has since identified that other sequences, such as *ATG32* (Kanki et al., 2009a; Okamoto et al., 2009) and *ATG33* (Kanki et al., 2009b) also encode proteins without which mitophagy cannot take place, further proving it to be a discretely regulated process.

Overall, these studies established that, in yeast, mitochondrial damage stimulates the specific degradation of mitochondria by mitophagy. This implies that the physiological role of mitophagy is that of a discretely controlled cellular surveillance mechanism which monitors mitochondrial functionality and specifically removes irreparably damaged mitochondria (Goldman et al., 2010). Given that such damage can be induced by ROS accumulation (Kissová et al., 2006), it is likely that mitophagy also constitutes an important defense against oxidative stress in yeast, in conjunction with the other antioxidant defenses described previously.

However, mitophagy's hypothetic role as a mitochondrial functionality control system in yeast is uncertain, given that mitophagy-deficient Δ*Atg32* mutants did not show any significant increase in ROS and did not exhibit reduced growth in comparison to wildtype cells, when grown on non-fermentable media (Kanki et al., 2009a; Okamoto et al., 2009). This might imply that mitophagy is a non-essential and redundant process in yeast, with respect to non-selective autophagy. This is surprising, given that mitophagy's role as a mitochondrial functionality control mechanism in mammalian cells has been established (Narendra et al., 2008; Geisler et al., 2010; Matsuda et al., 2010), naturally leading to the assumption that it has the same such role in yeast. Clearly, further studies of mitophagy's poorly understood mechanism and of its regulation are required for this issue to be resolved. Progress in this regard is already being made, given the recent discovery of two mitogen-activated protein kinases (MAPKs), Slt2p and Hog1p, which regulate mitophagy in yeast (Mao et al., 2011).

Also, the physiological role of mitophagy as a clearance mechanism of excess mitochondria has been confirmed by studies of stationary phase yeast cells grown under respiratory conditions (Kanki and Klionsky, 2008). Such clearance behavior serves to reduce the high metabolic cost of maintaining excess mitochondria, which are made redundant by the reduced growth demands of stationary phase cells, whilst concurrently lowering the risk of cellular ROS accumulation (Kanki et al., 2011).

PROGRAMMED CELL DEATH AND OXIDATIVE STRESS IN YEAST

In the event that yeast cells suffer prolonged or sudden exposure to harmful doses of ROS, it is often the case that their pro-survival response mechanisms and antioxidant defenses fail. This consequently elicits the yeast cell's final response to oxidative stress: the initiation of PCD (Madeo et al., 1999). This is essentially an "orderly" process of cell elimination, which can proceed via different pathways, depending on the physiological state of the cells and the nature of the oxidative insult. At least two main established PCD pathways have been observed in yeast in response to oxidative stress: the first is apoptosis, which is typically induced by exposure to low doses of oxidants such as H_2O_2 (Madeo et al., 1999). Alternatively, yeast cells can undergo necrosis, a PCD pathway which, until recently, was conveniently

dismissed as uncoordinated and accidental (Eisenberg et al., 2010; Galluzzi et al., 2011, 2012). Finally, even autophagic pathways such as mitophagy have been implicated in ROS-induced PCD in yeast (Bhatia-Kissová and Camougrand, 2010), which is intriguing given their main established role as pro-survival pathways.

OXIDATIVE STRESS AND APOPTOSIS IN YEAST

The first observation of an apoptotic phenotype in yeast cells was made by Madeo et al. (1997), who observed that mutant *S. cerevisiae* cells lacking the ATPase AAA cell division cycle gene *CDC48* (Latterich et al., 1995) showed key morphological hallmarks of mammalian apoptosis such as phosphatidylserine externalization, chromatin condensation, and DNA fragmentation. Since then, further markers characteristic of mammalian apoptosis have been discovered in yeast, including the release of mitochondrial cytochrome *c* (Manon et al., 1997; Ludovico et al., 2002; Sapienza et al., 2008), and the execution of conserved proteolytic pathways associated with mammalian apoptosis, such as Cdc6 protein degradation (Blanchard et al., 2002).

Undoubtedly, the most compelling evidence of apoptosis in yeast is derived from the discovery of several orthologs of key mammalian apoptotic regulators, such as the yeast metacaspase protein Yca1p/Mca1p (Madeo et al., 2002), apoptosis inducing factor Aif1p (Wissing et al., 2004), the Htr2A/Omi serine protease Nma111p (Fahrenkrog et al., 2004), inhibitor of apoptosis protein (IAP) Bir1p (Walter et al., 2006), and the mitochondrial endonuclease G Nuclp (Büttner et al., 2007). The yeast NADH dehydrogenase Ndi1p, located in the inner mitochondrial membrane, has also been implicated in cell death (Li et al., 2006). It is in fact a homolog of AIF-like mitochondrial-associated inducer of cell death (AMID; Li et al., 2006), a protein associated with caspase-independent apoptosis in humans (Wu et al., 2002). Similarly, the yeast protein Cdc48 also has a human ortholog that negatively regulates apoptosis (Shirogane et al., 1999). Yeast also possesses a true homolog of highly conserved (Chae et al., 2003) Bax-inhibitor BI-1 proteins called Bxi1p (Cebulski et al., 2011), anti-apoptotic Bax-suppressor homologs called Sno1p and Fyv10p (Khoury et al., 2008), and also a pro-apoptotic BH3 homolog called Ybh3p (Büttner et al., 2011). Furthermore, apoptotic markers have consistently appeared in aging yeast cells (Laun et al., 2001; Herker et al., 2004). ROS, which have a central role in regulating mammalian apoptosis, also act as crucial modulators of apoptosis in yeast (Madeo et al., 1999). We have confirmed this by specific ROS probes (unpublished observations). This wealth of evidence has largely confirmed the existence of a highly conserved apoptotic pathway in yeast, which seems to have evolved early on as a response to harmful stimuli that are in large part associated with ROS and oxidative stress.

Proponents of apoptosis in yeast have also presented a strong hypothesis to account for its physiological role in an organism which, despite being unicellular, naturally exists in the large, densely packed cell populations that make up yeast colonies (Váchová and Palková, 2007). With this paradigm, apoptosis in yeast was suggested to be an altruistic clearance mechanism of old or damaged cells, designed to free up nutrients and enhance the survival chances of younger, healthier clones in aging, or stressed yeast colonies (Fabrizio et al., 2004; Herker et al., 2004; Knorre et al., 2005). It has also been suggested that apoptosis-associated ROS accumulation may raise the chances of clone mutation in a yeast population, leading to an increased number of genetically varied individual cells, which are better adapted to altered environmental conditions (Fabrizio et al., 2004).

Still, the notion of apoptosis in yeast has been met with skepticism by some researchers, a number of whom have suggested that the standard flow-cytometric techniques used to assay Yca1p activity and ROS accumulation in yeast cells are artifact-prone, implying that previous evidence of yeast apoptosis was artifactual and that yeast cell death is independent of both ROS and Yca1p (Wysocki and Kron, 2004). Counter-arguments to these claims assert that these authors exposed their yeast cells to extreme death conditions, which either induced non-apoptotic cell death or caspase-independent apoptosis (LeBrasseur, 2004), the latter of which has since been observed in yeast cells, and which accounts for the rough majority of reported apoptotic death scenarios in yeast (Madeo et al., 2009).

Some have also proposed that Yca1p, the yeast ortholog of mammalian caspases (the cysteine-protease enzymes which mediate mammalian apoptosis) is not a true caspase at all and has no caspase-like activity. The argument behind this is that, whilst metacaspases and caspases probably share a common origin given the presence of a conserved caspase fold in both proteins, this does not justify putting them into the same protein family, since evolutionary conservation of protein structure does not necessarily mean that protein function is also conserved (Vercammen et al., 2007). The differing substrate affinity of metacaspases, attributed to their preference for protein cleavage-sites preceding basic arginine residues, as opposed to the preference of caspases for cleavage-sites preceding acidic aspartate residues, helped to strengthen the argument that the physiological function of metacaspases differs from that of caspases (Vercammen et al., 2007). However, it has since been shown that, despite their proteolytic discrepancies, metacaspases and caspases do indeed share a number of common substrates known to be involved in PCD, such as Tudor staphylococcal nuclease (TSN) derived from *Pinus abies* (Norway Spruce Tree; Sundström et al., 2009; Carmona-Gutierrez et al., 2010b).

Whilst many of its core aspects, such as the mechanics of yeast metacaspase activity, are still poorly understood (Abdelwahid et al., 2011), the paradigm of yeast apoptosis has by now been largely accepted within the research community, in spite of the lingering doubts expressed by some.

One aspect that leaves little room for doubt is the role of ROS as a key inducer of apoptotic cell death in yeast, as shown early on by Madeo et al. (1999) who observed significant $O_2^{\bullet -}$ accumulation in yeast cells undergoing H_2O_2-induced apoptosis – a cell death scenario which is exacerbated by deletion of *GSH1* and prevented by hypoxia or quenching of ROS using spin traps (Madeo et al., 1999) and antioxidants (Jeon et al., 2002). In fact, ROS accumulation and the involvement of mitochondria, where this usually takes place, are arguably the most unifying features shared by apoptotic yeast cells treated with different pro-apoptotic stimuli (Pereira et al., 2008). These include exogenous stimuli, ranging from metals such as copper (Liang and Zhou, 2007), lead (Bussche and Soares, 2011), iron (Almeida et al., 2008), and cadmium

(Nargund et al., 2008) to plant toxins (Narasimhan et al., 2005), amiodarone (Pozniakovsky et al., 2005), ethanol (Kitagaki et al., 2007), and anti-tumor drugs including paclitaxel (Foland et al., 2005), arsenic (Du et al., 2007), valproate (Mitsui et al., 2005), and aspirin which commits yeast cells lacking mitochondrial MnSOD to apoptosis by disrupting the cellular redox balance (Sapienza and Balzan, 2005; Sapienza et al., 2008). There are also several ROS-associated endogenous stimuli of apoptosis in yeast, which include aging (Laun et al., 2001; Herker et al., 2004) and the disruption of metabolic pathways involved in DNA metabolism (Weinberger et al., 2005), protein modification (Hauptmann et al., 2006), and actin dynamics (Gourlay and Ayscough, 2006).

Furthermore, yeast cell death induced by ROS-associated stress stimuli can proceed either via caspase-dependent apoptosis or caspase-independent apoptosis (Madeo et al., 2009), as described below.

Oxidative stress and caspase-dependent apoptosis in yeast

Caspase-dependent apoptosis in yeast was first reported by Madeo et al. (2002), who observed that yeast cells undergoing H_2O_2-induced apoptotic cell death exhibited an upregulation of caspase-like proteolytic activity, attributed to yeast caspase protein 1, Yca1p, encoded by the *YCA1* gene. The absence of Yca1p prevented H_2O_2-induced apoptosis entirely, whereas its overexpression exacerbated apoptosis, providing the first clear evidence of an apoptotic pathway in yeast which is caspase-dependent and induced by ROS.

Acetic acid-induced PCD of yeast cells, which bears typical markers of mammalian apoptosis, is also accompanied by an accumulation of ROS in yeast cell compartments such as the mitochondria (Ludovico et al., 2001, 2002) and can involve Yca1p (Madeo et al., 2002; Guaragnella et al., 2006). It has been suggested that H_2O_2 triggers acetic acid-induced PCD in yeast, given the early accumulation of H_2O_2 in acetic acid-induced cell death. This is corroborated by the observed absence of H_2O_2 buildup in acid-stress adapted yeast cells in which acetic acid-induced PCD is prevented (Giannattasio et al., 2005; Guaragnella et al., 2007). In fact, further studies have shown that the quenching of acetic acid-induced ROS, using antioxidants such as NAC, abrogates both cytochrome *c* release and Yca1p activity in wildtype yeast cells, preventing cell death altogether. However, it is worth noting that NAC does not prevent acetic acid-induced death in yeast knockout mutants deficient in Yca1p and cytochrome *c*, implying that acetic acid-induced PCD can also proceed along pathways that are Yca1p and ROS-independent (Guaragnella et al., 2010).

The accumulation of ROS is also implicated in yeast apoptosis induced by salt stress (NaCl; Wadskog et al., 2004), heat stress (Lee et al., 2007), and hyperosmotic stress (Silva et al., 2005) which, in all cases, cannot take place without Yca1p activity. Stress induced by disruption of iron homeostasis (Almeida et al., 2008), cadmium (Nargund et al., 2008), and arsenic (Du et al., 2007) can also result in a caspase-mediated apoptotic phenotype associated with significant accumulation of ROS. Likewise, both Yca1p activity and ROS accumulation are key requirements for apoptosis in yeast cells treated with low to moderate doses of virally encoded killer toxins (Reiter et al., 2005).

Apoptosis induced by the heterologous expression, in yeast, of Parkinson's disease-associated protein α-synuclein, also requires the activation of Yca1p. The latter's removal totally abolishes α-synuclein's ability to induce ROS accumulation and apoptotic cell death. Likewise, the addition of antioxidant GSH prevented α-synuclein-induced apoptosis in yeast, further demonstrating the important mediatory role of ROS in this pathway (Flower et al., 2005).

Treatment of yeast cells with drugs such as valproic acid, a teratogenic, short chain fatty acid with anticonvulsant and anti-tumor properties (Blaheta and Cinatl, 2002), also induces a significant accumulation of ROS that in turn upregulates the activity of Yca1p and initiates apoptotic cell death (Mitsui et al., 2005). Recent studies have also shown that propolis, a product derived from plant resins, which is utilized by bees to protectively seal the apertures in their hives, can elicit caspase-dependent apoptosis associated with ROS accumulation in yeast cells (de Castro et al., 2011). This corroborates the potential use of propolis as an antifungal agent.

Additionally, mutations that induce defects in yeast cellular processes such as those associated with mRNA stability (Mazzoni et al., 2005), initiation of DNA replication (Weinberger et al., 2005), ubiquitination (Bettiga et al., 2004), and mitochondrial fragmentation (Fannjiang et al., 2004) can induce Yca1p-mediated apoptotic cell death accompanied by ROS accumulation. A marked increase in ROS is also observed during pro-apoptotic chronological aging of yeast cells, the survival of which is initially improved by deletion of *YCA1* (Herker et al., 2004). However, aging Δ*yca1* yeast mutants later lose the ability of regrowth, as opposed to aged wildtype cells, which actually outlive their *YCA1*-deficient counterparts (Herker et al., 2004). This is likely because Δ*yca1* yeast mutants suffer greater accumulation of oxidized proteins than the wildtype cells (Khan et al., 2005), thus indicating the importance of Yca1p-dependent apoptosis as a clearance mechanism of aged or oxidatively damaged cells in a yeast colony (Herker et al., 2004; Madeo et al., 2009).

Finally, it has been argued that the caspase-like activity detected upon stimulation of apoptosis in yeast is not entirely attributable to Yca1p, this implicating the participation of other caspase-like proteases (Wilkinson and Ramsdale, 2011). One such protein is Esp1p, which plays an important pro-apoptotic role in yeast treated with ROS such as H_2O_2. Upon induction of apoptosis, Esp1p is released from anaphase inhibitor Pds1p, and cleaves Mcd1p (the yeast homolog of human cohesin subunit Rad21) such that the latter's C-terminal fragment migrates from the nucleus to the mitochondria. This induces disruption of the mitochondrial membrane potential and cytochrome *c* release, followed by apoptosis (Yang et al., 2008). Another yeast protein associated with caspase-like activity in yeast is Kex1 protease, which mediates apoptotic cell death induced by defective N-glycosylation, acetic acid and chronological aging (Hauptmann and Lehle, 2008).

Oxidative stress and caspase-independent apoptosis in yeast

Apoptotic cell death in yeast caused by ROS-associated stress stimuli, can also take place without the need for Yca1p activity. For instance, ROS accumulation and apoptosis caused by defective N-glycosylation in Ost2p-deficient yeast cells (Ost2p being the yeast homolog of mammalian defender of apoptosis-1 DAD1) are not prevented by deletion of *YCA1* (Hauptmann et al., 2006). Similarly,

absence of Yca1p activity does not impede ROS accumulation, mitochondrial degradation, and apoptosis of yeast cells treated with formic acid (Du et al., 2008). Caspase-independent apoptosis triggered by release of ammonia in differentiating yeast cell colonies (Váchová and Palková, 2005), in yeast cells treated with copper (which generates ROS via Fenton/Haber–Weiss pathways; Liang and Zhou, 2007), and in yeast cells treated with ceramide (Carmona-Gutierrez et al., 2011b) also share the common feature of ROS accumulation. In fact, the latter two apoptotic scenarios are both suppressed in mtDNA-deficient *rho*^{o} (ρ^{o}) "petite" mutant cells, where mitochondrial respiration is impaired. Likewise, functional mitochondria are a key requirement of caspase-independent apoptosis of aging yeast cells caused by heterologous expression of α-synuclein, and characterized by massive ROS accumulation (Büttner et al., 2008).

The requirement of functional mitochondria in caspase-independent yeast apoptosis is no surprise given that, aside from their major role as a source and target of ROS, mitochondria are also the source of important yeast apoptotic regulators capable of operating independently of Yca1p. One such regulator is the yeast AIF homolog Aif1p (Wissing et al., 2004). This protein can translocate from the mitochondrial intermembrane space to the nucleus, in response to oxidative pro-apoptotic stimuli such as H_2O_2, aging, acetic acid (Wissing et al., 2004), and fungal toxins such as bostrycin (Xu et al., 2010). Like its mammalian counterpart, Aif1p operates in tandem with cyclophilin A once it is inside the nucleus, inducing chromatin condensation, DNA fragmentation, and subsequent apoptotic cell death (Candé et al., 2004; Wissing et al., 2004). Similarly, the endonuclease G yeast homolog Nuc1p translocates from the mitochondria to the nucleus, in response to oxidative stimuli such as H_2O_2, resulting in a caspase-independent apoptotic phenotype involving ROS accumulation (Büttner et al., 2007).

Additionally, the yeast nucleus itself contains the Yca1p-independent death regulator Nma111p, the yeast homolog of pro-apoptotic mammalian serine protease HtrA2/Omi. In response to heat stress and oxidative stimuli such as H_2O_2, Nma111p starts to aggregate in the nucleus and induces yeast cell apoptosis in a manner that is dependent on its serine protease activity (Fahrenkrog et al., 2004). The pro-apoptotic effects of Nma111p are suppressed by its antagonistic substrate Bir1p, the yeast homolog of mammalian IAP, as demonstrated by the enhanced apoptotic phenotype of Δ*bir1* yeast cells exposed to oxidative stress (Walter et al., 2006).

Finally, yeast nuclei contain another trigger of caspase-independent apoptosis, which is induced by ROS such as H_2O_2. This involves the epigenetic modification of the chromatin histone structure H2B, the N-terminal tail of which is deacetylated at lysine 11 by histone deacetylase (HDAC) Hos3, and subsequently phosphorylated at serine residue 10 by Ste20 kinase (Ahn et al., 2005, 2006), which also plays a key role in pheromone-induced apoptotic cell death in yeast (Severin and Hyman, 2002). The resulting chromatin modification promotes apoptosis.

OXIDATIVE STRESS AND NECROSIS IN YEAST

Yeast can also undergo necrotic cell death in response to oxidative stress (Madeo et al., 1999). Necrosis is characterized by bioenergetic failure and morphological features such as random DNA fragmentation, an increase in cell volume or oncosis, swelling of organelles, loss of cell plasma membrane integrity, and subsequent leakage of intracellular contents (Zong and Thompson, 2006). Because of its seemingly disordered features, necrosis was long dismissed as an accidental form of cell death (Galluzzi et al., 2011) which generally occurs in response to extreme environmental stresses (Madeo et al., 1999). However, this paradigm has changed, given recent evidence that certain necrotic cell death scenarios are regulated by factors such as signaling and catabolic proteins (Baines, 2010). The fact that necrosis can be regulated by the active involvement of such proteins, provides strong evidence that it can indeed proceed as a programmed pathway referred to as "programmed necrosis" (Galluzzi et al., 2012). Furthermore, accumulating evidence suggests that, as in apoptosis, the accumulation of ROS and the presence of mitochondria are key requirements of this form of PCD, both in mammals and in yeast (Baines, 2010; Eisenberg et al., 2010).

In yeast cells, exposure to very high concentrations of pro-apoptotic stimuli including H_2O_2 (Madeo et al., 1999), acetic acid (Ludovico et al., 2001), and heavy metals (Liang and Zhou, 2007), generally causes accidental necrosis as a result of very severe oxidative damage to cell components. Acetic acid also causes disruption of pH homeostasis (Ludovico et al., 2001). However, considerable evidence suggests that yeast cells also possess a programmed necrotic cell death pathway similar to that of their mammalian counterparts, where many of the conditions and processes which regulate programmed necrosis in mammals have also been conserved (Galluzzi et al., 2011).

For instance, vacuolar dysfunction and subsequent cytoplasmic acidification can commit yeast cells to necrosis (Schauer et al., 2009), just like in mammalian cells (Hitomi et al., 2008). Necrosis in yeast is also regulated by yeast heat shock protein Hsp90 (Dudgeon et al., 2008), the homolog of human Hsp90p. The latter is a cytosolic chaperone of several kinases, one of which is the pro-necrotic signaling protein RIP1 kinase (Lewis et al., 2000; Vanden Berghe et al., 2003). Whilst a clear homolog of RIP1 kinase in yeast has not yet been elucidated, yeast Hsp90p has proved to be a key requirement for tunicamycin-induced necrosis of yeast cells deficient in calcineurin, a phosphatase enzyme that suppresses necrosis in yeast (Dudgeon et al., 2008). Additionally, necrotic death under these conditions is preceded by the accumulation of ROS, implying that oxidative stress is an important inducer of this death phenotype in yeast, as in mammals.

Further evidence of the involvement of ROS in programmed necrosis in yeast is the fact that yeast mitochondria have also been implicated as a key requirement of this pathway. For instance, necrosis induced by expression of proteinaceous elicitor harpin (Pss) derived from *Pseudomonas syringae*, was avoided altogether in *S. cerevisiae rho*$^{-}$ (ρ^{-}) "petite" cells containing dysfunctional mitochondria (Sripriya et al., 2009). Similarly, functional mitochondria are required for both apoptotic and necrotic cell death of aging yeast cells induced by heterologous expression of α-synuclein, which is accompanied by ROS accumulation and disruption of mitochondrial membrane potential (Büttner et al., 2008). Even unsaturated free fatty acid (FFA)-induced necrosis in yeast depends on the presence of functional mitochondria and

is accompanied by the accumulation of ROS (Rockenfeller et al., 2010).

Peroxisomes, which generate ROS such as H_2O_2, can also act as key regulators of necrosis in yeast. Deletion of *PEX6*, encoding a crucial component of the peroxisomal protein import machinery in *S. cerevisiae* cells, caused increased sensitivity to acetic acid-induced stress, accompanied by necrosis and ROS accumulation in cells approaching stationary phase growth (Jungwirth et al., 2008). Similarly, removal of peroxisomal peroxiredoxin Pmt20 induced massive necrotic cell death accompanied by pronounced ROS accumulation and lipid peroxidation in the yeast *Hansenula polymorpha* (Bener Aksam et al., 2008).

Another prominent regulator of necrosis in yeast is the mitochondrial yeast endonuclease G homolog Nuc1p, which also triggers apoptosis when released from the mitochondria. Deletion of its coding sequence *NUC1* was found to inhibit apoptotic death of yeast cells grown on glycerol medium, whilst enhancing necrosis of aging or peroxide-treated yeast cells grown on fermentative glucose medium (Büttner et al., 2007).

Similarly, a recent study has shown that deletion of the *PEP4* sequence, encoding the yeast ortholog of mammalian cathepsin D, Pep4p, upregulates necrotic and apoptotic death in chronologically aging yeast cells. Conversely, prolonged hyperexpression of Pep4p increased cell longevity by specifically inhibiting necrosis. This anti-necrotic effect involved epigenetic deacetylation of histones mediated by polyamines, the biosynthesis of which was enhanced by the expression of Pep4p (Carmona-Gutiérrez et al., 2011a). Polyamines are molecules with antioxidant properties, which suppress the oxidative and inflammatory stresses associated with aging (Løvaas and Carlin, 1991). In fact, polyamine cell content is known to decline as aging progresses in both yeast (Eisenberg et al., 2009; Carmona-Gutiérrez et al., 2011a) and mammals (Scalabrino and Ferioli, 1984; Nishimura et al., 2006).

Eisenberg et al. (2009) were the first to establish an association between age-induced decline of polyamine content and necrosis in yeast cells, reporting a large percentage of chronologically aging yeast cells that were necrotic and that depletion of natural yeast cell polyamines not only accelerated necrosis, but increased ROS accumulation and shortened cell lifespan. Conversely, addition of the polyamine spermidine actually prevented necrosis, reduced oxidative stress, and increased longevity. Intriguingly, the cytoprotective effects of spermidine were mediated by the activation of autophagy, and were in fact abolished upon deletion of crucial autophagy genes such as *ATG7*, further demonstrating the pro-survival role of autophagy under conditions of age-related stress. Furthermore, the study showed that spermidine-induced suppression of necrosis in aging yeast cells is mediated by epigenetic deacetylation of histones (Eisenberg et al., 2009).

In the light of all this evidence, it is clear that necrosis in yeast is indeed a highly regulated and therefore PCD pathway. Its involvement in highly physiological processes such as aging, along with its requirement of functional mitochondria and, in particular, the fact that it can be actively suppressed by mechanisms as intricate as epigenetic modulation, all strongly support this argument. Furthermore, given the near ubiquitous involvement of ROS in various yeast necrotic scenarios, it is evident that oxidative stress and ROS accumulation play an important, if not central role in programmed necrosis in yeast, just like in mammals (Eisenberg et al., 2010). At present, however, the full extent of this role remains to be elucidated.

From a physiological perspective, the role of programmed necrosis as opposed to apoptosis in yeast has been suggested to be that of a "noisy" response, which involves the release of damage-associated molecular patterns (DAMPs) that could act as warning signals for surviving cells in a colony under extreme stress (Galluzzi et al., 2011). This proposed hypothesis is corroborated by the observed release, from necrotic yeast cells, of Nhp6Ap (Eisenberg et al., 2009). This is the yeast ortholog of mammalian high-mobility group box 1 (HMGB1), which serves as a danger signal (Apetoh et al., 2007). However, whether Nhp6Ap shares the same function as HMGB1 remains to be elucidated (Galluzzi et al., 2011).

OXIDATIVE STRESS, AUTOPHAGY, AND CELL DEATH IN YEAST

Although primarily a homeostatic pro-survival response to stresses such as nutrient depletion and ROS accumulation, it has been suggested that autophagy might also act as a mediator of cell death when it occurs at high levels (Pattingre et al., 2005), given observed instances of numerous autophagic bodies present in the dying cells of various organisms (Tsujimoto and Shimizu, 2005; Kourtis and Tavernarakis, 2009). Amongst these organisms are yeast cells, where large scale autophagy induced by stimuli such as rapamycin is followed by growth arrest and a sharp decline in cell viability (Kissová et al., 2004). Also, as stated earlier, addition of rapamycin to respiring yeast cells induces selective mitophagy (Kissová et al., 2007). Furthermore, rapamycin-induced autophagy is accompanied by early ROS accumulation and early mitochondrial lipid peroxidation. Inhibition of these oxidative events by resveratrol largely impairs autophagy of cell components and delays rapamycin-induced cell death (Kissová et al., 2006). What this evidence collectively implies is that autophagic pathways, such as mitophagy, can have a pro-death role in yeast, driven by massive accumulation of ROS in the mitochondria. Furthermore, a recent study by Dziedzic and Caplan (2012) presented evidence suggesting that autophagy may accelerate cell death in *S. cerevisiae* under starvation conditions, given that deletion of autophagic proteins, encoded by *ATG8*, delayed cell death under leucine starvation.

Therefore, a complex interplay exists between autophagy and cell death, two distinct stress responses which, depending on the circumstances of the cells, can either compete against each other or cooperate (Carmona-Gutierrez et al., 2010a) in a manner which is probably regulated, at least in part, by the ROS-dependent mitophagic turnover of mitochondria. This is probably one of the few pro-death aspects of autophagy which is understood. Otherwise, the extent to which autophagy acts as a cell death response in yeast is largely unclear.

There has in fact been much heated debate as to whether autophagy is even a true cell death mechanism *per se*. Some argue that the very term "autophagic cell death" – a definition based solely on the morphological appearance of vacuolization in dying cells – can be misleading and often incorrectly used to refer to cases where cells actually die *with* autophagy but not *by* autophagy (Kroemer and Levine, 2008). Whilst the plausibility of cell death by autophagy has not been entirely excluded, there

seems to be a consensus that in the overwhelming majority of cases, autophagy constitutes a cytoprotective pathway, the removal of which accelerates rather than prevents cell mortality (Galluzzi et al., 2012).

OXIDATIVE STRESS AND AGING

The physiological process of aging in budding yeast is heavily associated with ROS accumulation and PCD (Laun et al., 2001; Fabrizio et al., 2004; Herker et al., 2004). Replicative aging is used as a model for aging in mammalian proliferating cells (particularly stem cells), and chronological aging is used as an aging model for mammalian post-mitotic cells (Kaeberlein, 2010).

Replicative aging in yeast is defined by the number of times a specific yeast mother cell can divide before it reaches senescence and dies (Mortimer and Johnston, 1959; Müller et al., 1980). Budding yeast cells replicate asymmetrically (Hartwell and Unger, 1977) in such a way that aging factors are retained by the mother cell. These include oxidized proteins (Aguilaniu et al., 2003; Erjavec and Nystrom, 2007), protein aggregates (Erjavec et al., 2007), and extrachromosomal ribosomal DNA circles (ERCs) believed to be generated by rDNA recombination (Sinclair and Guarente, 1997; Kaeberlein et al., 1999). The same mother cell can replicate several times until a certain threshold of damaged cell content is accumulated, at which time the cell dies via PCD pathways such as apoptosis (Laun et al., 2001).

One other prominent form of cell damage believed to contribute to replicative aging is the accumulation of dysfunctional mitochondria that are rich in ROS in old yeast mother cells (Laun et al., 2001). This clearly indicates that ROS promote replicative aging in yeast. In fact, several other studies have shown that genetic or environmental alterations which increase the ROS burden on yeast mother cells result in a shortening of their lifespan. Such alterations include the deletion of genes encoding SODs (Barker et al., 1999; Wawryn et al., 1999) and catalases, the increase of partial pressure of atmospheric oxygen and the modulation of GSH abundance (Nestelbacher et al., 2000).

Likewise, studies have shown that ROS originating from dysfunctional mitochondria contribute to chronological aging in yeast (Longo et al., 1996;Fabrizio et al., 2003, 2004). This aging model is defined by how long a yeast cell can survive once it has reached the post-diauxic, stationary phase of growth, when nutrients become scarce (Longo and Fabrizio, 2012). The pro-aging effects of ROS are corroborated by the observed accumulation of oxidatively damaged proteins (Reverter-Branchat et al., 2004) and ROS (Herker et al., 2004) in chronologically aged cells. Furthermore, yeast chronological lifespan can be extended by interventions that induce upregulated expression of *SOD2*, activation of Msn2p and Msn4p transcription factors, and activation of protein kinase Rim15p, all of which are involved in the stress response that mitigates oxidative stress (Fabrizio et al., 2001, 2003).

Further evidence of the role of oxidative stress in the aging of yeast cells lies in the manner by which, both replicative and chronological aging in yeast are regulated. Both aging types depend on the same set of nutrient-sensing kinases: Ras/cAMP-dependent PKA, Sch9, and Tor, the activities of which are, in turn, dependent on nutrient availability in the environment. Under nutrient-rich conditions, all three kinases naturally promote cell growth, cell division, and thus also aging of yeast cells, culminating in PCD. The pro-aging effect of these kinases is largely due to their suppressive influence upon the yeast stress response mechanisms, in the presence of nutrients (Fabrizio et al., 2001, 2003; Inoue and Klionsky, 2010; Longo and Fabrizio, 2012).

Conversely, when nutrients are scarce, the Sch9, Tor, and Ras/cAMP-dependent PKA pathways are downregulated and cell longevity is increased (Fabrizio and Longo, 2008). A similar longevity-inducing scenario of nutrient starvation called calorie restriction has consistently been shown to increase longevity of both replicatively (Lin et al., 2002) and chronologically aging cells (Reverter-Branchat et al., 2004). It is believed that the downregulation of Sch9, Tor, and Ras/cAMP-dependent PKA signaling is coupled with a concomitant upregulation of cytoprotective stress response regulators, which are activated downstream of the kinases. These include Msn2/4p and Gis1 transcription factors, along with Rim15 kinase (Wei et al., 2008) the collective activation of which enhances cytoprotective pathways such as autophagy (Inoue and Klionsky, 2010), a process associated with increased longevity and reduced ROS in aging yeast (Eisenberg et al., 2009).

Thus yeast cell aging is a conserved, genetically regulated process, where a cell's ability to upregulate its cytoprotective stress response mechanisms above a certain threshold is actively suppressed by a sustained baseline activity of nutrient-sensing Sch9, Tor, and Ras/cAMP-dependent PKA kinases (Longo et al., 2005). In this way, aging cells accumulate a certain amount of oxidative damage, without upregulating the current activity of their antioxidant defenses. They consequently accumulate ROS such as superoxide radicals (Fabrizio et al., 2004) and succumb to PCD, be it apoptosis (Herker et al., 2004) or necrosis (Eisenberg et al., 2009). Thus, in a physiological context, replicatively aged cells that are no longer capable of segregating damaged cell components from their daughter cells, are eliminated before they seriously compromise the integrity of a population. Likewise, chronologically aged cells, which have suffered too much oxidative damage to be worth maintaining, are eliminated to free up nutrients and enhance the survival chances of younger, fitter cells in a yeast population, especially during starvation. In the meantime, these same fitter individuals of a starved yeast population may further increase their chances of survival by actively suppressing the aforementioned kinase pathways to enhance their cytoprotective systems, until new nutrient sources are made available (Fabrizio and Longo, 2008).

CONCLUDING REMARKS

The evidence presented in this discussion clearly illustrates the remarkable ability of yeast to detect and appropriately respond to the constant threat of oxidative stress, using a diverse array of strategies such as ROS detoxification, autophagic degradation, and even cell death (**Figure 1**). Importantly, these defensive pathways are largely conserved in mammals. Thus, yeast has long proved itself to be a powerful research model, the use of which has thrown light upon many obscure aspects of important human pathologies that are harder to elucidate using other, more complex eukaryotic models.

For instance, the prevalent involvement of ROS and functional mitochondria among most pro-death stimuli in yeast has served

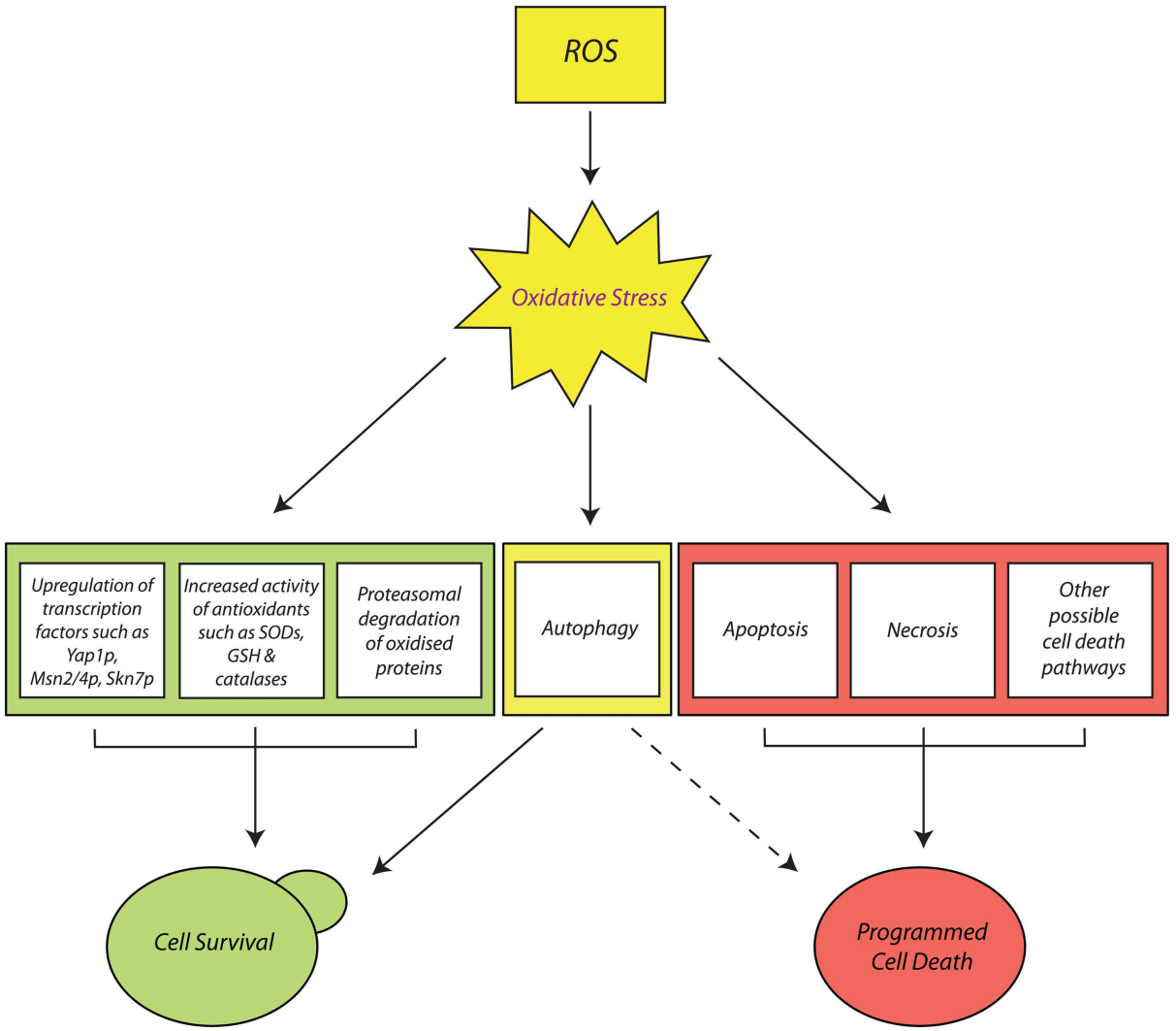

FIGURE 1 | Cellular responses to oxidative stress in *Saccharomyces cerevisiae*. Oxidative stress, induced by the accumulation of reactive oxygen species (ROS) such as $O_2^{\bullet-}$, H_2O_2, and $OH^{\bullet}$, can elicit a range of stress responses in budding yeast cells, that either result in cell survival (shown in green) or cell death (shown in red). Stress signals relayed by ROS themselves (such as H_2O_2) can activate transcription factors which upregulate the expression of genes encoding enzymatic (such as catalases) and non-enzymatic antioxidants (such as GSH). These response mechanisms, together with the targeted removal of small, oxidized proteins by the ubiquitin-dependent proteasome system (UPS), help ensure the survival of cells. Additionally, cells can activate cytoprotective autophagic pathways (bordered in yellow) that remove irreparably oxidized macromolecules or dysfunctional organelles, such as mitochondria. However, an abnormally high degree of autophagy might also mediate programmed cell death (PCD). Finally, exposure of cells to severe oxidative insults can elicit lethal response pathways such as apoptosis, necrosis, and possibly other forms of PCD which have yet to be discovered.

to highlight the central role of mitochondrial dysfunction and ROS accumulation in most important human pathologies such as aging (Laun et al., 2001) and Parkinson's disease (Büttner et al., 2008). Furthermore, the conserved metabolic machinery and simplicity of yeast has allowed researchers to perform very revealing mechanistic studies of anti-tumor drugs (Balzan et al., 2004; Mitsui et al., 2005; Aouida et al., 2007) and disease-inducing toxins (Sokolov et al., 2006; Franssens et al., 2010), paving the way toward enhanced therapeutic treatment and better understanding of human diseases. The yeast model can even be used to examine important aspects of mammalian aging, such as the contributive roles of DNA damage and genome instability (Wei et al., 2011). Finally, the chance of uncovering more conserved PCD pathways in yeast cannot be excluded, especially given the increasing number of newly elucidated PCD pathways in other eukaryotes (Galluzzi et al., 2012).

A potential example of these new pathways is "linker cell death" which has so far only been observed in *Caenorhabditis elegans*. This cell death pathway, which proceeds independently of known death genes and caspases, is characterized by non-apoptotic markers such as uncondensed chromatin, indentations of the nuclear envelope, organelle swelling, and accumulation of membrane bound

cytoplasmic structures (Abraham et al., 2007). It requires the expression of a *pqn-41* sequence, encoding a polyglutamine repeat protein that bears similarity to Huntingtin (Blum et al., 2012), the cause of Huntington's disease (Harper, 1999). The expression of *pqn-41*, which is itself dependent on the activity of a MAP kinase kinase called SEK-1, promotes cell death in parallel to a zinc-finger protein called LIN-29p.

In budding yeast, expression of expanded mammalian polyglutamines elicits physiological consequences similar to those of degenerating neurons in Huntington's disease patients. These include an apoptotic phenotype and nuclear polyglutamine aggregation, both of which are caspase-dependent (Sokolov et al., 2006; Bocharova et al., 2008). It is very tempting to speculate whether yeast cells also possess a conserved *pqn-41*-like polyglutamine protein, the expression of which might elicit an alternative caspase-independent PCD pathway that is similar or identical to linker cell death in *C. elegans*. If this is the case, what impact might this have on our understanding of neurodegenerative pathologies such as Huntington's disease? This is an intriguing question which has yet to be answered.

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The role of mitochondria in yeast programmed cell death

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Mammalian apoptosis and yeast programmed cell death (PCD) share a variety of features including reactive oxygen species production, protease activity and a major role played by mitochondria. In view of this, and of the distinctive characteristics differentiating yeast and multicellular organism PCD, the mitochondrial contribution to cell death in the genetically tractable yeast *Saccharomyces cerevisiae* has been intensively investigated. In this mini-review we report whether and how yeast mitochondrial function and proteins belonging to oxidative phosphorylation, protein trafficking into and out of mitochondria, and mitochondrial dynamics, play a role in PCD. Since in PCD many processes take place over time, emphasis will be placed on an experimental model based on acetic acid-induced PCD (AA-PCD) which has the unique feature of having been investigated as a function of time. As will be described there are at least two AA-PCD pathways each with a multifaceted role played by mitochondrial components, in particular by cytochrome *c*.

Keywords: yeast, programmed cell death, mitochondria, acetic acid, cytochrome *c*, protein trafficking, intracellular signaling

The unicellular yeast *Saccharomyces cerevisiae* has been established as a good model to elucidate molecular mechanisms underlying programmed cell death (PCD) pathways. *S. cerevisiae* PCD shares many morphological and biochemical features with apoptosis, the major form of mammalian PCD, although there are some peculiar differences. PCD have been described to occur in yeast in different physiological scenarios (Carmona-Gutierrez et al., 2010). Indeed, chromatin condensation, nuclear DNA fragmentation and phosphatidylserine externalization onto the cell surface are general markers of both mammalian and yeast PCD cells. A characteristic feature of mammalian apoptosis is the activation of caspases, proteases that initiate and execute cell death through degradation of cell components. Yeast contains only one gene homolog of caspases, named *YCA1*, encoding for yeast metacaspase (Madeo et al., 2002) which has substrate specificity different from caspases (Wilkinson and Ramsdale, 2011). Glyceraldehyde-*3*-phosphate dehydrogenase has been identified as the first *YCA1*-specific substrate degraded *en route* to H_2O_2-induced PCD (Silva et al., 2011), but yeast PCD mechanisms occurring both in *YCA1*-dependent and -independent manner as well as the role of other proteases in yeast PCD remain to be established (Madeo et al., 2009; Wilkinson and Ramsdale, 2011).

Both in yeast and in mammalian PCD mitochondria play a major role in final pro-survival or pro-death decision. Accordingly, the mitochondria-mediated PCD pathway in yeast resembles the mammalian intrinsic pathway, and shows remarkable complexity with respect to different proteins and pathways involved (Eisenberg et al., 2007; Pereira et al., 2008). Alterations in mitochondrial structure and function during PCD depend on a variety of specific triggers, respiratory or fermentative growth conditions, and on overall cell metabolism. First evidence for a mitochondria-dependent yeast PCD pathway was obtained in acetic acid-induced PCD (AA-PCD), with cells showing cytochrome *c* (cyt *c*) release into the cytosol and production of mitochondrial reactive oxygen species (ROS). Mitochondrial dysfunction occurs as shown by mitochondrial depolarization, and a large decrease in cyt *c* oxidase (COX) activity together with higher resistance to AA-PCD of respiratory-deficient cells, lacking either mtDNA or unable to form active cyt *c* or ATP synthase (Ludovico et al., 2002). Key regulators of mitochondrial metazoan apoptosis are the Bcl-2 family proteins which include both pro-apoptotic and anti-apoptotic members harboring multiple or single Bcl-2 homology (BH) domains (BH1-4). These proteins regulate mitochondrial outer membrane permeabilization (MOMP) followed by the release of pro-apoptotic factors including cyt *c* (Wang and Youle, 2009; Wasilewski and Scorrano, 2009). Recent discovery of a yeast BH3-only protein (Ybh3p) mediating both AA- and H_2O_2-induced PCD (Büttner et al., 2011) supports the hypothesis of the origin of the eukaryotic PCD systems through acquisition of several PCD effectors as a consequence of mitochondrial endosymbiosis (Koonin and Aravind, 2002). Indeed, yeast Ybh3p translocates to mitochondria inducing PCD and mitochondrial membrane depolarization through interaction with the mitochondrial phosphate carrier (Mir1p) and a core subunit of the respiratory complex III (Cor1p; Büttner et al., 2011). Thus, Ybh3p resembles mammalian Bax that can permeabilize mitochondria, whereas mammalian BH3-only proteins require Bax and Bak to release cyt *c*, suggesting that the most ancestral function of the BH3-like proteins may be to trigger changes in the IMM (Oettinghaus et al., 2011).

Whether yeast PCD does resemble and/or predate apoptotic death in multicellular organisms or is a distinct form of PCD in itself is still a matter of investigation. Indeed, it remains controversial as to whether metacaspases are distant relatives of caspases

or are more closely related to other classes of proteases. Moreover even if yeast encodes a BH3-only protein as recent studies suggest, yeast homologs of Bcl-2 proteins on which BH3-only proteins act are still unknown. Notwithstanding this, the central role of mitochondria in yeast PCD underlines the importance of dissecting the PCD process in this unicellular organism.

In this review we consider the mitochondrial proteins involved in yeast PCD execution and regulation (see **Table 1**). Most of them are involved in either electron transfer along the respiratory chain and oxidative phosphorylation, or mitochondrial dynamics, or mitochondrial permeabilization and protein trafficking from mitochondria to cytosol and vice versa. These points will be dealt with separately.

ELECTRON TRANSFER ALONG THE RESPIRATORY CHAIN AND OXIDATIVE PHOSPHORYLATION

Yeast internal NADH dehydrogenase (NDI1) is the homolog of metazoan AMID, the apoptosis-inducing factor (AIF)-homologous mitochondrion-associated inducer of death. Ndi1p overexpression can cause PCD, probably due to ROS production in mitochondria, only when cells are grown in glucose-rich media. However this occurs in yeast cells lacking mitochondrial superoxide dismutase both during fermentative and respiring growth (Li et al., 2006). Yme1p is a mitochondrial AAA-type protease involved in the coordinated assembly of COX. Yme1p activation results in a decrease of COX level *en route* to Bax-induced cell death; however since under fermentative conditions, when COX activity is strongly repressed, *YME1* deletion slightly delays Bax-induced cell death, some other unidentified Yme1p substrate could also play a role in this process (Manon et al., 2001). Analysis of the effect of oxidative phosphorylation inhibitors on yeast PCD has shown conflicting results depending on the PCD trigger. Although AA-PCD is insensitive to antimycin or oligomycin, myxothiazol and cyanide prevented amiodarone/α-factor-induced PCD (Ludovico et al., 2002; Pozniakovsky et al., 2005; Guaragnella et al., 2011b). Yeast cells grown in the presence of both antimycin and oligomycin and subsequently treated with acetic acid in the presence of both these compounds displayed a higher sensitivity to AA-PCD (Pereira et al., 2007). Yet, fully assembled and functional F_0F_1-ATPase and cyt *c* are required for Bax-induced PCD and AA-PCD to occur (Matsuyama et al., 1998; Ludovico et al., 2002; Guaragnella et al., 2011a).

Thus, complexes participating in oxidative phosphorylation have key roles in yeast PCD different from electron transport and ATP synthesis, likely ROS production. Interestingly, deletion of mitochondrial citrate synthase (*CIT1*) results in higher sensitivity to oxidative stress and PCD induction, due to impairment of reduced glutathione (GSH) biosynthesis (Lee et al., 2007), suggesting that other metabolic pathways are also involved in oxidative stress.

MITOCHONDRIAL DYNAMICS

Extensive mitochondrial fragmentation is recognized as a general feature in yeast PCD. Fis1p, Dnm1p, and Mdv1p/Net2p, which constitutes the machinery responsible for mitochondrial fission in healthy cells (Fannjiang et al., 2004), are involved in mitochondrial fragmentation/degradation and cell death induced by different stimuli (Fannjiang et al., 2004; Kitagaki et al., 2007; Bink et al., 2010). Indeed, *DNM1* gene deletion extends life span by increasing cellular resistance to PCD induction (Scheckhuber et al., 2007). In distinction from its pro-apoptotic function in mammals, yeast Fis1p is a mitochondrial protein which inhibits *DNM1*-mediated cell death by inhibiting the fission function of Dnm1p, differently from its role in mitochondrial fission during normal growth (Fannjiang et al., 2004). This inhibitory function of Fis1p can be functionally replaced by human Bcl-2 and Bcl-x_L, supporting the idea that Fis1p is a functional homolog of anti-apoptotic Bcl-2 family proteins (Cheng et al., 2008a) and, together with Ybh3p (Büttner et al., 2011), is a component of an ancestral mitochondrial PCD pathway. The pro-survival role of *FIS1* was confirmed in studies using different apoptotic triggers, such as virus-encoded toxin, ethanol, and fungicidal derivative BAR0329 (Ivanovska and Hardwick, 2005; Kitagaki et al., 2007; Bink et al., 2010). However, *FIS1* may have an additional long-term survival function which appears to be independent of *DNM1* and *MDV1*. Indeed, *FIS1* deletion results in acquisition of a secondary mutation in the stress-response gene *WHI2* that confers sensitivity to cell death (Cheng et al., 2008b).

Genetic screens have revealed the existence of two novel genes, named yeast suicide protein 1 (*YSP1*) and yeast suicide protein 2 (*YSP2*), required for mitochondrial fragmentation *en route* to amiodarone-induced PCD (Pozniakovsky et al., 2005; Sokolov et al., 2006). It has been proposed that Ysp2p acts downstream of ROS production due to intracellular acidification, following AA-PCD induction (Sokolov et al., 2006). No homologous genes have been found in higher organisms.

MITOCHONDRIAL PERMEABILITY AND PROTEIN TRAFFICKING FROM MITOCHONDRIA TO CYTOSOL AND VICE VERSA

As in mammals, the release of pro-apoptotic mitochondrial proteins occurs *en route* to yeast PCD. Cyt *c* was the first mitochondrial protein shown to have an apoptotic function different from its role as an electron carrier in the respiratory chain. Cyt *c* release from mitochondria occurs commonly in yeast PCD both in response to a variety of stimuli, including acetic acid (Ludovico et al., 2002; Giannattasio et al., 2008), amiodarone/α-factor (Pozniakovsky et al., 2005), H_2O_2 (Pereira et al., 2007), aspirin (Sapienza et al., 2008), salt stress (Gao et al., 2011), and as a result of heterologous expression of the mammalian BAX (Manon et al., 1997). Cyt *c* release was also observed in yeast strains lacking the histone chaperone *ASF1/CIA1* (Yamaki et al., 2001) and with a mutation in *CDC48* ($cdc48^{S565G}$; Braun et al., 2006). Deletion of cyt *c* isoforms or heme lyase, necessary for cyt *c* maturation, inhibits yeast PCD triggered by different stimuli (Ludovico et al., 2002; Severin and Hyman, 2002; Pozniakovsky et al., 2005; Silva et al., 2005; Yang et al., 2008; Gao et al., 2011), except ethanol (Kitagaki et al., 2007).

Mammalian AIF is a FAD-containing oxidoreductase localized in the mitochondrial intermembrane space whose specific enzymatic activity remains unknown (Sevrioukova, 2011). AIF is a caspase-independent death effector and also plays a vital mitochondrial role in healthy cells (Hangen et al., 2010). Similarly to AIF, the yeast homolog Aif1p translocates to the nucleus in

Table 1 | Yeast mitochondrial proteins involved in PCD regulation.

Gene (protein)	Mammalian homolog	PCD trigger	Role in PCD	Reference
AAC1/AAC2/AAC3 (ADP/ATP carrier isoforms)	ANT	Acetic acid, diamide, H_2O_2	MOMP	Pereira et al. (2007)
AIF1 (apoptosis-inducing factor)	AIF	Acetic acid, bostrycin, H_2O_2	Pro-apoptotic released factor translocating to the nucleus	Wissing et al. (2004), Xu et al. (2010)
ATP10 (ATP synthase assembly factor)	ATP synthase	Acetic acid	Pro-apoptotic factor	Ludovico et al. (2002)
CIT1 (citrate synthase)	CS	Aging, heat	GSH biosynthesis, antioxidant activity	Lee et al. (2007)
COR1 (complex III core subunit)	QCR1	Acetic acid + Ybh3 overexpression	ETC, YBH3 interaction	Büttner et al. (2011)
CYC1/CYC7 (cytochrome *c* isoforms 1, 2)	Cytochrome *c*	Acetic acid, amiodarone/α-factor, ASF1/CIA1 deletion, aspirin, $cdc48^{S565G}$, Bax heterologous expression, H_2O_2, hyperosmotic stress, salt stress	Pro-apoptotic released factor, ETC electron donor, ROS scavenger	Manon et al. (1997), Yamaki et al. (2001), Ludovico et al. (2002), Pozniakovsky et al. (2005), Silva et al. (2005), Braun et al. (2006), Pereira et al. (2007), Giannattasio et al. (2008), Sapienza et al. (2008), Gao et al. (2011)
CYC3 (cytochrome *c* heme lyase)	CCHL	Acetic acid, amiodarone, hyperosmotic stress	Cyt *c* holoenzyme formation	Ludovico et al. (2002), Pozniakovsky et al. (2005), Silva et al. (2005)
FIS1 (mitochondrial fission protein)	hFIS	Acetic acid, BAR0329, ethanol, heat shock, H_2O_2	Mitochondrial dynamics	Fannjiang et al. (2004), Kitagaki et al. (2007), Bink et al. (2010)
L14-A (mitochondrial 60S ribosomal protein)	–	Grapefruit seed extract	Unknown	Cao et al. (2012)
MIR1 (mitochondrial phosphate carrier)	PHC	Acetic acid + Ybh3 overexpression	Energetic metabolism, YBH3 interaction	Büttner et al. (2011)
NDI1 (internal NADH dehydrogenase)	AMID	NDI1 overexpression	ROS production	Li et al. (2006)
NUC1 (mitochondrial nuclease)	Endo G	Acetic acid, amiodarone, ethanol, H_2O_2	Pro-apoptotic released factor translocating to the nucleus	Büttner et al. (2007), Kitagaki et al. (2007)
POR1 (porin)	VDAC	Acetic acid, H_2O_2, diamide	Anti-apoptotic factor	Pereira et al. (2007)
RSM23 (mitochondrial 40S ribosomal protein)	hDAP-3	YCA1 overexpression	Pro-apoptotic factor	Madeo et al. (2002)
TIM18 (translocase of the inner mitochondrial membrane)	–	Arsenite	MOMP	Du et al. (2007)
YME1 (catalytic subunit of i-AAA protease complex)	–	Heterologous expression of Bax	Complex IV degradation	Manon et al. (2001)
YSP1 (yeast suicide protein 1)	–	α-Factor, amiodarone	Mitochondrial dynamics	Pozniakovsky et al. (2005)
YSP2 (yeast suicide protein 2)	–	Acetic acid, amiodarone	Mitochondrial dynamics	Sokolov et al. (2006)

The S. cerevisiae mitochondrial proteins reported in this table have been implicated in PCD induced by different triggers through biochemical and/or genetic studies. ANT, adenine nucleotide translocator; MOMP, mitochondrial outer membrane permeabilization; CS, citrate synthase; GSH, glutathione; QCR1, ubiquinol–cytochrome c reductase core protein; YBH3, yeast BH3-only; ETC, electron transport chain; CCHL, cyt c heme lyase; hFIS, human homolog of Fis1p; BAR0329, 4-{[3-(4-chlorobenzyl)-2-methoxyquinolin-6-yl]methyl}piperazine-1-carboximidamide; PHC, phosphate carrier; AMID, apoptosis-inducing factor-homologous mitochondrion-associated inducer of death; Endo G, endonuclease G; VDAC, voltage-dependent anion channel; hDAP-3, human death associated protein.

response to apoptotic stimuli (Wissing et al., 2004). *AIF1* disruption rescues yeast cells from oxygen stress and delays age-induced PCD. Conversely, overexpression of *AIF1* strongly stimulates H_2O_2-induced PCD; this effect is attenuated by disruption of *YCA1*. Contrarily, *AIF1*-dependent bostrycin-induced cell death was shown to be independent of *YCA1* (Xu et al., 2010).

Nuc1p is the yeast homolog of metazoan endonuclease G (EndoG), a mitochondrial protein with DNase/RNase activity involved in apoptotic DNA degradation (Li et al., 2001). Overexpression of Nuc1p promotes yeast PCD. Nuc1p-mediated PCD is shown to be *AIF1*- and *YCA1*-independent, which favors the existence of multiple, redundant pathways regulating cell death. Nuc1p translocates from mitochondria to the nucleus upon death induction. Nuc1p-dependent death depends on its interaction with *AAC2*, as well as with histone H2B and *KAP123*, coding for karyopherin involved in nuclear import, indicating that the pro-death role of Nuc1p requires nuclear import and chromatin association (Büttner et al., 2007). When mitochondrial respiration is increased *NUC1* deletion inhibits apoptotic death, whereas under respiration repressing conditions, *NUC1* deletion sensitizes yeast cells to non-apoptotic death, this showing a dual, pro-life and pro-death role for *NUC1* (Büttner et al., 2007; Kitagaki et al., 2007).

The yeast genome also harbors a gene, called *NMA111*, homologous to vertebrate HtrA2/Omi mitochondrial serine protease, which mediates apoptosis once released to the cytosol where it can antagonize the inhibitor of apoptosis protein XIAP (Vande Walle et al., 2008). Differently from HtrA2/Omi, yeast Nma111p is a nuclear protein that, under cellular stress conditions such as H_2O_2-induced PCD, tends to aggregate inside the nucleus without its expression level being upregulated, suggesting that aggregation of Nma111p is correlated to its death-mediating character (Fahrenkrog et al., 2004).

Mitochondrial protein release and MOMP are crucial events in yeast PCD. Certain mitochondrial proteins possibly involved in MOMP *en route* to yeast PCD have been identified. Yeast possesses the homologous genes of the putative core components of mammalian permeability transition pore, ADP/ATP carrier proteins (*AAC1,2,3*), yeast voltage-dependent anion channel (*POR1*), and a mitochondrial cyclophilin (*CPR3*). While Por1p was proposed to have a pro-survival role and Cpr3p had no effect on yeast PCD, only deletion of AAC proteins was shown to protect cells from AA- but not H_2O_2-induced PCD, and to inhibit cyt *c* release (Pereira et al., 2007). In addition, the AAC proteins and the vacuolar protease Pep4p have been shown to have a role in mitochondrial degradation *en route* to AA-PCD; Pep4p is released from the vacuole upon AA-PCD induction, suggesting a vacuole-mitochondrial cross-talk during yeast PCD (Pereira et al., 2010).

The mitochondrial inner membrane translocase, Tim18, was shown to be involved in arsenic-induced yeast cell death (Du et al., 2007), this raising a question about the possible involvement of this translocase in MOMP. Tim18 is part of the Tim54–Tim22 complex, Tim22 being a mitochondrial receptor for the pro-apoptotic protein Bax (Kovermann et al., 2002).

Two other proteins, Mmi1p and Mcd1p, have been shown to translocate to mitochondria *en route* to yeast PCD. The former functionally links microtubules and mitochondria (Rinnerthaler et al., 2006). The latter causes the decrease of mitochondrial membrane potential amplifying PCD in a cyt *c*-dependent manner (Yang et al., 2008).

A CASE STUDY: THE ROLE OF CYTOCHROME *c* IN YEAST PCD

Although cyt *c* release occurs *en route* to yeast PCD, so far in *S. cerevisiae* there is no evidence of the existence of a functional homolog of the apoptosome (Huttemann et al., 2011). Accordingly, yeast cyt *c* is unable to activate caspases in cytosolic extracts from metazoan cells (Kluck et al., 2000; Bender et al., 2012). Thus, some questions need to be answered: which event/s triggers cyt *c* release? Is cyt *c* released from damaged mitochondria? What is the role of the released cyt *c en route* to PCD, and is it strictly required for PCD to occur? In this regard, the definition of the sequence of events leading to the death cascade turns out to be useful.

After the discovery of the occurrence of AA-PCD in yeast (Ludovico et al., 2001, 2002), in a series of papers a detailed time course of certain events was investigated (Giannattasio et al., 2005, 2008; Guaragnella et al., 2006, 2007, 2008, 2010a; Ribeiro et al., 2006; Pereira et al., 2007). These events can be classified as pre- and post-cyt *c* release (**Figure 1**). Loss of cell viability is complete after 200 min of acetic acid treatment with accumulation of cells with fragmented nuclear DNA. The earliest event (15 min) following acetic acid challenge is ROS production, with a different role for H_2O_2 and superoxide anion, whose levels are modulated by catalase and superoxide dismutase. *En route* to death cyt *c* starts to be released at 60 min from coupled and intact mitochondria; maximum release is reached at 150 min. Later on cyt *c* is degraded, possibly by yet unidentified proteases. The latest event of AA-PCD is caspase-like activation occurring at 200 min from death induction. Mitochondria are functionally implicated in this death scenario. In fact, up to 150 min released cyt *c* can act both as an electron donor as well as a ROS scavenger. However, *en route* to death a progressive impairment of mitochondrial functions, evidenced by a decrease of the respiratory control index, a collapse of the mitochondrial membrane potential, a decrease in COX activity and in cytochromes $a + a_3$ levels, have been observed.

The AA-PCD time course clearly shows that ROS accumulation and caspase-like activation occur upstream and downstream of cyt *c* release, respectively. Functional genomics and biochemical studies on knock-out cells lacking *YCA1* and/or the genes encoding the two yeast cyt *c* isoforms allowed the elucidation of causal relationships among ROS levels, cyt *c* release and caspase-like activation and two separate pathways activated by acetic acid have been identified. Particularly, it has been found that ROS and *YCA1* are required for cyt *c* release, since both prevention of ROS production by the antioxidant *N*-acetyl cysteine (NAC) and *YCA1* disruption result in the inhibition of cyt *c* release (Guaragnella et al., 2010a,b). How *YCA1* is related to cyt *c* release remains to be elucidated. Nevertheless, a recent report suggests that *YCA1* has a role in mitochondrial respiratory functions (Lefevre et al., 2012). Interestingly, AA-PCD still occurs, although with a lower death rate compared to wild type cells, without cyt *c* release in ADP/ATP carrier as well as *YCA1* and/or cyt *c* knock-out cells (Pereira et al., 2007; Guaragnella et al., 2010b). This confirms on one hand that

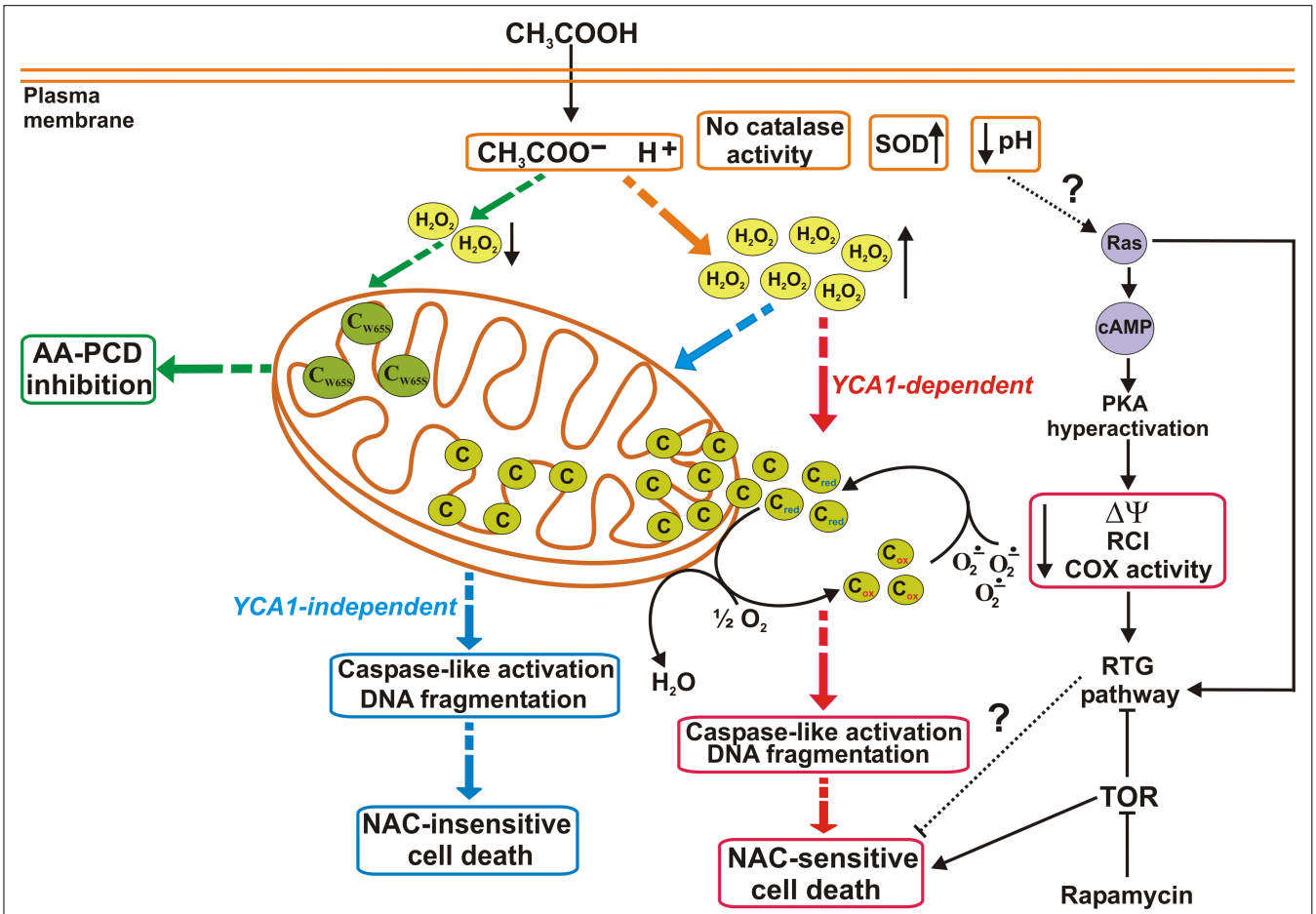

FIGURE 1 | Cytochrome *c* and mitochondrial dysfunction in AA-PCD pathways. At extracellular acidic pH values acetic acid enters yeast cells and dissociates into acetate and protons causing intracellular acidification. In NAC-sensitive AA-PCD (red dashed arrows) hydrogen peroxide (H_2O_2) accumulates early, superoxide dismutase (SOD) activity increases, while catalase activity is undetectable; cyt *c* is released to the cytosol in a YCA1-dependent manner as a functional protein, acting as an electron donor (c_{red}) to the electron transport chain and as a superoxide anion ($O_2^{-}\cdot$) scavenger; in a late phase, mitochondrial functions progressively decline, as revealed by a decrease in mitochondrial membrane potential ($\Delta\Psi$), respiratory control index (RCI), and cyt *c* oxidase (COX) activity. Caspase-like activity increases and DNA fragmentation occurs. The NAC-insensitive (blue dashed arrows) AA-PCD takes place in a YCA1-independent manner without cyt *c* release, yet caspase-like activation and DNA fragmentation occur in a late phase. In cells expressing a catalytically inactive form of iso-1-cyt *c* (C_{W65S}; green dashed arrows), no release of mutant cyt *c* occurs with inhibition of AA-PCD, and there is a decrease in H_2O_2 production. Possible involvement of certain signaling pathways in the interplay between PCD and cell adaptation is also shown: intracellular acidification caused by AA-PCD induction may stimulate RAS–cAMP–PKA signaling pathway, causing mitochondrial dysfunction, which can activate retrograde (RTG) pathway. The RTG pathway is positively and negatively regulated by Ras and TOR pathways, respectively. The TOR pathway is found at the crossroad of AA-PCD and RTG signaling, which may play a role in AA-PCD resistance.

YCA1 and cyt *c* act as pro-apoptotic proteins in yeast AA-PCD, but on the other hand that they are dispensable for PCD occurrence, showing the existence of *YCA1*/cyt *c*-independent AA-PCD pathway (**Figure 1**). In this pathway ROS accumulate early, caspase-like activity increase, and DNA fragmentation occurs. Importantly, *YCA1*/cyt *c*-independent AA-PCD is insensitive to NAC. This evidence suggests that cyt *c* still present in mitochondria might play a role in AA-PCD. Recent studies performed on yeast cells expressing a stable but catalytically inactive iso-1-cyt *c* (W65Scyc1) unable to reduce COX have shown inhibition of AA-PCD, with a decrease of ROS production, no cyt *c* release, this being independent of electron flow impairment, and an increase in caspase-like activation (**Figure 1**). Thus, cyt *c* release does not depend on cyt *c* function as an electron carrier and when still associated to the mitochondrial membrane, cyt *c* in its reduced form has a role in AA-PCD by regulating ROS production and caspase-like activity (Guaragnella et al., 2010a,b, 2011b). Regulation of ROS production by mitochondrial cyt *c* during AA-PCD may be exerted either directly by the cyt *c* peroxidase system able to scavenge both superoxide anion and H_2O_2 (Korshunov et al., 1999) or by a change in cyt *c*–cardiolipin interaction or inefficient cardiolipin peroxidation by ROS (Kagan et al., 2005; Bayir et al., 2006; Sinibaldi et al., 2010; Huttemann et al., 2011). These issues require further investigations.

CONCLUSIONS AND PERSPECTIVES

In the light of results emerging from research into yeast PCD we feel that there is consensus that the response to any stimulus

leading to PCD depends on the intrinsic status of the cells, for instance the growth phase or the metabolic and environmental conditions. Paradigmatic of this is that in response to acetic acid in cells with increased mitochondrial respiration yeast activates a Nuc1p-dependent PCD pathway (Büttner et al., 2007), whereas in stationary growth phase yeast is less sensitive to acetic acid (Ludovico et al., 2002), and after acid stress adaptation it is highly resistant to AA-PCD induction (Giannattasio et al., 2005; Ždralević et al., 2012).

Although a number of mitochondrial proteins participating in yeast PCD have been identified, how they work *en route* to PCD remains to be fully established. Further aspects also need to be investigated, including the fact that mitochondria are important organelles in the cross-talk between death- and life-promoting signaling pathways. Indeed, RAS–cAMP–PKA (Longo, 2003; Roosen et al., 2005; Gourlay et al., 2006; Leadsham and Gourlay, 2010), target of rapamycin (TOR) kinase (Almeida et al., 2009) and retrograde (Jazwinsky, 2003; Liu and Butow, 2006) signaling pathways have been shown to control yeast cell PCD and aging through mitochondrial function regulation (**Figure 1**).

Mitochondrial dysfunction and the mode of cell response to it underlie different pathological conditions such as neurodegeneration. Alterations in mitochondrial functions have long been observed also in cancer cells and targeting mitochondria as an anti-cancer therapeutic strategy has gained momentum recently (Gogvadze et al., 2009). Since yeast shares with cancer cells the metabolic features identified as the underlying causes of the Warburg effect (Ruckenstuhl et al., 2009; Diaz-Ruiz et al., 2010), it is a suitable model organism to identify cell compounds responsible for tumorigenesis for development of targeted cancer drugs.

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Endoplasmic reticulum involvement in yeast cell death

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Yeast cells undergo programed cell death (PCD) with characteristic markers associated with apoptosis in mammalian cells including chromatin breakage, nuclear fragmentation, reactive oxygen species generation, and metacaspase activation. Though significant research has focused on mitochondrial involvement in this phenomenon, more recent work with both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* has also implicated the endoplasmic reticulum (ER) in yeast PCD. This minireview provides an overview of ER stress-associated cell death (ER-SAD) in yeast. It begins with a description of ER structure and function in yeast before moving to a discussion of ER-SAD in both mammalian and yeast cells. Three examples of yeast cell death associated with the ER will be highlighted here including inositol starvation, lipid toxicity, and the inhibition of *N*-glycosylation. It closes by suggesting ways to further examine the involvement of the ER in yeast cell death.

Keywords: BXI1, endoplasmic reticulum, ER stress, IRE1, UPR, yeast cell death

INTRODUCTION

In recent years, it has become increasingly clear that yeast cells undergo programed cell death (PCD) in response to a variety of intrinsic and extrinsic stimuli, with characteristic markers associated with apoptosis in mammalian cells (Carmona-Gutierrez et al., 2010). Significantly, yeast orthologs of crucial metazoan apoptotic proteins, which include the metacaspase, Yca1p (Madeo et al., 2002), the yeast AIF1 homolog, Aif1p (Wissing et al., 2004), and the endonuclease G homolog, Nuc1p (Buttner et al., 2007), have been identified and linked to yeast cell death suggesting that a core machinery driving PCD is conserved in unicellular eukaryotes (Madeo et al., 2009).

Though significant research has focused on mitochondrial involvement in yeast PCD (Braun and Westermann, 2011), recent work with both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* has implicated the endoplasmic reticulum (ER) in yeast PCD. This minireview provides an overview of ER stress-associated cell death (ER-SAD) in yeast. It begins with a description of ER structure and function in yeast before moving to a discussion of ER-SAD in both mammalian and yeast cells. It closes by suggesting ways to further examine the involvement of the ER in yeast cell death.

ENDOPLASMIC RETICULUM STRUCTURE IN YEAST

The ER is the largest membrane-bound organelle in the eukaryotic cell (Friedman and Voeltz, 2011; Hu et al., 2011). It consists of the nuclear envelope and the peripheral ER, a single network of interconnected sheets and tubules, which in yeast is located close to the plasma membrane where it is referred to as the cortical ER. A recent study that imaged the budding yeast ER by transmission electron microscopy and dual-axis electron tomography revealed that it can divided into three structurally distinct major domains: the plasma membrane-associated ER (pmaER), the central cisternal ER (cecER), and the tubular ER (tubER; West et al., 2011).

Molecular components involved in regulating ER structure have recently been identified. Regions of high membrane curvature including the edges of the sheets and the tubules are stabilized by interactions between members of the reticulon family (Rtn1p and Rtn2p in budding yeast) and members of the DP1/Yop1p family of proteins (Yop1p in budding yeast; Voeltz et al., 2006; Shibata et al., 2010). It is thought that Rtn1p, Rtn2p, and Yop1p, like their mammalian counterparts, stabilize the high curvature of ER tubules by using their double hairpin structure to form wedges and arc-like scaffolds that mold the lipid bilayer into tubules (Shibata et al., 2009, 2010).

Once ER tubules are shaped and formed, they need to be connected to the ER network via homotypic fusion between two identical but apposing membranes. In yeast, homotypic fusion appears to be mediated by the Sey1p protein, the ortholog of the mammalian atlastins, a class of GTPases that belong to the dynamin family (Hu et al., 2009; Anwar et al., 2012). Given Sey1p's structure, the data suggest that the fusion reaction could be mediated by conformational changes in the Sey1p GTPase domains that pull the apposing membranes together forcing them to fuse (Orso et al., 2009; Anwar et al., 2012).

ENDOPLASMIC RETICULUM FUNCTION IN YEAST

In all eukaryotic cells, the ER performs a variety of functions including protein translocation and folding, lipid synthesis, and calcium homeostasis. In yeast cells, protein transport into the ER can occur via either the signal recognition particle (SRP)-dependent (cotranslational translocation) or the Hsp70p-dependent (post-translational translocation) pathway (Zimmermann et al., 2011). Membrane insertion and completion of translocation involve the heterotrimeric Sec61p complex and the ER-lumenal chaperone, Kar2p (or BiP). Kar2p appears to facilitate post-translational insertion of the polypeptide into the Sec61p complex and its translocation via a ratcheting mechanism (Lyman and Schekman, 1996). Once ER client proteins

have been translocated, they can be folded, modified, and packaged into ER-to-Golgi COPII transport vesicles that form at ER exit sites (ERES; Duden, 2003; Watanabe and Riezman, 2004; Watson and Stephens, 2005). In yeast, ER sheets have a markedly higher ribosome density than tubules suggesting that sheets may be better suited for ribosome binding and protein translocation (West et al., 2011).

Next, the ER serves as the main site for synthesis of three major classes of membrane lipids: sphingolipids, phospholipids, and sterols (Carman and Henry, 2007; Henry et al., 2012). For example, in yeast, most of the biosynthetic steps for triacylglycerols occur in the ER: Gat1p and Gat2p, which are the major glycerol-3-phosphate acyltransferases (GPATs) that catalyze the first step in the synthesis of almost all membrane phospholipids and neutral glycerolipids, localize to both the perinuclear and the cortical ER (Bratschi et al., 2009). Additionally, the two ER membrane proteins, Orm1p and Orm2p, have been shown to be involved in sphingolipid synthesis and phospholipid homeostasis (Han et al., 2010). These are only a few of numerous ER-localized gene products known to be involved in lipid biosynthesis in yeast.

Finally, the creation of a specifically targeted version of the Ca^{2+}-sensitive photoprotein, aequorin, to the lumen of the yeast ER revealed that this organelle is involved in calcium storage and homeostasis with a steady-state concentration of 10 μM free Ca^{2+} in wild-type yeast cells (Strayle et al., 1999). It is also known that two P-type ATPases are involved in regulating the levels of Ca^{2+} in the ER including the yeast high-affinity Ca^{2+}/Mn^{2+} P-type ATPase, Pmr1p, which pumps cytosolic Ca^{2+} into the ER and the Golgi (Sorin et al., 1997; Strayle et al., 1999), and the ER-localized P-type ATPase, Cod1p/Spf1p, which appears to work with Pmr1p to maintain ER function and homeostasis (Vashist et al., 2002). Significantly, Ca^{2+} levels in the ER have been implicated in retention of resident luminal proteins, in export of secretory proteins, in protein folding and degradation, and in the association of the ER chaperone Kar2p with misfolded proteins (Durr et al., 1998).

ER STRESS-ASSOCIATED DEATH IN MAMMALIAN CELLS

In mammalian cells, a diverse range of factors can disrupt ER function and lead to ER stress, which if left unchecked can trigger ER-SAD. These include increases in ER-lumenal protein levels that exceed the capacity of ER-resident chaperones, exposure to long-chain saturated fatty acids (SFA), alterations in calcium levels in the ER lumen, and disturbances to the ER redox balance (Ron and Walter, 2007; Parmar and Schroder, 2012). In animal cells, ER stress is sensed by three upstream signaling pathways driven by three effector proteins, IRE1, ATF6, and PERK, which are collectively called the unfolded protein response (UPR; Wang et al., 1998; Harding et al., 1999; Urano et al., 2000; Walter and Ron, 2011). Activation of the UPR can alleviate ER stress by synthesizing novel components of the protein folding machinery and by expanding the ER itself (Cox et al., 1997; Schuck et al., 2009). This activated UPR can be divided into three phases: the adaptive, the alarm, and the apoptotic phase. The adaptive phase begins with an immediate and fast response that decreases protein influx into the ER followed by a slower transcriptional response involving downstream transcription factors that upregulate genes encoding ER-resident chaperones, components of the ER-associated protein degradation (ERAD) machinery, and regulators of ER size (Trusina et al., 2008). In many scenarios, the adaptive phase can restore equilibrium between protein load and chaperone capacity within the ER, dampening the UPR. However, in cases of chronic or unresolved ER stress, the UPR continues to a second alarm phase that involves several signal transduction events that move the cell from a pro-survival to a pro-apoptotic state (Tabas and Ron, 2011; Woehlbier and Hetz, 2011). This ends in the final apoptosis phase involving the transcriptional and post-translational activation of the BH3-only proteins and other BCL2 protein family members that trigger the canonical mitochondrial cell death pathway. Additionally, the BCL2 proteins have been implicated in linking ER Ca^{2+} homeostasis and apoptosis (Oakes et al., 2003; Bassik et al., 2004).

Mechanistically, in mammalian cells, the ER-SAD program is mediated largely by the UPR signaling molecules and endonuclease IRE1α, which can transmit both pro-survival and pro-death signals. Indeed, in my view, ER-SAD can be defined as the cell death process that involves IRE1 function. In light of this, it is significant that there are pro- and anti-apoptotic effectors assembled around IRE1α that are able to control the amplitude and duration of IRE1α signaling (Woehlbier and Hetz, 2011). For example, IRE1α signaling can be enhanced at the ER membrane by the formation of a complex between the cytosolic domains of IRE1α and the BAX–BAK complex, two pro-apoptotic members of the BCL2 family of proteins (Scorrano et al., 2003; Zong et al., 2003). In contrast, IRE1α signaling can be attenuated by the binding of the ER-localized anti-apoptotic protein, BAX inhibitor-1 (BI-1; Lisbona et al., 2009; Bailly-Maitre et al., 2010; Castillo et al., 2011). Late-phase UPR signaling of IRE1α can lead to changes in the expression and activity of BCL2 protein family members and therefore to the activation of apoptosis.

Finally, we should note two emerging areas of inquiry involving the role of the ER in cell death. First, several studies in mammalian cells have begun exploring the links between ER and mitochondrial function during PCD. Both organelles form interconnected membrane networks that can influence various cellular processes including cell death (Csordas et al., 2006). More recently, Cardenas and colleagues have shown that Ca^{2+} transport between the ER and the mitochondria is regulated by the inositol triphosphate receptor, IP_3R, to modulate mitochondrial bioenergetics (Cardenas et al., 2010), a process involving BI-1, an ER-resident protein known to be involved in autophagy and apoptosis (Sano et al., 2012). This may explain the earlier observation that mitochondria preferentially accumulate Ca^{2+} in regions called mitochondria-associated microdomains (MAMs) where the ER and mitochondria are found in close proximity (Rizzuto et al., 1998). Next, there have been a few published reports that describe the fragmentation of the ER during ER stress and ER-SAD in mammalian cells (Brough et al., 2005; Kucharz et al., 2011a,b; Howarth et al., 2012). Though there is evidence that the loss of the GTPase atlastin 1 can cause ER fragmentation in *Drosophila* (Orso et al., 2009), the mechanism behind the ER fragmentation associated with ER stress in the mammalian system is still not known.

ER STRESS-ASSOCIATED DEATH IN YEAST CELLS

As they do in mammalian cells, a range of intrinsic and extrinsic triggers can disrupt ER function and lead to ER stress and to ER-SAD in yeast (**Table 1**). Notably, however, in yeast cells, ER stress is only sensed by a single signaling pathway driven by Ire1p, which is the most ancient of the three parallel UPR pathways found in the metazoan (Sidrauski and Walter, 1997). Three examples of yeast cell death associated with the ER will be highlighted here, including inositol starvation, lipid toxicity, and the inhibition of *N*-glycosylation.

First, Guerin et al. (2009) reported that the fission yeast, *S. pombe*, undergoes cell death with apoptotic-like features when it is deprived of inositol, a precursor of numerous phospholipids and signaling molecules. Deleting either *pca1*$^+$, the gene for the only caspase-like protein in *S. pombe*, or *ire1*$^+$, the gene for the only IRE1 homolog, enhanced cell survival in media lacking inositol, suggesting that both genes are involved in the cell death pathway. Interestingly, the ER transmembrane chaperone, calnexin, encoded by the *cnx1*$^+$ gene, has also been implicated in inositol-starvation-induced ER-SAD since overexpressing different portions of the Cnx1p protein can alter the number of dying cells in inositol-starved conditions as measured by several assays (Guerin et al., 2009).

Next, feeding the budding yeast, *S. cerevisiae*, with extracellular SFA like palmitate (C16:0) triggers ER stress and leads to growth arrest and death (Pineau et al., 2009). Addition of palmitate to the cell culture also alters ER morphology with swelling of the organelle, detachment of the pmaER from the plasma membrane, and in certain cells, the replacement of pmaER by electron-lucent clefts extending throughout the cytoplasm that are visible in the electron microscope. Both the induction of the UPR and the cell death associated with lipid toxicity were abrogated with the addition of the molecular chaperone 4-phenyl butyrate, suggesting that lipid-induced ER stress overburdens the folding machinery in the ER. Notably, lipid toxicity is known to induce apoptosis in mammalian cells (Kharroubi et al., 2004; Diakogiannaki et al., 2008). One paper reports that palmitate induces apoptosis in pancreatic beta cells by activating the IRE1α, PERK, and ATF6 pathways (Cunha et al., 2008).

Finally, Hauptmann et al. (2006) reported that preventing the *N*-glycosylation of yeast proteins, either by mutating critical subunits of the oligosaccharyltransferase (OST) complex in the ER lumen, or by treating the cells with tunicamycin, a drug known to block the ER enzyme UDP-*N*-acetylglucosamine-1-*P* transferase (Alg7p) that is necessary for *N*-glycosylation, induced an apoptotic-like death in wild-type *S. cerevisiae* cells. The dying cells contained condensed nuclei, fragmented DNA, and externalized phosphatidylserine. Defects in *N*-glycosylation also led both to the appearance of a caspase-like activity that did not require functional yeast metacaspase, Yca1p, and to the production of reactive oxygen species (ROS) that could be diminished by heterologous expression of the human anti-apoptotic protein, Bcl-2. Two years later, the same team reported that the Golgi-localized Kex1p protease is involved in the apoptotic-like cell death linked to defects in *N*-glycosylation (Hauptmann and Lehle, 2008). Deletion of *KEX1* diminished the appearance of the caspase-like activity and decreased ROS accumulation in cells cultured in tunicamycin. Notably, the cell death described in these experiments was blocked by the addition of osmotic stabilizers to the culture media.

Strikingly, these studies disagreed with previous findings that had shown that tunicamycin does not induce cell death in wild-type cells unless the calcium-dependent phosphatase, calcineurin, had previously been inactivated (Bonilla et al., 2002; Bonilla and Cunningham, 2003). To resolve this disagreement, Dudgeon et al. (2008) used improved staining methods using both propidium iodide (PI) and FITC-VAD-FMK together instead of FITC-VAD-FMK alone, to analyze the cell death associated with growth in media containing tunicamycin. Their data showed that tunicamycin can induce cell death, but only in cells grown in low osmolyte yeast–peptone–dextrose (YPD) media, and not in cells grown in synthetic media. They also demonstrated that this dying process is not apoptotic in nature. The dying cells lacked two critical hallmarks of apoptosis – both chromatin fragmentation and phosphatidylserine externalization – suggesting that there may have been methodological problems in past efforts to characterize the cell death induced by tunicamycin in *S. cerevisiae*.

Instead, tunicamycin appeared to trigger two different forms of death in wild-type budding yeast cells, one that is partially dependent on a functional electron transport chain (ETC) and another that is independent of ETC function. Active calcineurin signaling could prevent the former, which is why it has been called calcineurin-less death, but not the latter form of cell death. Significantly, calcineurin-less death in response to tunicamycin

Table 1 | ER-Associated Cell Death in Mammalian and Yeast Cells.

ER function	Organism	Triggers of cell death (select list)	ER proteins involved
Protein translocation and folding	Mammalian	Protein aggregation	IRE1α
		Ischemia reperfusion	ATF6
		Beta-mercaptoethanol (BME)	PERK
		Tunicamycin	BI
		Dithiothreitol (DTT)	Grp78/BiP
	Yeast	Heat shock	Ire1p
		Beta-mercaptoethanol (BME)	Bxi1p
		Tunicamycin	Kar2p/BiP
		Dithiothreitol (DTT)	
Lipid synthesis	Mammalian	Elevated free fatty acids (FFA)	IRE1α
			ATF6
			PERK
	Yeast	Elevated saturated fatty acids	Ire1p
		(SFA) inositol starvation	Cnx1p
Calcium dynamics	Mammalian	Thapsigargin	PERK
		Calcium overload	SERCA
			Grp78/BiP
	Yeast	Calcium starvation	Pmr1p
		Calcium chelators	

did not involve any of the apoptosis-associated factors, Yca1p, Nuc1p, Nma111p, or Ste20p, strengthening the claim that this death is not apoptotic in nature. Rather, the dying process was regulated by Cmk2p, one of the two Ca^{2+}/calmodulin-dependent protein kinases, and by Hsp90p, one of the major heat shock chaperones in budding yeast. Finally, a very recent study from the same research team has concluded that tunicamycin leads to cell death by permeabilizing the vacuolar membranes of yeast cells and that this cell death program involves the V-ATPase that acidifies the vacuole (Kim et al., 2012). This tunicamycin-induced cell death involving vacuole membrane permeabilization could be blocked by calmodulin.

In light of the cumulative evidence, in my view, it is clear that tunicamycin leads to cell death, but that this dying process is not apoptotic in nature as had been previously reported. One possible reason for the discrepancy in the literature is the earlier study's failure to distinguish living apoptotic cells from dead necrotic cells, which are known to stain non-specifically with FITC-VAD-FMK (Wysocki and Kron, 2004; Hauptmann et al., 2006). Moreover, the observation reported in the earlier paper that the addition of an osmotic stabilizer like sorbitol is able to block some of the *N*-glycosylation-linked cell death suggests that this cell death may be lytic rather than apoptotic in nature (Hauptmann and Lehle, 2008). This is not surprising since protein *N*-glycosylation is crucial for cell wall assembly (Lesage and Bussey, 2006; Levin, 2011). Defects in this biochemical pathway would be expected to weaken the cell's ability to maintain its structural integrity.

Finally, can this tunicamycin-induced calcineurin-less cell death in *S. cerevisiae* properly be called ER stress-associated cell death? It is not clear. As I noted above, in mammalian cells, ER-SAD has been linked to the activation of the UPR and IRE1 function. Though tunicamycin does induce the UPR in budding yeast, it is also apparent that Ire1p is not required for either the pro-death activity of tunicamycin in calcineurin-deficient cells or the anti-death activity of calcineurin in these cells (Bonilla et al., 2002). This is in stark contrast to the single published report suggesting that tunicamycin induces an apoptotic-like cell death in *S. pombe* that appears to partially require the fission yeast Ire1p (Guerin et al., 2008).

FUTURE DIRECTIONS

The study of the ER's role in yeast cell death is in its infancy. It would be interesting to determine if other physiological triggers of yeast cell death like acetic acid or ethanol – triggers not directly linked to ER function – involve the ER. Recently, my laboratory has begun characterizing the yeast BI protein, Bxi1p, an ER-localized protein that links the UPR to PCD in *S. cerevisiae* (Cebulski et al., 2011).Cells lacking *BXI1* are not only more sensitive both to ethanol-induced and to glucose-induced PCD, but also have a diminished UPR. Studies linking Bxi1p and Ire1p function in yeast are ongoing in my lab. Finally, it will be important to check if PCD alters the yeast's ER structure, and conversely, if mutants that modify ER structure alter the dynamics of yeast cell death. These investigations should uncover the mechanistic links between ER structure, ER function, and cell death in yeast.

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The impact of peroxisomes on cellular aging and death

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Peroxisomes are ubiquitous eukaryotic organelles, which perform a plethora of functions including hydrogen peroxide metabolism and β-oxidation of fatty acids. Reactive oxygen species produced by peroxisomes are a major contributing factor to cellular oxidative stress, which is supposed to significantly accelerate aging and cell death according to the free radical theory of aging. However, relative to mitochondria, the role of the other oxidative organelles, the peroxisomes, in these degenerative pathways has not been extensively investigated. In this contribution we discuss our current knowledge on the role of peroxisomes in aging and cell death, with focus on studies performed in yeast.

Keywords: aging, autophagy, cell death, peroxisome, yeast

INTRODUCTION

The production and accumulation of reactive oxygen species (ROS) is a profound stress factor in living cells due to the fact that ROS can oxidize and therefore damage vital macromolecules such as nucleic acids, proteins, and lipids. Intracellular accumulation of these damaged components leads to aging – a process defined as the deterioration of cells in time which is accompanied by gradual loss of cell viability. Until recently, mitochondria were considered as the main players in ROS production and hence in aging of eukaryotic cells. However, recent reports proposed that peroxisomes also produce significant amounts of ROS. Therefore, like mitochondrial dysfunction, peroxisomal dysfunction may also contribute to cell death and aging. The role of mitochondria in cell death pathways such as apoptosis and necrosis is well established. However, the importance of peroxisomes in these processes is much less understood. In this contribution we summarize findings on the role of peroxisomes in aging and cell death processes. We focus on data obtained in yeast and discuss the relevance of these findings for higher eukaryotes including man.

PEROXISOMES: STRUCTURE AND FUNCTION

Peroxisomes are highly dynamic organelles: their morphology, abundance, and function depending on species, developmental stage, and external stimuli (Oku and Sakai, 2010; Schrader et al., 2011). The predominant feature of peroxisomes is the presence of H_2O_2 producing oxidases and the antioxidant enzyme catalase to detoxify this ROS species. In yeast, peroxisomes are predominantly involved in the primary metabolism of unusual carbon sources, such as oleic acid in *Saccharomyces cerevisiae* and methanol in methylotrophic yeasts. In man, peroxisomes are involved in the α- and β-oxidation of very long chain fatty acids, biosynthesis of ether phospholipids and bile acids (Wanders and Waterham, 2006). Recently also novel, non-metabolic functions have been identified for mammalian peroxisomes, among which anti-viral innate immunity and anti-viral signaling (Dixit et al., 2010).

THE PEROXISOME LIFE CYCLE AND IMPLICATION FOR AGING

Like mitochondria, peroxisomes can multiply by division of pre-existing ones (**Figure 1**). However, so far no evidence has been obtained that peroxisomes fuse. The initial stage of peroxisome fission is organelle elongation mediated by the peroxisomal membrane protein Pex11p. Dynamin-related proteins (DRPs) are responsible for the final scission event. In *S. cerevisiae* the DRPs Vps1p and Dnm1p are involved in peroxisome fission (Hoepfner et al., 2001; Kuravi et al., 2006), whereas in the yeast *Hansenula polymorpha* peroxisome fission entirely depends on Dnm1p (Nagotu et al., 2008). Recruitment of Dnm1p to the peroxisomal membrane is mediated by the peroxisomal membrane protein Fis1p. Interestingly, Fis1p also recruits Dnm1p to mitochondria for mitochondrial fission (Mozdy et al., 2000). Similarly, mammalian Fis1 and Drp1 are both involved in peroxisome and mitochondrial fission. Hence, peroxisomes and mitochondria share key components of their fission machineries.

In two fungal model systems for aging, the filamentous ascomycete *Podospora anserina* and baker's yeast down-regulation of mitochondrial fission by deletion of the *DNM1* gene leads to a robust increase in replicative lifespan (Scheckhuber et al., 2007, 2008). Moreover, deletion of *DNM1* also has a positive effect on

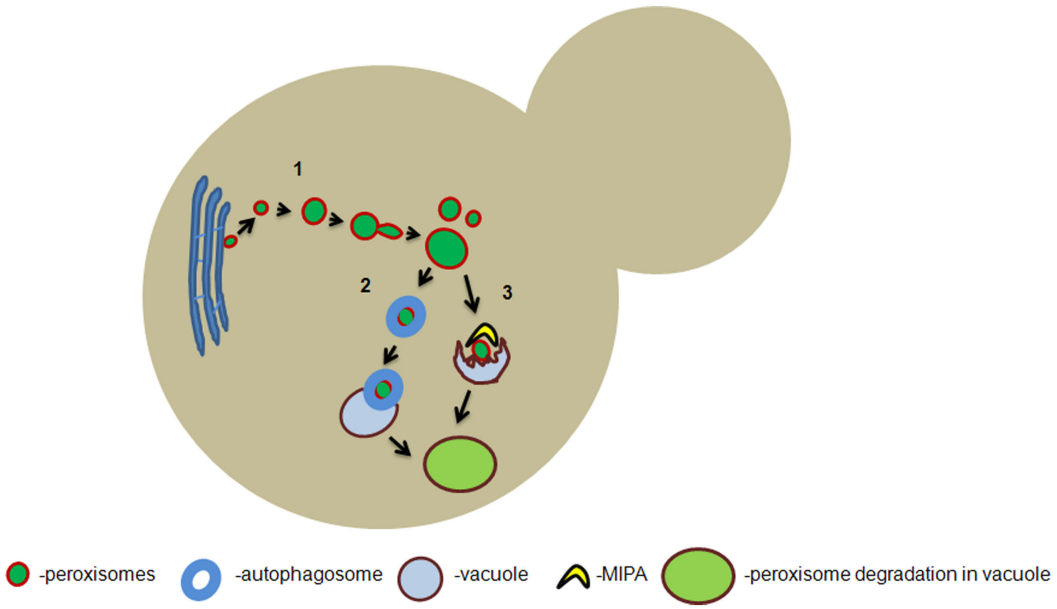

FIGURE 1 | Hypothetical model of peroxisome formation and degradation in yeast. *De novo* formation of peroxisomes from the endoplasmic reticulum takes place in yeast cells which are devoid of pre-existing peroxisomes (1). In wild-type yeast cells, the predominant mode of peroxisome proliferation occurs via fission of the pre-existing organelles which involves Pex11p dependent elongation and dynamin-related protein (DRP) dependent scission of the peroxisomal membrane. When peroxisomes are targeted for macropexophagy they are engulfed by autophagosomes which ultimately fuse with the vacuolar membrane to deliver the organelle for degradation (2). Peroxisomes can also be targeted for micropexophagy in which the protrusion of the vacuolar membrane engulfs the organelle and they are subsequently degraded (3). MIPA: micropexophagy-specific membrane apparatus.

chronological aging in baker's yeast (Palermo et al., 2007). These beneficial effects might be based on improved content mixing of mitochondria so that molecular damage to proteins, lipids, and mtDNA can be ameliorated more efficiently. However, it has to be stressed that the effect of *DNM1* deletion on peroxisome fission was not investigated in these studies. Hence, the observed effects may also be partially due to defects in peroxisomal fission.

Several data suggest that peroxisomes divide asymmetrically, resulting in larger mature organelles and small, nascent ones (Koch et al., 2003, 2010; Cepinska et al., 2011; Huber et al., 2012). As a consequence cells contain a heterologous population of peroxisomes, ranging from relatively young and vital nascent organelles to relatively old, mature ones, in which dysfunctional components accumulate in time due to damage caused by products of peroxisomal metabolism.

In addition to fission, peroxisomes can also be formed *de novo*, a process that is most prominent in yeast *pex3* or *pex* mutants that lack pre-existing peroxisomes, upon reintroduction of the corresponding genes. Several data suggested that these new organelles originate from the endoplasmic reticulum (ER; Hoepfner et al., 2005; Zipor et al., 2009).

Data in yeast, data in yeast indicate that the major pathway of peroxisome proliferation is fission. However, possibly in other species the formation of peroxisomes from the ER is a more prominent process (Geuze et al., 2003; Tabak et al., 2003; Kim et al., 2006; Yonekawa et al., 2011).

Autophagy can result in a reduction in the number of peroxisomes per cell. Autophagy is the pivotal cellular housekeeping process that can eliminate redundant or unwanted components or entire organelles from the cell. Selective degradation of peroxisomes by autophagy is designated "pexophagy," a phenomenon mainly studied in the methylotrophic yeast species *H. polymorpha* and *Pichia pastoris*.

In *H. polymorpha*, macropexophagy is induced when methanol-grown cells are shifted to glucose (**Figure 1**). Under these conditions, the key peroxisomal enzymes of methanol metabolism become redundant for growth. The surplus in organelles is then selectively degraded by macropexophagy (Monastryska et al., 2004), a process that involves sequestration of individual peroxisomes from the cytosol and subsequent fusion with the vacuole for degradation (Veenhuis et al., 1983).

When methanol-grown cells of *P. pastoris* are shifted to glucose, peroxisomes are degraded by micropexophagy, which involves the formation of finger-like protrusions by the vacuole and subsequent engulfment of clusters of peroxisomes from the cytosol with ultimate degradation in the vacuole. Before this takes place, a double membrane flattened sac-like structure termed micropexophagy-specific membrane apparatus (MIPA) is synthesized at the peroxisome surface to complete the sequestration process (Tuttle and Dunn, 1995; Mukaiyama et al., 2004).

In addition, data obtained with *H. polymorpha* revealed a constant removal of peroxisomes by autophagy (Aksam et al., 2007). This process most likely prevents the accumulation of damaged peroxisomal components, which would be potentially hazardous for the cells. Hence, at peroxisome-inducing conditions, organelle proliferation and degradation occur simultaneously to maintain

tightly controlled organelle homeostasis. This mechanism is consistent with the view that timely rejuvenation of peroxisomes is vital for cell viability and survival. Most likely the oldest, mature organelles are most susceptible for autophagic degradation. A central question that offers interesting perspectives for future research is how these peroxisomes are recognized by the pexophagy machinery. The peroxisomal membrane proteins Pex3p and Pex14p have been shown to play a role in the early stage of pexophagy (Bellu et al., 2001, 2002; van Zutphen et al., 2008) and may play a role in this recognition process. Indeed Pex3p was shown to interact with Atg30p, a phosphoprotein that is necessary for the presentation of the compromised peroxisome to the vacuole for its subsequent degradation (Farre et al., 2008).

PEROXISOMAL DAMAGE AND REPAIR MECHANISMS

PEROXISOMAL ROS AND ANTIOXIDANT ENZYMES

Peroxisomes counteract the compromising effects of ROS by antioxidant enzymes. Among these are peroxisomal catalase, glutathione peroxidase, peroxiredoxin I and Pmp20p to degrade hydrogen peroxide and CuZnSOD and MnSOD to detoxify superoxide anions (Bonekamp et al., 2009).

Saccharomyces cerevisiae contains two catalases, namely peroxisomal Cta1p and cytosolic Ctt1p. Unexpectedly, inactivation of both catalases in this yeast was shown to result in an increase of the chronological lifespan (Mesquita et al., 2010). This surprising finding could be explained by the activation of superoxide dismutases by the elevated hydrogen peroxide levels. In this view elevated levels of hydrogen peroxide in catalase-deficient *S. cerevisiae* cells prolong chronological lifespan.

However, earlier studies indicated that deletion of the gene encoding the peroxisomal catalase in *S. cerevisiae* resulted in a shortened lifespan (Petriv and Rachubinski, 2004). These seemingly contradictory results may be possibly related to differences in the experimental procedures to determine the chronological lifespan (i.e., use of different cultivation conditions such as media composition and growth temperatures; Mesquita et al., 2010: SC medium, 26°C; Petriv and Rachubinski, 2004: YNBD medium, 30°C). The same authors also reported that in *Caenorhabditis elegans* the deletion of peroxisomal catalase (*Ctl-2*) in the genetic background of the long lived Δ*clk-1* mutant shortens the maximum lifespan as well. The shortened lifespan was accompanied by altered peroxisome morphology that might point to compromised peroxisomal function with increased production of ROS (Petriv and Rachubinski, 2004).

Similar to catalase, the peroxisomal peroxiredoxin Pmp20p is also involved in degradation of H_2O_2. Peroxiredoxins are thiol-specific evolutionary conserved antioxidant enzymes. In addition to H_2O_2 breakdown they are also involved in degradation of organic hydroperoxides (ROOH) and therefore important for maintaining the integrity of lipid membranes (Yamashita et al., 1999).

Studies in the methylotrophic yeasts *H. polymorpha* and *C. boidinii* demonstrated that Pmp20p is important for cell viability during growth on methanol due to its capability to repair ROS generated damages (i.e., lipid peroxidation) at the peroxisomal membrane surface (Horiguchi et al., 2001; Aksam et al., 2007). In the fission yeast *Schizosaccharomyces pombe* it was shown that Pmp20p (in addition to thioredoxin peroxidase and glutathione peroxidase) inhibited thermal aggregation of citrate synthase at a high temperature (43°C) *in vitro*. This suggests that at least in *S. pombe* peroxisomal Pmp20p may have a second function as a molecular chaperone which could be important for organelle quality control (Kim et al., 2010).

In man catalase gene mutations have been identified and linked to detrimental conditions like diabetes, hypertension, macular degeneration, cataracts, cancer, and skin pigmentation disorders (Goth et al., 2004). A severe decrease of catalase activity is found in clinical cases of hypo- or acatalasemia and has been predominantly identified in Japan and certain European countries (e.g., Hungary, Switzerland). Clinical symptoms are severe (among them hemolytic anemia), illustrating the importance of functional catalase for health. Re-direction of functional catalase to peroxisomes in catalase-deficient cell lines led to increased detoxification of H_2O_2 and also restoration of cellular plasmalogen and fatty acid levels (Sheikh et al., 1998).

Reactive oxygen species are usually associated with their potent damaging capabilities but they are also involved in crucial cellular signaling processes. With regard to aging, low levels of ROS might induce a hormesis response which increases chances of cellular survival by up-regulating pathways dedicated to high-stress adaptation like the retrograde response (Jazwinski, 2012) and the target of rapamycin (TOR) and adenosine mono phosphate kinase (AMPK) pathways (Gems and Partridge, 2008). Although it is clear that mitochondria are supposedly the main players in this regard, it is likely that also peroxisomes integrate into the signaling network as important mediators of aging processes.

PEROXISOMAL LON PROTEASE

So far one conserved peroxisomal protease is known that plays a role in peroxisomal protein quality control, namely the peroxisomal Lon protease, Pln (Kikuchi et al., 2004; Aksam et al., 2007). Pln belongs to the ATPases associated with diverse cellular activities (AAA) protein family which is supposed to be involved in degradation of unfolded and non-assembled peroxisomal matrix proteins (Aksam et al., 2007; Ngo and Davies, 2007). Studies in *H. polymorpha* showed that the deletion of the gene encoding Pln leads to a pronounced decrease in cell viability accompanied by enhanced ROS production (Aksam et al., 2009). One possible explanation could be that the accumulation of unfolded proteins leads to the formation of protein aggregates, resulting in a disturbance of ROS homeostasis which ultimately leads to cell death. Indeed, electron dense aggregates are often observed in peroxisomes of *H. polymorpha* cells lacking Pln (Aksam et al., 2007). However, the precise molecular mechanism and interplay between protein aggregation, ROS production, and cell death still remains unclear and needs to be studied in more detail.

Several studies have investigated the function of Pln in mammalian cells after the identification of this enzyme in rat liver peroxisomes (Kikuchi et al., 2004). In mammals Pln also seems to play a role in peroxisomal matrix protein import as catalase import is compromised when Pln is overproduced (Omi et al., 2008).

THE ROLE OF PEROXISOMES IN YEAST AGING

Mechanistic insights into the role of peroxisomes in aging have been gained mainly from studies conducted in *S. cerevisiae*. When this yeast is grown in glucose rich media, neutral lipids (e.g., triacylglycerols) are produced in the ER and incorporated in lipid bodies (Olofsson et al., 2009).When these storage lipids need to be mobilized, peroxisomes and lipid bodies come into close contact to allow uptake of the neutral lipids for subsequent oxidation of the non-esterified fatty acids (free fatty acids; Binns et al., 2006). An intriguing model has been put forward linking life span determination and the synthesis and degradation of lipids in the ER, lipid bodies, and peroxisomes (Goldberg et al., 2009a,b; Titorenko and Terlecky, 2011). According to this model, repression of essential components of fatty acid β-oxidation by ethanol (produced as a side-product of glucose fermentation) leads to the accumulation of free fatty acids. This challenges the cell with detrimental effects, i.e., stimulation of necrotic cell death (Aksam et al., 2008; Jungwirth et al., 2008) and lipoapoptosis (shown in the fission yeast *S. pombe*; Low et al., 2005). Moreover, as a result of impaired β-oxidation in peroxisomes diacylglycerol accumulates in the ER and mediates the induction of protein kinase C-dependent signaling which ultimately affects cellular pathways involved in various stress responses (as demonstrated in the nematode *C. elegans*; Feng et al., 2007).

Peroxisomes and peroxisomal enzymes also play a vital role in the phenomenon of retrograde response (RTG) in baker's yeast (Butow and Avadhani, 2004; Jazwinski, 2012). RTG is activated in yeast cells that are confronted with mitochondrial respiratory dysfunction. Interestingly, induction of RTG leads to an increased replicative lifespan (Kirchman et al., 1999). The more pronounced RTG induction is, the larger the beneficial effect on aging. RTG leads to the induction of transcription of several nuclear genes that help to promote the survival of the cell despite mitochondrial dysfunction (**Table 1**). Genes encoding Cit1p, Aco1p, Idh1/2p (first enzymes of the citric acid cycle), Ald4p and Acs1p (enzymes for cytosolic biosynthesis of acetyl-CoA), Crc1p, Ctp1p, Dic1p, and Odc2p (membrane transporters for shuttling metabolites between mitochondria, peroxisomes, and the cytosol), and Pex11p, Pxa1p,

Table 1 | Overview of genes mentioned in this article.

Name of gene	*S. cerevisiae*	*H. polymorpha*	Mammals	Description of protein	Pathway
ACO1	+	+	+	Aconitase	Krebs cycle/RTG in *S. cerevisiae*
ACS1	+	+	+	Acetyl-CoA synthetase	Acetate utilization/RTG in *S. cerevisiae*
ALD4	+	+	+	Aldehyde dehydrogenase	Glucose fermentation/RTG in *S. cerevisiae*
ATG30	−	+	−	Peroxisomal receptor	Pexophagy
CIT1	+	+	+	Citrate synthase 1 (mito.)	Krebs cycle/RTG in *S. cerevisiae*
CIT2	+	−	−	Citrate synthase 2 (peroxi.)	RTG in *S. cerevisiae*
CRC1	+	+	−	Carnitine carrier	Fatty acid metabolism/RTG in *S. cerevisiae*
CTA1	+	+	+	Peroxisomal catalase	ROS detoxification
CTP1	+	+	+	Citrate transport protein	Mitochon. transporter/RTG in *S. cerevisiae*
CTT1	+	−	−	Cytosolic catalase	ROS detoxification
DIC1	+	+	+	Dicarboxylate carrier	Mitochon. transporter/RTG in *S. cerevisiae*
DNM1/Drp1	+	+	+	Dynamin-related protein (DRP) 1	Mitochondrial/peroxisomal division
Ephx2	+	+	+	Epoxide hydrolase	Detoxification of epoxides
FIS1/hFis1	+	+	+	Binding partner for DRP 1	Mitochondrial/peroxisomal division
FOX1-2	+	+	+	Enzymes involved in β-oxidation of fatty acids	Fatty acid oxidation/RTG in *S. cerevisiae*
IDH1/2	+	+	+	Isocitrate dehydrogenase	Krebs cycle/RTG in *S. cerevisiae*
ODC2	+	+	+	Oxodicarboxylate carrier	Amino acid metabolism/RTG in *S. cerevisiae*
PEX3	+	+	+	Peroxisomal membrane protein	Peroxisome biogenesis/inheritance
PEX6	+	+	+	AAA-peroxin	Recycling of peroxisomal signal receptor Pex5p
PEX11	+	+	+	Peroxisomal membrane protein	Peroxisome proliferation
PEX14	+	+	+	Peroxisomal membrane protein	Peroxisomal protein import
PLN	−	+	+	Peroxisomal LON protease	Protein degradation
PMP20	−	+	+	Peroxisomal peroxiredoxin	ROS detoxification
POT1/Acaa1a	+	+	+	3-ketoacyl-thiolase	Fatty acid oxidation/RTG in *S. cerevisiae*
PXA1	+	+	+	Peroxisomal ABC transporter	Fatty acid transport/RTG in *S. cerevisiae*
VPS1	+	+	−	Vacuolar protein sorting 1	Vacuolar sorting/peroxisomal division (*S. cerevisiae*)

+, homolog present; −, homolog not present; RTG, retrograde response.

Cit2p, Fox1p, Fox2p, Pot1p (peroxisomal proteins) belong to this group. Collectively, these (and further) proteins enable yeast cells to enhance oxidation of fatty acids and to synthesize essential metabolic intermediates of the Krebs cycle that otherwise would not be available.

THE ROLE OF PEROXISOMES IN AGING IN MAMMALS

In rat hepatocytes subjection to conditions of hyperinsulinemia leads to an inhibition of β-oxidation and a concomitant acceleration of aging in these animals (Xu et al., 1995). Clearly, the activity of the peroxisomal marker enzyme catalase decreases by 30–40% in liver samples isolated from old mice and rats (Haining and Legan, 1973; Semsei et al., 1989; Perichon and Bourre, 1995, 1996; Xia et al., 1995). Decreased levels/activity of this H_2O_2-decomposing protein is contributing to the phenomenon of peroxisome senescence. This process leads to diminished regulation of organelle maturation and division, protein import and overall dysfunction (Legakis et al., 2002; Koepke et al., 2008). A comparative study with the goal to identify differences between liver subproteomes from young and old mice revealed several up-regulated peroxisomal proteins. One of the up-regulated enzymes was epoxide hydrolase 2 (Ephx2), which detoxifies epoxides and converts these to excretable dihydrodiols (Amelina et al., 2011). This may constitute a counteracting mechanism in old animals. Another up-regulated enzyme, peroxisomal 3-ketoacyl-thiolase A [Acaa1a, Pot1p (Fox3p) in baker's yeast], was correlated to increased cholesterol levels in old animals (Amelina et al., 2011).

Recently, an inter-organelle crosstalk between mitochondria and peroxisomes regarding the production of ROS was described (Ivashchenko et al., 2011). Enhanced formation of these compounds in peroxisomes profoundly disturbs the redox balance within mitochondria which results in fragmentation of these organelles. This finding proves that peroxisome dysfunction can have a pronounced effect on mitochondrial structure and function. It is thus conceivable that certain scenarios of mitochondrial dysfunction involving elevated cellular ROS levels can also be linked to peroxisomes.

PEROXISOMES AND CELL DEATH IN YEAST AND MAMMALS

Cellular death has many different faces. Two of the most common ones, apoptosis and necrosis, have been studied in much detail over the past few years (Golstein and Kroemer, 2007; Taylor et al., 2008). Apoptosis is a ubiquitous mode of programmed cell death, strictly regulated and conserved in eukaryotes (Madeo et al., 2004). This highly organized process is manifested by condensation and cleavage of nuclear DNA, release of pro-apoptotic proteins from mitochondria and, at later stages, "blebbing" of the plasma membrane. Mitochondria are key factors when it comes to the execution of apoptosis. Necrosis, on the other hand, is accompanied by rupture of organelles and the plasma membrane. Recent evidence demonstrates that necrosis can be also tightly controlled, similar to apoptosis (Golstein and Kroemer, 2007). Peroxisomes are involved in regulation of necrosis. For example, *S. cerevisiae PEX6* deletion cells display hallmarks of necrosis and strongly elevated formation of ROS (Jungwirth et al., 2008). Moreover, in the methylotrophic yeast *H. polymorpha* deletion of *PMP20* leads to pronounced induction of necrosis when the cells are grown on methanol-containing medium (Aksam et al., 2008). Interestingly, matrix proteins of the peroxisomes were found to be leaking into the cytosol of *PMP20* deletion cells during necrosis. This is reminiscent of protein release by mitochondria during apoptosis and constitutes an interesting parallel between mitochondria and apoptosis on the one hand and peroxisomes and necrosis on the other hand (Eisenberg et al., 2010).

PERSPECTIVES

Research on the role peroxisomes play during cellular degeneration is supposed to unravel exciting new mechanisms and will likely integrate these fascinating organelles into the network of cellular pathways mediating aging. Some of the interesting lines of research that will yield promising insights into the role of peroxisomes might comprise (i) identification and characterization of a peroxisomal unfolded protein response (UPR) as a mechanism to signal organelle dysfunction (and subsequent degradation), (ii) studying selective inheritance of peroxisomes during replicative aging so that the daughter cells receive "healthy" peroxisomes (Cepinska et al., 2011), and (iii) investigating the role of *de novo* formation of peroxisomes vs. fission in maintaining a functional peroxisomal population during chronological and replicative aging.

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Apoptotic-like programed cell death in fungi: the benefits in filamentous species

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Studies conducted in the early 1990s showed for the first time that *Saccharomyces cerevisiae* can undergo cell death with hallmarks of animal apoptosis. These findings came as a surprise, since suicide machinery was unexpected in unicellular organisms. Today, apoptosis in yeast is well-documented. Apoptotic death of yeast cells has been described under various conditions and *S. cerevisiae* homologs of human apoptotic genes have been identified and characterized. These studies also revealed fundamental differences between yeast and animal apoptosis; in *S. cerevisiae* apoptosis is mainly associated with aging and stress adaptation, unlike animal apoptosis, which is essential for proper development. Further, many apoptosis regulatory genes are either missing, or highly divergent in *S. cerevisiae*. Therefore, in this review we will use the term apoptosis-like programed cell death (PCD) instead of apoptosis. Despite these significant differences, *S. cerevisiae* has been instrumental in promoting the study of heterologous apoptotic proteins, particularly from human. Work in fungi other than *S. cerevisiae* revealed differences in the manifestation of PCD in single cell (yeasts) and multicellular (filamentous) species. Such differences may reflect the higher complexity level of filamentous species, and hence the involvement of PCD in a wider range of processes and life styles. It is also expected that differences might be found in the apoptosis apparatus of yeast and filamentous species. In this review we focus on aspects of PCD that are unique or can be better studied in filamentous species. We will highlight the similarities and differences of the PCD machinery between yeast and filamentous species and show the value of using *S. cerevisiae* along with filamentous species to study apoptosis.

Keywords: apoptosis, botrytis, fungi, PCD, *Saccharomyces*

OVERVIEW

Organisms in the fungal kingdom can be separated into two distinct morphotypes: unicellular (yeasts) and multicellular (filamentous), with some species having a dimorphic appearance. Although this separation does not have a phylogenetic basis, the different in morphology also extends to the molecular level. Yeasts are the better studied group due to their long association with human civilization and ease of use; the combination of eukaryotic single cell type, genetic tractability, and the ability to easily quantify cell populations, make yeasts excellent research systems. In particular, the baker's yeast *Saccharomyces cerevisiae* has been developed as an eukaryotic model to study cellular and developmental processes, including programed cell death (PCD). Originally, *S. cerevisiae* was used as a system to evaluate and search for human apoptotic proteins (Sato et al., 1994; Xu and Reed, 1998). These studies lead to the discovery and study of PCD in *S. cerevisiae* (Madeo et al., 1997). Research of PCD was later extended to additional fungi, including filamentous species. These studies revealed substantial variability in the regulation and manifestation of PCD in different species, and especially between *S. cerevisiae* and filamentous fungi. Most significantly, processes such as multicellular development and pathogenicity, in which PCD may play a significant role, cannot be studied in *S. cerevisiae*. We will compare the current status of knowledge on PCD in *S. cerevisiae* and filamentous species, and highlight the advantages of using *S. cerevisiae* along with filamentous species in the study of PCD.

PCD IN *S. CEREVISIAE*

In metazoans there are two major apoptotic pathways: the extrinsic pathway, composed of a so called death receptors and ligands of the TNF family, and the intrinsic pathway culminating in mitochondrial outer membrane permeability. In mammals the extrinsic pathway is mediated by the death-inducing signaling complex (DISC), which contains a death receptor trimer, FADD adaptor proteins and caspases 8 and 10. The intrinsic pathway is initiated by the release of cytochrome *c* from the mitochondria following apoptotic stimuli, which along with Apaf-1 and procaspase 9 form a heptameric complex known as the apoptosome (Mace and Riedl, 2010). Pro- and anti-apoptotic members of the Bcl-2 family of proteins, which function upstream of or at the mitochondria membrane, are central regulators of PCD in animals (Chipuk et al., 2010).

Programed cell death is induced in yeast by a variety of triggers and is accompanied by most if not all the typical characteristics of animal apoptosis (Xu and Reed, 1998; Rockenfeller and Madeo,

2008; Schmitt and Reiter, 2008; Carmona-Gutierrez et al., 2010). Nevertheless, the yeast apparatus bears significant differences compared to apoptotic apparatus in animals. Most significantly, the entire extrinsic pathway is not found in fungi. Furthermore, important regulators of the intrinsic pathway, including Bcl-2 proteins, P-53, FLIP, poly ADP-ribose polymerase (PARP), and even caspases do not have clear homologs in *S. cerevisiae*. Interestingly, homologs of some of the proteins that are not present in *S. cerevisiae* can be found in filamentous species (see below). Such differences at the molecular level are indicative of significant functional differences and should be taken into consideration when comparing fungal and animal PCD.

The most highly represented apoptosis-related proteins found in yeast are mitochondria-associated proteins. In particular, a significant portion of the apoptosis-promoting, mitochondria-secreted proteins have been identified, including homologs of genes encoding for cytochrome *c*, the endonucleases apoptosis-inducing factor (AIF) and EndoG, and the IAP-inhibiting serine protease Omi/HrtA2. In addition, several orthologs of non-mitochondrial proteins have been analyzed (for a review, see Carmona-Gutierrez et al., 2010). Interestingly, the only known executor of apoptosis in *S. cerevisiae* is the metacaspase Yca1/Mca1, which mediates the final stages of cell death following a wide range of stimuli (Madeo et al., 2009). Likewise, Bir1p, a class II IAP protein and homolog of human survivin, is the only known inhibitor of apoptosis in yeasts (Owsianowski et al., 2008). In addition to homologs of apoptosis proteins, a number of mitochondria proteins that are involved in mitochondria fusion, fission, and homeostasis also affect yeast apoptosis (Fröhlich et al., 2007). Deletion of the *S. cerevisiae* dynamin related protein Dnm1p, which is responsible for mitochondrial fission caused elongation of mitochondria and subsequent increase of life (Scheckhuber et al., 2007; Carmona-Gutierrez et al., 2010). Mutants in Fis1p, an anchor protein for Dnm1p, increased sensitivity of the yeast cells to apoptosis, probably due to selection for a *whi2* mutation (Teng et al., 2011). The microtubule and mitochondria interacting protein Mmi1p, an ortholog of human Tctp, shuttles from the cytoplasm to mitochondria upon an apoptosis stimulus and promotes PCD in yeast cells (Rinnerthaler et al., 2006).

Despite the absence of a significant portion of the animal apoptotic network, *S. cerevisiae* has proven a viable system to study human apoptosis. These studies stem from the first observation that expression of human Bcl-2 pro- and anti-apoptotic proteins promote or suppress PCD in yeast cells, respectively (Sato et al., 1994; Xu and Reed, 1998). Nevertheless, as a single cell organism, the results obtained in *S. cerevisiae* are limited to cellular processes and relevance to situations in animals is not always clear; primarily, multicell level development cannot be studied in *S. cerevisiae*. In addition, certain PCD-related processes such as aging and autophagy might be significantly different in the context of multicellular organisms compared with unicellular organisms. In these instances filamentous fungi might be useful in complementing and augmenting the results obtained in yeasts.

PCD IN FILAMENTOUS FUNGI

Filamentous fungi combine the genetic simplicity and short life cycle of yeast with the morphological complexity of multicellular organism. They typically form a network of interconnected hyphae, which are defined as "colonies" that grow by hyphal tip extension, branching, and fusion. In higher fungi (Ascomycotina and Basidiomycotina, subkingdom Dikarya), the septa along the hyphae are incomplete, leaving a pore through which cytoplasm and organelles can move (Glass and Fleissner, 2006). PCD has been observed in higher fungi during sexual and asexual development, for example during gills formation in mushrooms or formation of sclerotia in some Ascomycetes (Georgiou et al., 2006). This type of coordinated cell death echoes developmental PCD in higher eukaryotes. In addition, and similar to the situation in yeasts, PCD in filamentous fungi is also associated with stress adaptation, spore formation, antagonistic interactions, and aging (Sharon et al., 2009). However, some aspects of fungal PCD are significantly different between single cell and filamentous species. These differences might stem from the different lifestyles of single cell and multicellular organisms. In addition to differences due to unicellular and multicellular organization, there are processes related to PCD that either cannot be analyzed in *S. cerevisiae*, e.g., pathogenicity, or are significantly different in multicellular species. The use of filamentous species in these cases is of special importance.

PCD AND AGING

Aging is a process of progressive decline in the ability to withstand stress, damage, and disease. Aging processes have been extensively studied in various model organisms including *S. cerevisiae*. In addition, the filamentous fungus *Podospora anserina* has been used as a model to study aging in multicellular eukaryote (Osiewacz, 2002, 2011). In fact, study of aging in *P. anserina* started already in the 1950s, and the connection of mitochondria and aging was demonstrated for the first time in this fungus (Rizet, 1953). In *P. anserina*, senescence is characterized by an age related decrease in mycelium growth rate, reduction in formation of aerial hyphae, increased pigmentation, and eventual death of peripheral hyphae (Albert and Sellem, 2002; Scheckhuber and Osiewacz, 2008). At the microscopic level, the peripheral hyphae show abnormal branching and swelling. In wild-type isolates of *P. anserina*, aging is correlated with accumulation of mutated mtDNA leading to mitochondrial genome instability (Stahl et al., 1978; Kuck et al., 1985; Osiewacz and Borghouts, 2000; Albert and Sellem, 2002). The instability of the mitochondria genome correlates with appearance and accumulation of a 2.5-kb DNA fragments that correspond to an integral part of the 95-kb mtDNA and to the first intron (pl-intron) of the *PaCOX1* gene, the first subunit of cytochrome *c* oxidase (Cox) in the respiratory chain. Strains selected for increased lifespan were found to be deficient in Cox activity due to deletion of the first exon of the *PaCOX1* gene. Deletion of *PaCOX5* (encoding subunit V of Cox) led to severe decrease in growth rate, along with decreased ROS production, drastic reduction in the rearrangement of mtDNA, and a 30-fold increased lifespan of the fungus (Dufour et al., 2000). Mutants with deletions in genes encoding other Cox subunits had a similar phenotype (Lorin et al., 2006). In these mutants, respiration was carried out via alternative oxidase (Aox)-dependent pathways, an enzyme of the inner mitochondrial membrane. Genetic manipulation that restored ROS production to wild-type levels also reversed

the amount of mutated mtDNA to wild-type levels and reversed lifespan of the strains to wild-type levels. Deletion of *PaDNM1* forced increased lifespan and reduced sensitivity to the apoptosis-inducing compound etoposide, further demonstrating the central role of mitochondrion-mediated PCD in aging (Scheckhuber et al., 2007). Collectively, these results indicate that increased ROS levels during aging trigger mitochondria-dependent PCD in senescent cultures of *P. anserina*. Deletion of putative AIFs also leads to lifespan extension, providing evidence that aging in *P. anserina* is programed and tightly connected with PCD (Hamann et al., 2007; Brust et al., 2010).

Similar to all other systems, the final stages of PCD in fungi are carried out by cysteine proteases exhibiting caspase activity. At least one, but usually two or three caspase-related genes are found in fungi. While the enzymes encoded by these genes recognize the typical substrates of caspases, the encoded proteins show limited homology to animal caspases. Furthermore, they lack a cas domain, the most significant signature of caspases. It has been proposed that these proteins represent an ancient form of caspases and therefore they were termed metacaspases (Savoldi et al., 2008; Tsiatsiani et al., 2011). A caspase-independent pathway also exists in fungi, which (similar to situation in human) involves homologs of AIF and AIF-homologous mitochondrion-associated inducers of death (AMID; Modjtahedi et al., 2006).

Functional analyses of the metacaspase-dependent and -independent pathways were conducted by deletion of either the metacaspases or AIF members in *P. anserina*. Deletion of either of the two putative metacaspases, *PaMCA1* and *PaMCA2*, in *P. anserina* reduced sensitivity to PCD-promoting conditions and had a lifespan extending effect on the fungus (Hamann et al., 2007). The AIF family in *P. anserina* includes at least five members that are divided to cytosolic and mitochondria species. Deletion of the mitochondria-residing members, either *PaAIF2* or *PaAMID2*, caused reduced sensitivity to oxidative conditions and extended lifespan of the fungus. In contrast, deletion of the cytosolic isoforms of AIF, *PaAIF1* and *PaAMID1*, had no effect on lifespan and on sensitivity of the fungus to oxidative stress (Brust et al., 2010).

Together, *S. cerevisiae* and *P. anserina* form an excellent system for unraveling the role of mitochondria in aging. Both species are capable of adjusting their metabolism in case of mitochondria dysfunction, but *S. cerevisiae* does not have the Aox pathway, which is used by *P. anserina* to compensate for Cox deficiency. *S. cerevisiae* also lacks complex I of the mitochondria respiratory chain and therefore this complex can only be studied in *P. anserina* (Osiewacz and Scheckhuber, 2006). Likewise, *S. cerevisiae* can grow under anaerobic conditions, and hence is useful in studying processes that might be lethal in strict aerobes such as *P. anserina*.

PCD AND FUNGAL PATHOGENESIS

During pathogenic interaction both the host and pathogen are exposed to PCD-inducing conditions and compounds (Sharon and Finkelshtein, 2009). Interestingly, all plant pathogenic fungi are filamentous in nature. While not as strict, most human fungal pathogens also are either filamentous or dimorphic. Furthermore, dimorphic pathogenic species, such as the human pathogen *Candida albicans* or the maize pathogen *Ustilago maydis*, switch from a yeast to a filamentous state during transition from a latent to a pathogenic state (Garber and Day, 1985; Lo et al., 1997). Hence, filamentous fungi can be used to study the role of pathogen PCD in plant and animal diseases.

In plants, the manipulation of the host apoptotic response, either enhancement (by necrotrophic pathogens) or suppression (by biotrophic pathogens) of PCD, is a common strategy used by fungi to weaken the host (Sharon and Finkelshtein, 2009). This phenomenon was demonstrated in transgenic plants expressing anti-apoptotic genes, which suppressed PCD and enhanced or reduced plants' susceptibility to either biotrophic or necrotrophic pathogens, respectively (Dickman et al., 2001; Huckelhoven et al., 2001, 2003; del Pozo and Lam, 2003; Eichmann et al., 2004). A number of studies demonstrated limited necrosis and restricted spreading of the model necrotrophic pathogen *Botrytis cinerea* in plants that over-express anti-apoptotic genes or in hypersensitive response (HR)-deficient mutant plants that do not produce ROS, whereas accelerated cell death mutant plants are more susceptible to this pathogen (Govrin and Levine, 2000; Imani et al., 2006; Van Baarlen et al., 2007). Dihydrosphingosine-induced cell death was shown to mediate phytotoxicity of AAL toxin. This toxin is produced by the necrotrophic pathogen *Alternaria alternata* and belongs to a class of host-selective fungal mycotoxins that are structurally related to sphinganine, a precursor in plant sphingolipid biosynthesis. AAL toxin kills the cells of sensitive host plants by inducing PCD (Brandwagt et al., 2000). Administration of AAL toxin to sensitive tissues blocks sphingolipid biosynthesis and leads to accumulation of dihydrosphingosine. AAL-insensitive plants contain the *ASC-1* resistance gene, a homolog of the yeast longevity assurance gene (*LAC1*). Asc1p modifies sphingolipid metabolism in AAL-treated cells, thereby preventing accumulation of dihydrosphingosine and induction of apoptosis (Brandwagt et al., 2000; Spassieva et al., 2002).

Several studies documented fungal cell death during infection and showed that it was essential for completion of pathogenic life cycle (Howard et al., 1991; Thines et al., 2000; Veneault-Fourrey et al., 2006). In contrast, Barhoom and Sharon (2007) reported on hyper virulence of a cell death-protected *Colletotrichum gloeosporioides* strain, over-expressing human Bcl-2. These studies hint to a link between fungal PCD and disease. Early studies showed that some plant compounds, for example the tobacco pathogenesis related protein osmotin, can induce PCD in *S. cerevisiae* (Narasimhan et al., 2001). Additional antifungal peptides from other organisms were found, which can induce PCD in different fungi (Ramsdale, 2008), however the relevance of these results to pathogenesis remains unclear. More recent studies provided new and more direct evidences that plant defense compounds induce PCD in fungi during plant colonization. The saponin α-tomatine, a sesquiterpene glycoside produced by tomato, has antifungal activity. Initially, α-tomatine was considered to promote fungal death by disruption of membrane integrity (Friedman, 2002). A more recent study showed that α-tomatine induces PCD in the plant pathogen *Fusarium oxysporum*. Moreover, PCD was found necessary for antifungal activity of the compound (Ito et al., 2007). Treatment with either ROS scavengers (ascorbic acid and dimethylthiourea) or a caspase inhibitor (Z-VAD-FMK) reduced fungal cell death in a dose-dependent manner, suggesting that α-tomatine-induced cell death in *F. oxysporum* is ROS

and caspase-dependent. In addition, the fungicidal action of α-tomatine was suppressed by the mitochondrial electron transport inhibitor oligomycin, suggesting a role for mitochondria in the process.

A more recent example demonstrated the role of PCD in pathogenicity of *B. cinerea*. Camalexin, the major phytoalexin produced in *Arabidopsis*, belongs to a group of secondary metabolites with anti-microbial activity that are produced in plants upon microbial attack (collectively called phytoalexins) and form a line of defense against potential pathogens (Kliebenstein et al., 2005; Lazniewska et al., 2010). Similar to other phytoalexins, camalexin has growth inhibiting activity against a wide range of microorganisms (Ferrari et al., 2003; Kliebenstein et al., 2005; Rowe et al., 2010). Micromolar concentrations of camalexin were found to induce PCD in *B. cinerea*, but at higher concentrations of camalexin the apoptotic markers were reduced, indicating that at these concentrations necrotic cell death was induced (Finkelshtein et al., 2011; Shlezinger et al., 2011b). Similar results were also observed following treatment of *B. cinerea* with hexanoic acid, another plant defense compound (Finkelshtein et al., 2011). These results suggest that when exposed to plant defense molecules during the early phase of infection, *B. cinerea* might be subjected to host-induced PCD. In this event, fungal anti-apoptotic machinery might be necessary for survival and pathogenicity. In order to investigate this possibility, Shlezinger et al. (2011b) tested the role of *B. cinerea* anti-apoptotic BcBir1 protein in disease. This study revealed that following germination and formation of first contact with the plant, the fungus undergoes massive PCD [between 30 and 48 h post-inoculation (PI)], and then fully recovers at 72 h PI, when spreading lesions start to develop. PCD-modified strains were produced by manipulation of the *BcBIR1* gene; over-expression strains were less sensitive, and knockdown strains were hypersensitive to apoptosis induction, respectively. Plant infection assays showed enhanced and reduced virulence of the *BcBIR1* over-expression and knockdown strains, respectively. Importantly, the levels of PCD in *BcBIR1* over-expression strains was markedly reduced between 30 and 48 h PI compared to almost complete elimination of the wild-type cells at this time point. In contrast, in the knockdown strain there was early and intense PCD and it remained high also at 72 h PI, when the wild-type cells showed complete recovery. On *Arabidopsis thaliana* mutant plants that are impaired in defense responses and are hypersensitive to *B. cinerea*, PCD levels were reduce in all strains, confirming that the amount of fungal PCD is negatively correlated with plant susceptibility to the fungus. Specifically, the phytoalexin-deficient *pad3* mutant, which does not produce camalexin, was highly susceptible to *B. cinerea*, and disease was produced on this line also following infection with the *Bcbir1* knockdown strain. As pointed out, camalexin induced PCD in *B. cinerea* wild-type strain *in vitro*. In accordance with the PCD-promoting effect of camalexin, the *BcBIR1* over-expression and knockdown strains showed reduced or enhanced sensitivity to camalexin, respectively, along with reduced PCD on the *pad3* plants.

PCD IN CELL–CELL INTERACTIONS: HETEROKARYON INCOMPATIBILITY

In filamentous fungi, vegetative hyphae commonly fuse. These hyphal fusions occur during colony formation as well as between hyphae of different strains as part of parasexual reproduction (Saupe et al., 2000; Glass and Kaneko, 2003; Glass and Dementhon, 2006). The fusion between hyphae from different strains leads to formation of a heterokaryon, a situation in which cells contain nuclei of different genetic background. Specific heterokaryon-incompatibility (HI) loci determine fusion compatibility between hyphae from different strains (Leslie and Zeller, 1996; Glass et al., 2000). When hyphae that are not vegetative compatible fuse, a rapid, localized cell death is activated that specifically kills the fusion cell and prevents heterokaryon formation (Glass and Kaneko, 2003).

In many ways, HI resembles the HR in plants, during which localized PCD prevents pathogen spreading (Lam et al., 2001). Both HI and HR are accompanied by classical apoptotic markers and have been widely studied (del Pozo and Lam, 1998; Jacobson et al., 1998; Glass et al., 2000; Saupe et al., 2000; Marek et al., 2003; Glass and Dementhon, 2006; Paoletti and Clave, 2007; Williams and Dickman, 2008). During HI, the fusion hyphae undergo a series of apoptosis-associated morphological changes, including cytoplasm condensation, vacuolization, and shrinkage of the plasma membrane (Glass and Kaneko, 2003; Marek et al., 2003; Glass and Dementhon, 2006). Nuclear fragmentation and positive TUNEL staining have also been documented. Data from whole genome microarrays of *Neurospora crassa* showed that ROS, phosphatidylinositol and calcium signaling, are all involved in HI and PCD. However, homologs of apoptotic genes, such as caspases (metacaspases) and AIF were not required for HI in *N. crassa* (Hutchison et al., 2009).

Severin and Hyman (2002) showed that in the absence of an appropriate mating partner, exposure of yeast cells to pheromones of the opposite mating type leads to ROS accumulation, DNA degradation, and cell death. It should be noted however that pheromone-induced cell death was observed at pheromone concentrations that were 10-fold higher than physiological concentrations; no cell death was induced by physiological concentrations of the mating pheromone. Unlike the case of yeast pheromones, PCD is a general phenomenon of HI and occurs naturally. The widespread occurrence and high number of HI loci in filamentous fungi argues for their importance. Therefore, HI represents an important process in which PCD plays major role. This system can be used in functional and mechanistic studies of heterologous apoptotic proteins and has several advantages over other systems, including budding yeasts. Mainly, the induction of PCD during HI is very rapid and it does not require application of exogenous substances (Garnjobst and Wilson, 1956; Biella et al., 2002; Glass and Kaneko, 2003; Sbrana et al., 2007). Thus, apoptosis can be studied under natural conditions in a short time period, in contrast to PCD induced by aging or starvation.

ANTIFUNGAL DRUGS AND PCD

Recognition in the importance of PCD in fungi has led to re-evaluation of the mode of action of leading antifungal drugs. Surprisingly, it was found that a range of well-known antifungal compounds induce PCD in fungi. For many years amphotericin B (AmB) has been the most common drug used to treat fungal infections (Brajtburg et al., 1990). Similar to other polyene antibiotics, AmB has high affinity to sterols, particularly ergosterol.

The antifungal activity of AmB was attributed to formation of pores in the cell membrane and hence distortion of the fungal cell integrity (Liao et al., 1999). More recently it was found that AmB induces PCD in fungi, including the human pathogens *C. albicans* and *A. fumigatus* (Phillips et al., 2003; Mousavi and Robson, 2004). Notably, at concentrations of 1 mg/ml AmB or higher, cell death shifted from apoptotic to necrotic, as determined by increased and decrease propidium iodine- and TUNEL-positive cells, respectively. Similar to HI PCD, appearance of apoptotic markers could not be blocked or reduced by caspase inhibitors, nor were any changes recorded in caspase activity, suggesting a caspase-independent process. Additional antifungal drugs of different chemical groups have been reported to induce PCD in fungi, suggesting that induced PCD might be a common mode of action for many antifungal compounds (Ramsdale, 2008). The induction of apoptosis by AmB might be mediated by sphingolipids that are released from the plasma membrane. Sphingolipid metabolism is associated with a wide range of cellular activities, including stress response, apoptosis, inflammation, cell-cycle regulation, and cancer development (Dickson, 1998; Kolesnick and Krönke, 1998; Hannun and Luberto, 2000; Hannun et al., 2001). Two major sphingoid bases of fungi – dihydrosphingosine and phosphosphingosine, induced ROS accumulation and cell death with typical markers of apoptosis in *Aspergillus nidulans* (Cheng et al., 2003).

Greater understanding of PCD in pathogenic fungi may offer a chance of exploiting the fungal death machinery to control fungal infections. Clearly identifiable differences between the death machineries of pathogens and their hosts make this a feasible task.

THE FUNGAL PCD MACHINERY

As pointed out earlier, the complete extrinsic apoptosis pathway and major signaling components upstream of the mitochondria (intrinsic) pathway, are not found in fungal genomes. This raises the question if there are functional homologs of these proteins, which do not share sequence similarity. A number of studies showed that expression of Bcl-2 protein members triggers (e.g., Bax) or prevents (e.g., Bcl-2) PCD in fungi (Longo et al., 1997; Fröhlich and Madeo, 2000; Polcic and Forte, 2003; Barhoom and Sharon, 2007). Thus, despite the lack of Bcl-2 homologs, proteins of the Bcl-2 family are recognized in fungi and specifically activate (pro-apoptotic members) or block (anti-apoptotic members) PCD. Studies in *B. cinerea* revealed a number of proteins that interact with the human Bcl-2 protein and might mediate the effect of this protein in the fungus. Moreover, a yeast two-hybrid screen of a *B. cinerea* expression library that was performed with some of these candidates led to identification of proteins that interact with the same Bcl-2-interacting proteins (Oren-Young and Sharon, unpublished results).

Filamentous fungi have larger genomes and more complex development programs compared to *S. cerevisiae*. It is therefore intuitive to assume that PCD pathways in filamentous species will include a larger number of proteins and would be more complex compared with *S. cerevisiae*. Indeed, a few homologs of animal apoptotic proteins that are not found in *S. cerevisiae* can be identified in genomes of filamentous species using a simple BLAST search. Some processes, such as the HI response are restricted to filamentous species and therefore genes that are involved in regulation of these processes are not present in unicellular species. More than 50 putative human and mouse apoptosis-associated genes that are not found in *S. cerevisiae* were described in *Aspergillus* and represent a potentially filamentous-specific PCD regulators (Fedorova et al., 2005). In addition to protein homologs of components of the metazoan apoptotic machinery, this list includes many fungal-specific genes, such as het loci, and species-specific protein families. Functional analyses were performed only on a small number of candidates, and therefore it is unclear how many proteins on this list are true regulators of PCD.

Neurospora crassa HET-C2 is probably the best characterized HI gene. *HET-C2* orthologs were identified in genomes of all filamentous species and are also present in many animals and plants, but they are not found in *S. cerevisiae* or in the fission yeast *Schizosaccharomyces pombe*. The high level of conservation among Het-C2 family members is consistent with the important role of these proteins in glycosphingolipid and sphingosine metabolism, and possibly in regulation of cellular stress responses. Het-C2 shows significant similarity to human Gltp (Rao et al., 2004) and *A. thaliana* Acd11 (Brodersen et al., 2002), which catalyze intermembrane transfer of glycosphingolipids and sphingosines, respectively. *P. anserina* Het-C2 was proposed to act as a glycolipid metabolite sensor in addition to its role in glycolipid transfer, regulation of ascospore maturation, and triggering of HI (Saupe et al., 1994; Mattjus et al., 2002). The high level of sequence conservation in this family, suggests that the role of Het-C2 orthologs in *Aspergilli* PCD is likely similar.

Interestingly, many of the identified PCD-related proteins from *Aspergillus*, such as Amid, Bir1, HtrA, and CulA, are more similar to their human counterparts than to the yeast homologs. Moreover, a small number of animal apoptotic proteins, including PARP, have homologs in filamentous fungi but are not found in *S. cerevisiae*. PARPs catalyze the NAD(+)-dependent modification of proteins with poly (ADP-ribose), which play key roles in a plethora of processes including DNA repair, tumor progression, and aging. PARP is one of the known target proteins inactivated by caspase degradation in animal cells (Schlegel et al., 1996). *A. nidulans* PARP-like protein is broken down by caspase activity during sporulation-induced PCD (Thrane et al., 2004). *P. anserina* genome encodes a single protein with a PARP catalytic domain. Over-expression of the gene caused increased sensitivity to apoptosis inducers, impaired growth and pigmentation, sterility, and a shorter lifespan (Müller-Ohldach et al., 2011).

The availability of a large number of fungal genomes provides new opportunities to search for additional PCD-associated fungal genes. In many cases, homology with the entire animal ortholog is rather low or restricted to a specific domain and hence simple BLAST searches might not be sensitive enough to recognize the homology. In order to obtain a deeper coverage of the putative fungal PCD orthologs, a computer-guided approach was developed, which enables automatic searches of all available fungal genomes for presence of homologs of apoptotic proteins or domains (Shlezinger et al., 2011a). Using this approach, it is possible to identify all the fungal genes that are putative homologs of known apoptotic genes or that contain a putative apoptotic domain. Searches conducted with this program

revealed that except for BIR, all other conserved apoptotic domains were absent from fungal genomes, including Bcl-2 homology (BH) domains (BH1–4), caspase recruitment domain (CARD), cellular apoptosis-susceptibility (CAS) protein, death domain (DD), death effector domain (DED), CIDE [cell death-inducing DNA fragmentation factor 45 kDa α (DFFA)-like effector], or death receptors. Likewise, homologs of many central apoptosis regulators, such as P53, Flip, Smac/Diablo, Apaf1, and even caspases are not readily found in fungi. It should be noted that putative homologs for some of these proteins have been reported in filamentous species, including P53 (Katz et al., 2006) and PARP (Fuchs and Steller, 2011). However in most instances homology centers around parts of the proteins that are associated with general functions, such as protein interaction, while the domain known to mediate apoptosis is usually absent. A unique exception is the *S. cerevisiae* Bxi1, a homolog of the human lifeguard 4 protein. Similar to all members of the lifeguard family of proteins, Bxi1 contains a Bax inhibitor 1 (BI-1)-like domain, and therefore was assumed to represent a yeast homolog of BI-1 (Chae et al., 2003; Cebulski et al., 2011). However, recent work has shown that this protein contains a BH3-like signature at the carboxy part of the protein. Remarkably, functional analyses confirmed a pro-apoptotic activity in these residues (Buttner et al., 2011). Search of fungal genomes using the domain-centered approach revealed a single homolog of lifeguard 4/Bxi1 in all fungi. However, in filamentous species members of the subkingdom Dikarya (Ascomycetes and Basidiomycetes), a true homolog of plant and human BI-1 was also found (Goldfinger and Sharon, unpublished). These new findings indicate that additional "missing" fungal homologs of animal apoptotic proteins and domains might be found using more robust bioinformatic approaches.

SUMMARY

The realization in the early 1990s that yeast cells contain a suicide mechanism led to intense research of PCD in *S. cerevisiae*. The PCD response was characterized in great detail, *S. cerevisiae* homologs of mammalian apoptotic genes were identified, and the relevant proteins analyzed. Based on these studies it is now generally accepted that yeast cells contain a PCD machinery, which resembles the animal apoptosis machinery. Studies of PCD in additional fungi lagged behind the work in budding yeasts, and a more intense research was initiated only in the past decade. As expected, the machinery is similar to the one found in *S. cerevisiae*, however some differences were also revealed. Most significantly, it was realized that PCD is important for fungal pathogenicity and multicellular-level development. Furthermore, filamentous species contain more PCD-related genes, including a few homologs of animal apoptosis proteins, which are absent in yeasts, and some that are fungal specific, such as the HI proteins encoding genes. The expansion of the research to additional species also led to better mapping of apoptosis networks in fungi. Using robust bioinformatics, it was possible to not only identify more components of the PCD apparatus in fungi, but also to exclusively show what parts of the animal machinery are missing or significantly altered. From such analyses it is now clear that the entire death receptors-mediated extrinsic pathway is missing in the fungal kingdom. Further, the main regulators of the intrinsic pathway that are responsible to initiate mitochondria-related apoptosis also seem to be largely absent in fungi. These discoveries put fungal PCD in a new light; while the pioneering studies in *S. cerevisiae* uncovered the presence of PCD machinery that is highly similar to animal apoptosis, the expansion of the research to additional fungal species shows that the molecular machinery bears significant differences compared with the animal apoptotic machinery. These differences probably also reflect differences in the execution and role of PCD in fungi compared to animals. We expect that research of fungal PCD will intensify and extend to an even wider range of species, leading to a deeper understanding of the regulation of this process and the physiological roles that it has in fungal life cycles.

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The non-death role of metacaspase proteases

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The activation of caspase proteases and the targeting of protein substrates act as key steps in the engagement and conduct of apoptosis/programmed cell death. However, the discovery of caspase involvement in diverse non-apoptotic cellular functions strongly suggests that these proteins may have evolved from a core behavior unrelated to the induction of cell death. The presence of similar proteases, termed metacaspases, in single cell organisms supports the contention that such proteins may have co-evolved or derived from a critical non-death function. Indeed, the benefit(s) for single cell life forms to retain proteins solely dedicated to self destruction would be countered by a strong selection pressure to curb or eliminate such processes. Examination of metacaspase biology provides evidence that these ancient protease forerunners of the caspase family also retain versatility in function, i.e., death and non-death cell functions. Here, we provide a critical review that highlights the non-death roles of metacaspases that have been described thus far, and the impact that these observations have for our understanding of the evolution and cellular utility of this protease family.

Keywords: metacaspase, caspase, non-death, cell cycle, proteostasis

INTRODUCTION

The conserved family of clan CD proteases, caspases, has been extensively characterized in programmed cell death or apoptosis, a function that is vital for homeostasis of complex organisms. Despite the well established death centric role, there is increasing evidence for caspase involvement in non-apoptotic scenarios, such as terminal differentiation of numerous cell types, non-death cellular remodeling events and immune system adaptation. In addition to the study of caspase function in multi-cellular life forms, the discovery of functional caspase orthologs in lower eukaryotes, such as fungi and protozoa (termed metacaspases) suggests these proteins emerged early in the evolutionary record (Uren et al., 2000; Aravind and Koonin, 2002).

Interestingly, recent investigations of metacaspase function have revealed these enzymes play a role in various non-apoptotic or "non-death" processes, in a manner analogous to the metazoan caspase family. Here, we critically review the literature and the latest studies which examine the physiologic function of metacaspase proteases. We conclude that the versatility displayed by the caspase protease family may simply reflect primordial death and non-death functions that initially evolved from para- and metacaspase activity, a functional diversity that is clearly present in unicellular organisms such as yeast and trypanosomatids.

METACASPASES IN CELL CYCLE REGULATION

One of the earliest reports indicative of a non-death role for metacaspases was derived from observations in the protozoan *Trypanosoma brucei*. Here, the expression of several metacaspases (MCA2/3/5) was shown to be critical for the viability of the bloodstream form of the parasite. RNAi induced knockdown of the expression of these metacaspase genes was accompanied by severe growth retardation and cell cycle defects of the circulating *Trypanosoma* (Helms et al., 2006). Furthermore, the Δ*mca2/3*Δ*mca5* mutants showed no significant difference in cell death kinetics in response to prostaglandin D_2 treatment, observations which support a cellular role for *Trypanosoma* metacaspase beyond the apoptosis cascade.

A subsequent study in the related kinetoplast protozoan, *Leishmania major*, further supported the role of metacaspases in cell cycle dynamics. The *L. major* metacaspase (LmjMCA), which is syntenic to MCA5 in *T. brucei*, was observed to be a critical component that regulated stage progression during cellular division (Ambit et al., 2008). For example, during logarithmic growth, LmjMCA expression increased when compared to stationary phase cultures. Moreover, the association of LmjMCA with mitotic spindles during cellular division provided convincing evidence that this metacaspase impacted cell cycle progression. Accordingly, the overexpression of LmjMCA resulted in severe growth retardation with concurrent defects in kinetoplast segregation, multiple mitotic nuclei, and changes in ploidy with a reduced number of cells undergoing cytokinesis. Attempts to create an LmjMCA null strain also resulted in striking cell cycle defects, leading to lethality. Together, these observations suggest that LmjMCA plays a critical role in the management of cell cycle progression.

The mechanism by which a protozoan metacaspase exerts cell cycle control is not entirely clear, although a number of studies suggest that the subcellular localization of the enzyme as well as the level of expression may dictate this non-death activity. First, the RAB11 marker for recycling endosomes was observed to co-localize with a large proportion of the metacaspases in a distinctive compartment between the nucleus and the kinetoplast (Helms et al., 2006). However, the recycling process of VSG was observed to be independent of the metacaspases.

RAB11 positive endosomes are known to be involved in kinetoplast division leading to cytokinesis in the procyclic form of *T. brucei* (Jeffries et al., 2001; Kohl et al., 2003). Thus, the role of these metacaspases in cytokinesis of the bloodstream form of *T. brucei* may argue a non-death role in cell cycle progression, yet specific experiments to support this contention have yet to be undertaken. More recently, overexpression of the *Trypanosoma cruzi TcMCA3* has been linked to a non-death biologic activity, resulting in a reduced growth rate and a transient G1/S block. Additionally, overexpression of TcMCA5 lacking the Ct region (pro, gln, and tyr rich region) led to increase in hypodiploid cells, which implicates the Ct region in dictating metacaspase function (Laverrière et al., 2012). Of note, MCA5 is syntenic in the three protozoa species (Mottram et al., 2003); however, the ability of the Ct region to mediate metacaspase function has yet to be explored in *L. major* and *T. brucei*. Together, these observations in related protozoa species argue that metacaspases regulate cell cycle progression, a function that appears to be independent of promoting cell death.

The metacaspase involvement in cell cycle control appears to be a well conserved phenomenon that extends across phyla. In *Saccharomyces cerevisiae*, deleting the single metacaspase Yca1 (Δ*yca1*) or altering the proteolytic activity of the enzyme leads to altered DNA content and growth rate, which is marked by a slowed G1/S transition (Lee et al., 2008). A similar trend has also been reported for *T. cruzi* (Laverrière et al., 2012). In addition, yca1 null cells failed to respond to a nocodazole-induced mitotic G2/M checkpoint in conditions that favored cell growth. Taken together, these observations implicate Yca1 in regulation of cell cycle checkpoints. Similarly, the metacaspase of the related yeast species, *Schizosaccharomyces pombe* also impacts cell cycle dynamics. In this instance, overexpression of the fission yeast metacaspase, Pca1, led to accelerated growth, a feature which was much improved upon cadmium induced oxidative stress (Lim et al., 2007). The precise mechanism by which a metacaspase protease regulates cell cycle progression remains unknown yet is of considerable interest.

METACASPASE REGULATION OF PROTEOSTASIS AND PROTEIN AGGREGATE FORMATION

The ability of the pombe metacaspase to promote cell cycle advance during oxidative stress strongly suggests that this clade of enzymes may have a cytoprotective role, a feature that appears contrary to the well-described death centric behavior described to date. Consequently, in a subsequent study in *S. cerevisiae* we identified the regulation of protein aggregates as a function by which Yca1 may confer improved fitness and survival (Lee et al., 2010). A genome wide proteomic analysis showed that Δ*yca1* cells are enriched for the Hsp70 family of chaperones (Ssa1, Ssa2, and Ssa4) as well as Hsp104 remodeling chaperone that is involved in the disaggregation of insoluble protein aggregates (Parsell et al., 1994). Furthermore, the normally cytosolic YCA1-GFP was observed to co-localize with Hsp104-RFP, a marker for protein aggregates, under heat stress independent of its catalytic activity. Consequently, filter-trap analyses showed that the loss of Yca1 or its catalytic activity was synonymous with increased levels of insoluble protein aggregates (Lee et al., 2010). Truncated forms of Yca1 lacking the polyQ region were observed to shift from the insoluble protein fraction to a more equitable distribution, with the truncated Yca1 contained in both the soluble and insoluble protein fractions. These observations would suggest that the polyQ region is responsible (in part) for the targeting of Yca1 to aggregated material/proteins and that the stability and/or dissipation of protein aggregates are controlled by the yeast metacaspase Yca1. This unexpected feature of Yca1 appears to be independent of invoking cell death and is associated with maintaining proper cell cycle progression.

As noted with cell cycle regulation and metacaspase activity, the apparent role of a metacaspase(s) in regulating protein levels may also be a conserved molecular function for this otherwise death centric protein. In support of this contention, a study in the filamentous fungus, *Aspergillus fumigatus* revealed that loss of metacaspase expression led to a blunted response to endoplasmic stress (ER) induction (Richie et al., 2007). Specifically, the induction of ER stress in cells lacking functional metacaspase using 2-deoxy-D-glucose (2-DG), tunicamycin (TM), and dithiothreitol (DTT) displayed retardation in growth. Moreover, the increased sensitivity to the glucose analog, 2-DG induced stress in Δ*casA*/Δ*casB* cells was particularly highlighted in the study. 2-DG is known to induce the unfolded protein response (UPR) which delays protein synthesis in order to allow for either the successful re-folding or degradation of misfolded proteins to ensure ER homeostasis (Wu and Kaufman, 2006). In addition to regulating protein homeostasis, these authors also observed that apoptosis induction proceeded independent of metacaspase activity. For example, there was no significant change in the number of PI-positive protoplasts, an observation that is strikingly similar to the observations of metacaspase independent cell death that have been reported in *S. cerevisiae* (Madeo et al., 2009). Although not definitive, the protein homeostatic behavior attributed to metacaspases in yeast and fungi species in the above mentioned studies imply an ancient non-death regulatory role for these enzymes.

Given the observations above it is tempting to speculate that all caspase/metacaspase enzymes have evolved a proteostasis function. Nevertheless, a separate study in *A. nidulans* suggests that unlike in *A. fumigatus*, where loss of both metacaspases had an additive impact on stress outcomes, the metacaspases in *A. nidulans* may actually retain inhibitory or antagonistic functions related to maintaining protein stability (Colabardini et al., 2010; Tsiatsiani et al., 2011). Here, ER stress was induced by treating cells with farnesol, which is also known to induce the UPR, as well as DTT and 2-DG. Spotting assays with the different treatments showed that the loss of *cas*B had a much more significant effect on growth in comparison to the Δ*cas*A cells. Overexpression of pkcA in Δ*cas*A cells restored the sensitivity to farnesol-induced apoptosis. These observations led the authors to speculate that in *A. nidulans* metacaspases may function antagonistically with *cas*A promoting death while *cas*B has a protective role during ER stress (Colabardini et al., 2010).

FUNCTIONAL OVERLAP BETWEEN CASPASES AND METACASPASES

The cellular behavior of metacaspases described thus far provides substantial evidence that these proteases are physiologically active and retain critical function(s) independent of apoptosis.

We have previously argued that death and differentiation may be of a common origin and that different stimuli/substrates may dictate the outcome (Fernando and Megeney, 2007). The physiological functions of metacaspases discussed thus far which are mirrored by their metazoan counterparts lend further support to the hypothesis (**Figure 1**). For example, the ability of metacaspases to regulate cell cycle events have also been observed for their metazoan counterparts; human hepatoma cells that lack caspase 3 activity have also been shown to bypass the G2/M mitotic checkpoint in response to nocodazole treatment (Hsu et al., 2006) and thus implicating an evolutionarily conserved function for caspase and metacaspases between different phylogeny (Tsiatsiani et al., 2011). To date, it remains unclear whether metacaspases have the ability to alter cell fate in a manner similar to metazoan caspases. With reference to mammalian caspase enzymes, upregulation of caspase 3 activity is a critical step in promoting cell differentiation in virtually all progenitor cell types examined from skeletal muscle, to neurons, hematopoietic cell lineages and ES cells (Fernando et al., 2002, 2005; Fujita et al., 2008; Janzen et al., 2008). Moreover, the role of caspase 3 in determining cellular differentiation is conserved across the phyla, from *Drosophila* to humans (Abdul-Ghani and Megeney, 2008).

The importance of caspase 8 activity for trophoblast fusion during human placental development (Black et al., 2004) and caspase 9 as an initiator of lens fiber epithelial development (Weber and Menko, 2005) suggests that both initiator and executioner caspase enzymes have the ability to function in non-death scenarios. The metacaspases involved in non-death functions described here thus far belong to or are known to resemble the type I category, which is thought to be similar to the initiator or pro-caspases in metazoans; both sets possess a regulatory region in the N-terminal. As for type II metacaspases, which are predominantly present in plants (Tsiatsiani et al., 2011), they have yet to be reported in processes other than cell death. Given these observations it is tempting to speculate that the non-death function of initiator caspases may have evolved from the non-death targeting activity of type I metacaspase enzymes.

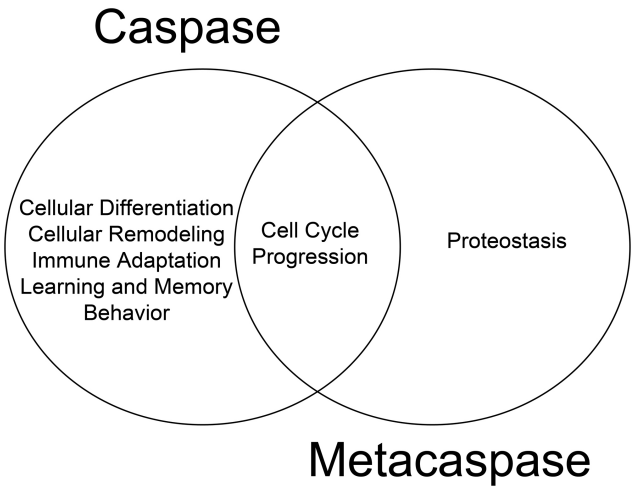

FIGURE 1 | Functional overlap of caspase and metacaspase function. Regulation of cell cycle progression is a conserved function for both proteases, suggesting a common evolutionary origin. Additional non-death functions of metacaspases such as the role of Yca1 in proteostasis has yet to be confirmed as a feature of caspase biology.

The role of metacaspases in maintaining protein homeostasis is a more recent discovery that is unique to *S. cerevisiae* and has yet to be explored extensively in other organisms. Nonetheless, the novel findings generated from the yeast studies provide support for the postulation that Yca1 regulation of protein aggregates may be a mechanism by which the cell increases fitness and adaptation to stress (Lee et al., 2008, 2010). The beneficial role of Yca1 in proteostasis is largely in contrast to the negative role ascribed to mammalian caspases in the same context. Here, caspase proteases have garnered considerable interest for as causative agents in various neurodegenerative/neuromuscular disease conditions such as Alzheimer's disease, Huntington's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), and inclusion body myopathies. In these instances, caspase activation is believed to contribute to the development of a proteotoxic environment by cleaving various proteins that in turn promote aggregate formation, leading to cell stress and eventual cell death (Rothstein, 2009; Rohn, 2010; Graham et al., 2011).

In contrast to the well-accepted contention that activated caspases are synonymous with deleterious activity in neurons, a number of studies suggest that caspase activity may be required for neural cell adaptation and may counteract proteotoxicity. First, caspase activation has been shown to mediate long-term potentiation, learning, dendrite, and axon remodeling, all of which are independent of cell death (Huesmann and Clayton, 2006; Fernando and Megeney, 2007; D'Amelio et al., 2010; Li et al., 2010). More recently, caspase 3 has been reported to cleave TDP-43 in mouse primary cortical neurons, a response which attenuates TDP-43-induced apoptosis (Suzuki et al., 2011). Abnormal aggregated forms of hyperphosphorylated TDP-43 are the major components of ubiquitinated inclusion bodies (IBs) that characterize ALS and frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U; Neumann et al., 2006). The study of Suzuki et al. (2011) demonstrated that ER stress or staurosporine treatment led to caspase cleavage of TDP-43 and generation of C-terminal fragments (CTFs). The death inducing ability of the resulting CTF aggregates were lower than the wildtype TDP-43, and of particular note a caspase cleavage resistant mutant of TDP-43 showed a magnified death response compared to the wildtype protein; an observation that emphasizes a cytoprotective response of the caspase cleavage event (Suzuki et al., 2011). Contrary to the study by Suzuki et al. (2011), other groups have shown that CTFs of TDP-43 can itself be toxic and induce cell death (Johnson et al., 2008; Zhang et al., 2009). However, the cell death in these latter studies may be simply a reflection of a caspase activation pattern that is unrestrained and is coincident with the TDP-43 modifications, rather than disease causing *per se*. Indeed, a reasonable supposition may be that the caspase activation that accompanies protein aggregation in neurodegenerative disease conditions is an adaptive response to rid the cell of toxic materials rather than a disease propagating alteration (**Figure 2**). The corollary to this model would suggest that caspase mediated cell

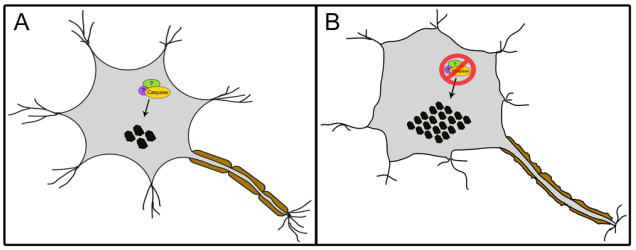

FIGURE 2 | A proposed model of a caspase-dependent mechanism involved in maintaining proteostasis. The interaction of caspases, possibly with yet unidentified additional cofactors, facilitates the regulation of protein aggregates (black circles) within mammalian cells such as neurons **(A)**. The loss of this mechanism leads to increase in aggregate levels resulting in stress **(B)**.

death ensues from excess activation of an otherwise beneficial response.

Interestingly, a recent structural comparison between the *T. brucei* metacaspase, MCA2 and caspase 7 suggests that despite overall structural similarity, metacaspases and caspases differ in their internal design (McLuskey et al., 2012). In addition, both proteases contain specific residues that facilitate substrate binding to the S1 pocket. Albeit these residues are not conserved between the proteases, the authors suggest that proteases within this family may share a common mechanism for substrate recognition. However, the Y31 residue involved in substrate binding and recognition is only conserved in *T. brucei* metacaspases (McLuskey et al., 2012). Thus, future structural analyses of other metacaspases are required to determine whether all metacaspases contain an amino acid residue equivalent to the Y31 residue within their N-terminal region. Of note, caspase 7 lacks an equivalent N-terminal region that is present in MCA2 and thus a comparison to initiator or pro-caspases such as caspases 8 or 10 may be favorable. Nonetheless, the structural similarities exhibited in this study reinforce the functional overlap between metacaspases and caspases in both death and non-death scenarios and further support the argument that both classes of proteases are evolutionarily conserved. Overall, the current evidence presented here suggests that metacaspase proteins act beyond their well described role in apoptosis. The non-death activity of metacaspase proteases reflect an ancient and conserved function that appears to extend to metazoan caspases, and may well represent the evolutionary origin of the death and non-death roles for these same proteins. Future studies to address proteostasis activity in the metazoan caspase family will be a critical step to evaluate and support such a hypothesis.

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Bir1 deletion causes malfunction of the spindle assembly checkpoint and apoptosis in yeast

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Cell division in yeast is a highly regulated and well studied event. Various checkpoints are placed throughout the cell cycle to ensure faithful segregation of sister chromatids. Unexpected events, such as DNA damage or oxidative stress, cause the activation of checkpoint(s) and cell cycle arrest. Malfunction of the checkpoints may induce cell death. We previously showed that under oxidative stress, the budding yeast cohesin Mcd1, a homolog of human Rad21, was cleaved by the caspase-like protease Esp1. The cleaved Mcd1 C-terminal fragment was then translocated to mitochondria, causing apoptotic cell death. In the present study, we demonstrated that Bir1 plays an important role in spindle assembly checkpoint and cell death. Similar to H_2O_2 treatment, deletion of BIR1 using a BIR1-degron strain caused degradation of the securin Pds1, which binds and inactivates Esp1 until metaphase-anaphase transition in a normal cell cycle. BIR1 deletion caused an increase level of ROS and mis-location of Bub1, a major protein for spindle assembly checkpoint. In wild type, Bub1 was located at the kinetochores, but was primarily in the cytoplasm in bir1 deletion strain. When BIR1 was deleted, addition of nocodazole was unable to retain the Bub1 localization on kinetochores, further suggesting that Bir1 is required to activate and maintain the spindle assembly checkpoint. Our study suggests that the BIR1 function in cell cycle regulation works in concert with its anti-apoptosis function.

Keywords: apoptosis, BIR1, cell cycle, oxidative stress, spindle assembly checkpoint

INTRODUCTION

Similar to many other organisms, cell cycle in the yeast *Saccharomyces cerevisiae* is highly regulated. Various checkpoints are in place throughout the cell cycle to ensure faithful segregation of sister chromatids. Unexpected events, such as DNA damage or oxidative stress, cause the activation of checkpoint(s) and cell cycle arrest (Lew et al., 1997). Malfunction of the checkpoints may induce cell death. UV-radiation, for instance, causes alteration of cell cycle and apoptosis in yeast (Del Carratore et al., 2002). Mis-regulation of cell cycle is also found in apoptotic cell death of neuronal cells. Under normal conditions, neuronal cells are maintained in a quiescent G0 state. In the ischemia/reperfusion stroke model of both mice and rats, cerebral neurons were observed to re-enter the cell cycle prior to cell death (Osuga et al., 2000; Katchanov et al., 2001).

Cell cycle regulation under oxidative stress is well studied in both mammalian and yeast cells. Studies show that oxidative stress causes cell cycle arrest, due to the reactive oxygen species (ROS) induced DNA damage (Migliore and Coppede, 2002; Shapira et al., 2004). ROS also induces apoptosis in yeast (Ghibelli et al., 1995; Madeo et al., 1999). It is less clear under what conditions the ROS-exposed cells undergo cell cycle arrest, or apoptosis. One report shows that the H_2O_2 treated human fibroblasts undergo either cell cycle arrest or apoptosis. It depends, at least in part, on where the cell resides in the cell cycle. The majority of the apoptotic fibroblasts were in S phase, whereas growth-arrested cells were predominantly in G1 or G2/M phase (Chen et al., 2000). Another factor that may affect the fate of the cells is the level of the oxidative stress. In yeast, for example, low dose of H_2O_2 induces cell cycle arrest, while high dose causes cell death (Madeo et al., 2004; Shapira et al., 2004).

Failure of the cell cycle checkpoint activation may play an important role in stress-induced cell death (Sczaniecka and Hardwick, 2008). We previously reported that under oxidative stress, the yeast cohesin protein Mcd1, a human Rad21, was cleaved by the caspase-like protease Esp1. The cleaved Mcd1 C-terminal fragment was then translocated to mitochondria, causing apoptotic cell death (Yang et al., 2008). The Esp1 is a cell cycle regulated protein. It is activated by the anaphase promoting complex (APC), which degrades the anaphase inhibitor Pds1. The cleavage of Mcd1 under oxidative stress suggests the interruption or mis-regulation of the spindle assembly checkpoint, which regulates the APC activation.

Bir1 is a chromosomal passenger protein involved in coordinating cell cycle events for proper chromosome segregation (Widlund et al., 2006). It has been shown that Bir1 is required for recruiting condensin. Deletion of BIR1 in *Schizosaccharomyces pombe* inactivates the spindle assembly checkpoint and causes the destruction of securin in the absence of normal anaphase (Morishita et al., 2001). Bir1 also exhibits anti-apoptotic activity. Bir1 deletion in yeast causes apoptotic cell death and is hypersensitive to oxidative stress (Walter et al., 2006). It is not clear whether the anti-apoptosis function of yeast Bir1 is related to its function in

cell cycle regulation (Yang et al., 2008), or if yeast Bir1 functions as an inhibitor of apoptosis, similar to the caspase inhibitors found in mammalian cells (Silke and Vaux, 2001).

In the present study, we demonstrated that Bir1 plays an important role in spindle assembly checkpoint and cell death. We showed that deletion of BIR1 caused elevated level of ROS and apoptotic cell death. We further demonstrated that the apoptosis was likely induced by the mis-regulation of spindle assembly checkpoint, as indicated by the degradation of the anaphase inhibitor Pds1 and the mis-localization of the spindle assembly checkpoint protein Bub1.

MATERIALS AND METHODS

YEAST STRAINS AND CULTURE CONDITIONS

Yeast strains were derivatives of the BY4742 (MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ). The plasmids for making heat-inducible BIR1-degron were purchased from EUROSCARF[1]. The BIR1-degron strain was constructed as described previously (Sanchez-Diaz et al., 2004). The C-terminal BUB1-HA tag was constructed as described before (Janke et al., 2004).

Cells were grown at 30˚C in YPD (1% yeast extract, 2% peptone, and 2% glucose), except where noted in the text. For BIR1-degron induction, cells were first grown YPDCu (YPD with 0.1 mM CuSO4) at 24˚C to a concentration of 1×10^8 cells/ml. Cells were then diluted into YPRCu medium (1% yeast extract, 2% peptone, 2% raffinose, and 0.1 mM CuSO4) and continued to grow at 24˚C to 1×10^7 cells/ml. Cells were then transferred to YPG medium pre-warmed to 37˚C to induce the BIR1-degron.

SEMI-QUANTITATIVE RT-PCR

Total RNA from yeast was extracted using RNeasy Protect Mini Kit (QIAGEN, CA, USA). The reverse transcript (RT)-PCR and the amplification procedure were performed as described previously (Gao et al., 2009). Yeast actin gene (*ACT1*) was used as control.

WESTERN BLOT ANALYSIS

Cells were collected by centrifugation and lysed in lysis buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.1% SDS, and protease inhibitor cocktail. Two-hundred micrometers of glass beads were added and vortexed vigorously. Sample was boiled, spun down and the supernatant was used to run the SDS-gel. Lysates were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were then blocked in 4% non-fat milk and then incubated with anti-HA antibody (clone 3F10, Roche Diagnostics, IN, USA).

CONFOCAL MICROSCOPY

Nuclear spread preparation of BUB1-HA was according to Zhang et al. (2005). The primary and secondary antibodies used for immunostaining were rat anti-HA (Roche Diagnostics, IN, USA) and Alexa Fluor 488 conjugated goat anti-rat IgG (Molecular Probes), respectively. Nucleus was counterstained with 4,6-diamidino-2-phenylindole (DAPI; 0.5 mg/ml). Images were taken using a Zeiss 710 Laser Scanning Confocal Microscope (Jena, Germany).

TRANSMISSION ELECTRON MICROSCOPY

Transmission electron microscopy (TEM) sample was prepared according to Wright (2000). Briefly, cells cultured in either the non-inducing medium (YPDCu at 24˚C) or the inducing medium (YPG at 37˚C) and then were harvested by gentle centrifugation, washed in phosphate buffered saline (PBS; pH = 7.2), resuspended in 2.5% (v/v) glutaraldehyde in PBS and fixed 40 min at room temperature. Cells were further fixed by 2% potassium permanganate in water for 1 h at room temperature. Fixed cells were dehydrated with 30, 50, 75, 85, 95, and 100% ethanol. Cells were transitioned with propylene oxide, infiltrated in Spurr resin (Electron Microscopy Sciences, PA, USA) Resin was polymerized at 65˚C overnight. Sixty nanometers of ultrathin sections were cut with a diamond knife, stained with 2% uranyl acetate and lead citrate, examined using a Hitachi H-7000 electron microscope, and equipped with a high resolution (4 K × 4 K) cooled CCD digital camera (Gatan, Inc.).

DETECTION OF REACTIVE OXYGEN SPECIES

The detection of ROS was performed according to Ren et al. (2005). Briefly, cells were grown at either the non-inducing or inducing media to early log phase. Cells were then washed with PBS for three times and stained for 10 min with dihydroethidium (5 μM; Sigma), viewed with a Zeiss 710 laser scanning confocal microscope with excitation at 514 nm. The fluorescence intensity of about 300 cells from three different experiments was measured using ImageJ software.[2]

STATISTIC ANALYSIS

Data were presented as the mean ± SD from three independent experiments. Statistical significance was determined by Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

BIR1-DEGRON CAUSES DEGRADATION OF BOTH BIR1 mRNA AND PROTEIN

To further understand the role of Bir1 in cell cycle regulation and cell death, we constructed a heat-inducible BIR1-degron strain (Sanchez-Diaz et al., 2004). RT-PCR and Western blot were used to check the changes of BIR1 mRNA and protein levels. When cells were shifted from normal growing condition (YPDCu, 24˚C) to inducing condition (YPG, 37˚C) for 1 h, RT-PCR showed the expression level of Bir1 mRNA was dramatically decreased. Western blot using anti-HA, showed that Bir1 protein was completely depleted (**Figure 1**).

BIR1 DELETION CAUSES DEFECT ON SPINDLE ASSEMBLY CHECKPOINT

Next, we asked if deletion of BIR1 via the BIR-degron affects the function of spindle assembly checkpoint. When nocodazole (10 μg/ml), which blocks the polymerization of microtubules, was added to the culture medium of wild type cells for 2 h, about 90% of the cells were blocked at the metaphase, which was judged by (1). A cell had a daughter cell similar to the size of the mother cell; and (2). The nuclei were in the middle of the dividing cells

[1] http://web.uni-frankfurt.de/fb15/mikro/euroscarf/.

[2] http://imagej.nih.gov/ij/.

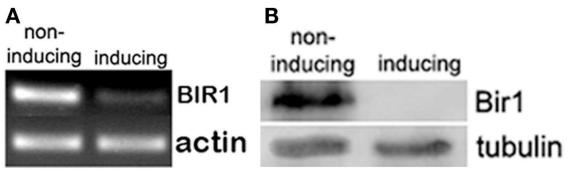

FIGURE 1 | BIR1-degron causes degradation of both BIR1 mRNA (A) and protein (B). BIR1-degron strain was grown in the non-inducing condition (YPDCu, 24˚C) to early log phase. Cells were then transferred to inducing condition (YPG, 37˚C) for 1˚h. PT-PCR **(A)** or Western blot **(B)** was used to examine the mRNA or protein level.

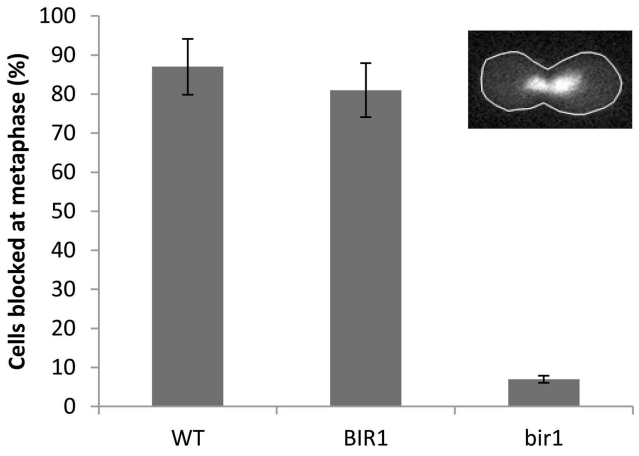

FIGURE 2 | Bir1 deletion causes spindle checkpoint defects. Wild type was grown in YPD at 30˚C (WT), or BIR1-degron strain was grown in either non-inducing condition (YPDCu, 24˚C; BIR1), or in inducing condition (YPG, 37˚C; bir1) to log phase. 10 μg/ml of nocodazole was added to culture media for 2 h. Cells were stained with DAPI and examined using a fluorescence microscope. Cells were considered as metaphase when (1). A cell had a daughter cell similar in size to the mother cell; and (2). The nuclei were in the middle of the dividing cells (inset). About 300 cells were counted from two independent experiments.

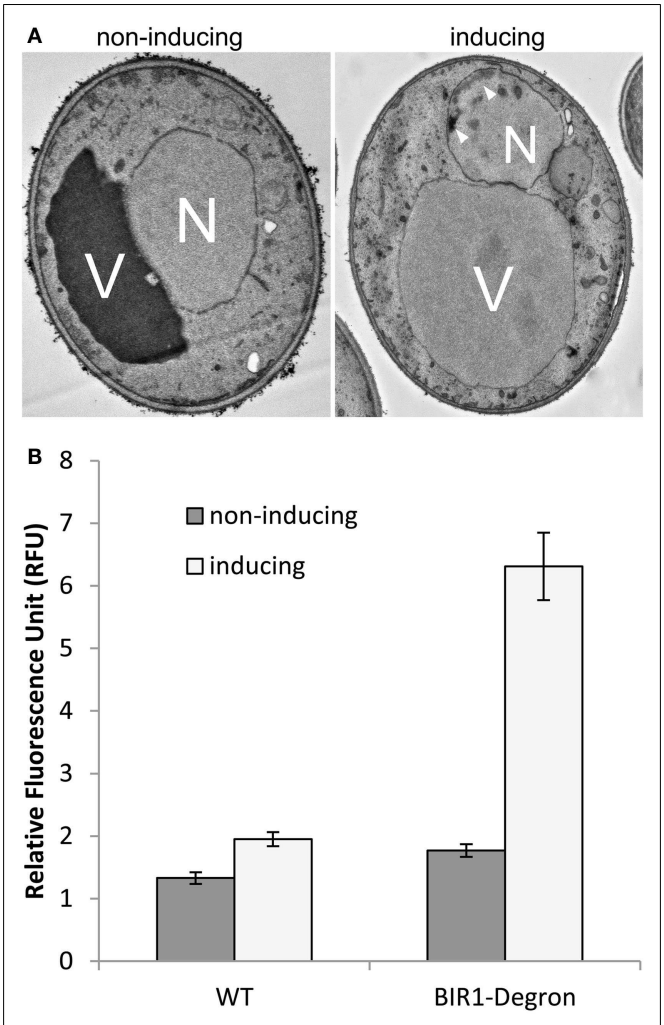

FIGURE 3 | Bir1 deletion causes apoptotic cell death. (A). TEM images showing bir1 deletion caused chromatin condensation (arrowheads) in the nucleus. V, vacuole; N, nucleus. **(B)**. Quantitative analysis of ROS production.

(**Figure 2**, inset). A similar result was obtained with BIR1-degron strain growing at the non-inducing condition (YPDCu, 24˚C; data not shown). When growing at the inducing condition, however, less than 10% of the bir1-degron cells were blocked at metaphase (**Figure 2**), suggesting that Bir1 is required for activating the spindle assembly checkpoint.

BIR1 DELETION INDUCES APOPTOTIC CELL DEATH

Previous study has shown that deletion of BIR1 causes apoptotic cell death (Walter et al., 2006). Using TEM, we showed the chromatin condensation in the nucleus, when BIR1-degron is activated, further confirming the occurrence of apoptosis in the bir1-degron strain (**Figure 3A**). The level of ROS, another marker of the apoptotic cell death, was also much higher when BIR1-degron was growing in the inducing medium (**Figure 3B**). The slight increase of ROS in wild type was likely caused by the temperature change, from the non-inducing condition (24˚C) to inducing condition (37˚C). To see if the malfunction of spindle assembly checkpoint is involved in the cell death, we used nocodazole (10 μg/ml) to block the cells to pro-metaphase prior to the induction of BIR1-degron or added H_2O_2 (4 mM), which has shown to induce apoptotic cell death in yeast (Madeo et al., 1999). As shown in **Figure 4**, when cells were first blocked at pro-metaphase by nocodazole, the cell survival rate was significantly higher either in the presence of H_2O_2 (**Figure 4A**), or during the induction of BIR1-degron (**Figure 4B**), compared to cells without nocodazole treatment. These results further suggest that spindle assembly checkpoint plays an important role in bir1 deletion or oxidative stress-induced cell death.

BIR1 DELETION CAUSES THE DEGRADATION OF THE ANAPHASE INHIBITOR Pds1

To further confirm the defect of spindle assembly checkpoint caused by BIR1 deletion, Western blot was used to examine the levels of Pds1, the anaphase inhibitor. In a normal cell cycle, Pds1 is degraded only during metaphase to anaphase transition, by APC.

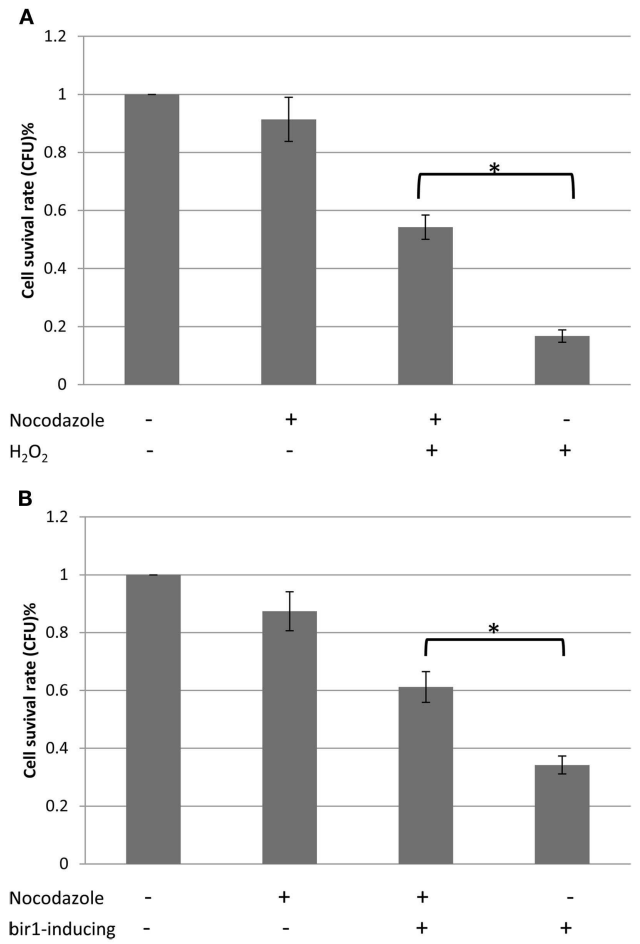

FIGURE 4 | Cell survival rates with or without nocodazole. (A). Wild type cells were grown to early log phase. 10 μg/ml nocodazole was then added to the culture medium for 2˚h, to block cells to pro-metaphase. Four millimolars of H_2O_2 was added for 1˚h and 500 cells were plated on YPD plate. Colony forming unit (CFU) was calculated against control. **(B)**. BIR1-degron cells were grown in YPDCu (24˚C) to early phase. 10 μg/ml nocodazole was then added to the culture medium for 2˚h, to block cells to pro-metaphase. Cells were then transferred to YPG (37˚C) for 1˚h, followed by plating assay. $^*P < 0.05$.

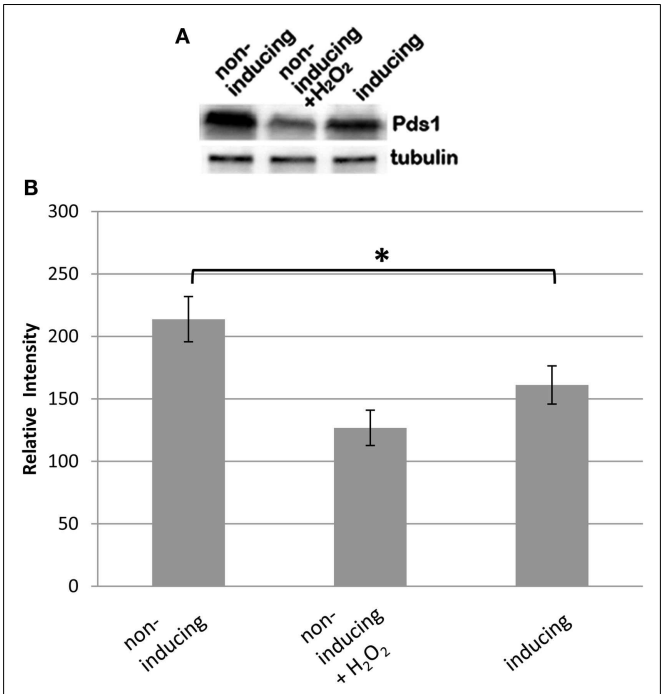

FIGURE 5 | Bir1 deletion causes the degradation of the anaphase inhibitor Pds1. BIR1-degron cells were grown in YPDCu (24˚C) to early phase. Four millimolars of H_2O_2 was added for 1˚h (middle lane), or cells were transferred to YPG (37˚C) for 1˚h. **(A)** Western blot analysis of Pds1 degradation and **(B)** quantitative analysis of the Western blot ($n = 3$). β-Tubulin was used as control. $^*P < 0.05$.

We showed previously that oxidative stress caused cleavage of the cohesin protein Mcd1 by the caspase-like protease Esp1 (Yang et al., 2008). Western blot showed that the addition of H_2O_2 caused the degradation of Pds1 (**Figure 5A**), confirming the activation of APC under oxidative stress. When BIR1 was deleted via BIR1-degron, Pds1 was also degraded (**Figure 5A**), further suggesting the disruption of the spindle assembly checkpoint. The Pds1 degradation caused by H_2O_2 is greater than bir1 deletion (**Figure 5B**), suggesting that oxidative stress has a more severe effect on cell cycle disruption.

Bub1 FAILS TO BE LOCALIZED ON CENTROMERES IN THE PRESENCE OF H_2O_2 OR bir1 DELETION

Bub1 is a protein kinase that is involved in the spindle assembly checkpoint. Bub1 localizes at the centromeres throughout mitosis in fission yeast (Bernard et al., 1998) and mammalian cells (Howell et al., 2004). Using an HA-tagged BUB1 strain and immunocytochemistry, we showed that in log phase cells, Bub1 formed small foci inside the nucleus, similar to the pattern of centromere staining (Bernard et al., 1998). When cells were treated with H_2O_2, however, no Bub1 foci were observed inside the nucleus. Bub1 was diffused into cytoplasm. A similar phenotype was observed when bir1 was deleted by the bir1-degron (**Figure 6**). These results suggest that Bir1p is required for Bub1 localization on centromeres. Another possibility is that H_2O_2 or bir1 deletion induces apoptosis which causes centromeres to be disrupted therefore Bub1 cannot localize to centromeres.

DISCUSSION

Eukaryotic cells have evolved a complex network, known as cell cycle control system, to regulate the progression of cell cycle and cell division. One important component of the regulation network is the checkpoint control, which senses flaws in critical events, such as DNA replication and chromosome segregation (Lew et al., 1997). When checkpoints are activated, cell cycle is delayed, until damages are repaired or removed. Malfunction of checkpoint control could induce cell death, or cancer.

Two kinds of BIR-containing proteins have been identified; one functions as inhibitor of apoptosis and the other is involved in

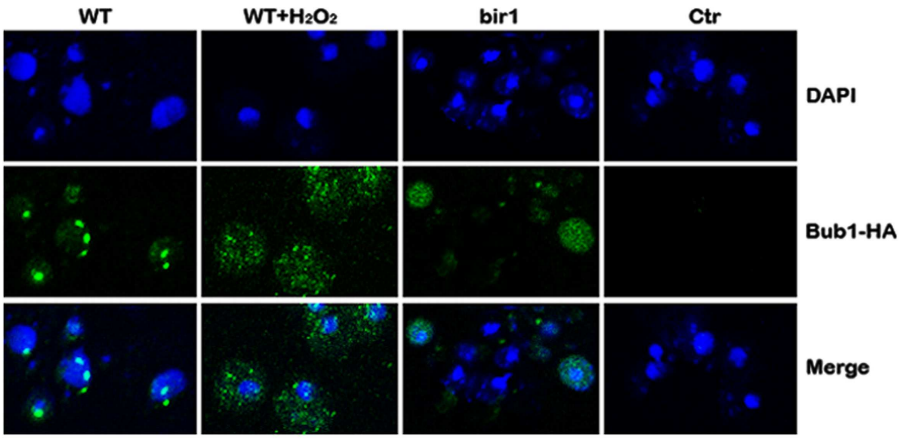

FIGURE 6 | Bub1 fails to be localized on centromeres in the presence of H_2O_2 or bir1 deletion. Wild type (WT) cells were grown in YPD to log phase. Four millimolars of H_2O_2 was added to the medium for 1˚h (WT + H_2O_2); or Bir1-degron strain was grown to log phase at the non-inducing condition (YPDCu, 24˚C); Cells were then transferred to inducing medium (YPG, 37˚C) for 1˚h (bir1). Bub1 localization was assayed by immunostaining and confocal microscopy. Control (Ctr) was conducted by replacing primary antibody (anti-HA) with buffer.

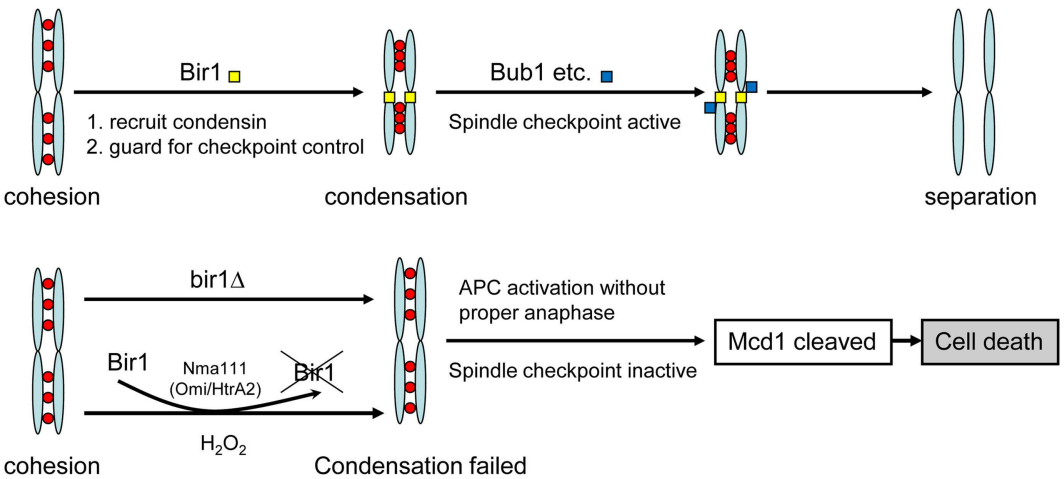

FIGURE 7 | Model of cell death induced by bir1 deletion or oxidative stress. Under normal condition, Bir1 is localized at the centromeres as part of the spindle assembly checkpoint complex. In the lack of Bir1 (mutation or by removed by Nma111 under oxidative stress Walter et al., 2006), the spindle checkpoint is inactivated, causing improper APC activation and the consequent cell death.

cell cycle regulation. Similar to mammalian survivin (also known as Birc5), the budding yeast Bir1 contains a single RING-finger domain. It is cell cycle regulated and localizes to the centromeres until metaphase-anaphase transition but remains in the equatorial zone as the sister chromatids separate (Uren et al., 1998, 2000; Widlund et al., 2006). Anti-apoptotic function has also been reported for survivin and the yeast homolog Bir1. Du et al. (2000) revealed that survivin bound to Diablo/Smac, a mitochondrial protein that promotes apoptosis by activating caspases in the cytochrome c/Apaf-1/caspase-9 pathway. Madeo and his colleagues (Walter et al., 2006) showed that the yeast Bir1 is a substrate of Nma111, the homolog of the human pro-apoptotic serine protease Omi/HtrA2. Under oxidative stress, Bir1 was degraded by Nma111, causing apoptotic cell death. In this study, we further demonstrated that deletion of yeast BIR1 induced apoptotic cell death. However, the cell death induced by deletion of bir1 is not due to its anti-apoptosis function, but rather its function in cell cycle regulation. We demonstrated that Bir1 is required for the activation of the spindle assembly checkpoint. This notion is supported by (1). BIR1 deletion causes defect on spindle assembly checkpoint (**Figure 2**); and (2). The spindle checkpoint protein Bub1 fails to localize on centromeres when BIR1 is deleted (**Figure 6**). As a result, the anaphase inhibitor Pds1 is degraded in bir1 deletion in the absence of normal anaphase. The degradation of Pds1 causes the cleavage of the cohesin protein Mcd1 and the C-terminal fragment of Mcd1 induces apoptotic cell death (**Figure 7**; Yang et al., 2008). A similar pathway may also occur during the oxidative stress-induced apoptotic cell death, where

under oxidative stress, Bir1 is degraded by the serine protease Omi/HtrA2, and Pds1 is consequently degraded (**Figure 7**; Walter et al., 2006). It is worth noting that other pathways may also be involved, especially in the oxidative stress-induced cell death (Mazzoni et al., 2005; Almeida et al., 2007; Lu et al., 2011).

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The splicing mutant of the human tumor suppressor protein DFNA5 induces programmed cell death when expressed in the yeast *Saccharomyces cerevisiae*

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DFNA5 was first identified as a gene responsible for autosomal dominant deafness. Different mutations were found, but they all resulted in exon 8 skipping during splicing and premature termination of the protein. Later, it became clear that the protein also has a tumor suppression function and that it can induce apoptosis. Epigenetic silencing of the *DFNA5* gene is associated with different types of cancers, including gastric and colorectal cancers as well as breast tumors. We introduced the wild-type and mutant *DFNA5* allele in the yeast *Saccharomyces cerevisiae*. The expression of the wild-type protein was well tolerated by the yeast cells, although the protein was subject of degradation and often deposited in distinct foci when cells entered the diauxic shift. In contrast, cells had problems to cope with mutant DFNA5 and despite an apparent compensatory reduction in expression levels, the mutant protein still triggered a marked growth defect, which in part can be ascribed to its interaction with mitochondria. Consistently, cells with mutant DFNA5 displayed significantly increased levels of ROS and signs of programmed cell death. The latter occurred independently of the yeast caspase, Mca1, but involved the mitochondrial fission protein, Fis1, the voltage-dependent anion channel protein, Por1 and the mitochondrial adenine nucleotide translocators, Aac1 and Aac3. Recent data proposed DFNA5 toxicity to be associated to a globular domain encoded by exon 2–6. We confirmed these data by showing that expression of solely this domain confers a strong growth phenotype. In addition, we identified a point mutant in this domain that completely abrogated its cytotoxicity in yeast as well as human Human Embryonic Kidney 293T cells (HEK293T). Combined, our data underscore that the yeast system offers a valuable tool to further dissect the apoptotic properties of DFNA5.

Keywords: DFNA5, *Saccharomyces cerevisiae*, yeast, cell death, apoptosis, hearing impairment, tumor suppressor

INTRODUCTION

Evasion of apoptosis is known to be an important factor in tumorigenesis, but the underlying mechanisms are often not well understood. Therefore, more research is required on the factors that govern cellular decisions between malignant outgrowth or programmed cell death, as this may eventually lead to the design of more efficient anti-cancer therapies (Fulda and Debatin, 2004; Bremer et al., 2006; Call et al., 2008). Here, we describe our findings on the heterologous expression in yeast of a human protein that has an important role in controlling the switch between cell survival and cell death, i.e., DFNA5.

DFNA5 was originally identified in a Dutch family with autosomal dominant non-syndromic hearing impairment (Van Laer et al., 1998). This association with hearing loss was later confirmed with the description of *DFNA5* mutations in a Korean family, two Chinese families and a second Dutch family (Yu et al., 2003; Bischoff et al., 2004; Cheng et al., 2007; Park et al., 2010). Although the mutations in these families are different, they all lead to exon 8 skipping during splicing, thereby causing a frameshift and premature termination of the protein. Another type of mutation was reported in an Iranian family, where an insertion of a cytosine at nucleotide position 640 truncates the protein at a position corresponding to exon 5 of the gene. However, this mutation does not segregate with the hearing loss phenotype and is even present in persons with normal hearing (Van Laer et al., 2007). Hence, it appears that *DFNA5*-associated hearing loss is caused by a gain-of-function mutation due to exon 8 skipping. To date, the exact function of the DFNA5 protein is still unknown, but recent evidence suggests that the protein harbors a pro-apoptotic function (Op de Beeck et al., 2011a). Analysis of the protein revealed that it consists of two globular domains separated by a hinge region, whereby the first domain, consisting of the amino acid residues 1–256, displays pro-apoptotic activity, while the second domain, corresponding to residues 282–496, may serve as a regulator that shields the apoptosis-inducing function of the first domain (Op de Beeck et al., 2011b).

Some studies also associated DFNA5 with several types of cancer, including gastric, colorectal as well as breast cancer. Emerging evidence from the past years suggest that DFNA5 plays a role as a tumor suppressor protein and that the corresponding gene is epigenetically silenced through methylation (Thompson and Weigel, 1998; Lage et al., 2001; Akino et al., 2007; Kim et al., 2008a; Fujikane et al., 2010). Consistent with this role are the observations that siRNA-mediated knock-down of *DFNA5* in non-malignant breast epithelial cell lines enhances colony numbers, colony size and cell growth (Kim et al., 2008b), while forced expression of *DFNA5* in gastric cancer cell lines suppresses colony formation (Akino et al., 2007). Also in support of a tumor suppressor function are the reports that expression of the *DFNA5* gene is controlled by p53 (Masuda et al., 2006) and that silencing of the gene correlates with tumor cell resistance to chemotherapeutic drugs (Lage et al., 2001).

Supported by the observation that the exon 8 splicing mutant of DFNA5 (mutDFNA5), but not the wild-type allele (wtDFNA5), triggers cell cycle arrest when expressed in the fission yeast *Schizosaccharomyces pombe* (Gregan et al., 2003), we decided to use yeast as a model to investigate the role of DFNA5 in more detail. We expressed wtDFNA5 and mutDFNA5 in the budding yeast *Saccharomyces cerevisiae* and analyzed the repercussions on growth, oxidative stress, and the induction of programmed cell death. Data obtained from the wild-type strain and a series of deletion mutants confirmed that mutDFNA5 strongly induces programmed cell death, a phenomenon being dependent on mitochondrial integrity, but independent of the yeast caspase, Mca1. In addition, the yeast model proved to be an ideal tool to identify point mutants in the apoptosis-inducing domain of DFNA5 that abrogated its ability to induce cytotoxicity.

MATERIALS AND METHODS

STRAINS, PLASMIDS, AND GROWTH ANALYSIS

In this study, we used the BY4741 wild-type strain (Brachmann et al., 1998) and isogenic deletion strains of the genome-wide collection (EUROSCARF, Frankfurt, Germany) lacking proteins involved in programmed cell death as listed in **Table 1**. The C-terminally HA-tagged full-length wtDFNA5 and mutDFNA5 cDNAs, the C-terminally EGFP fusion proteins and the wtDFNA5 first and second globular fragments were isolated and amplified as previously described (Gregan et al., 2003; Op de Beeck et al., 2011a,b) using the primers listed in **Table 2**. All amplified products were ligated into the pYX212 plasmid using either *Eco*RI and *Bam*HI or *Eco*RI and *Sal*I restriction sites, thereby placing the inserts under expression control of the constitutive *TPI1* promoter. The mutants HCA-F2R and HCA-A3R at the N-terminal end of the first globular domain were generated by site directed mutagenesis (Agilent, Santa Clara, CA, USA) in combination with the custom designed primers listed in **Table 2**. All constructs were verified by bidirectional sequencing on an ABI genetic analyser 3130xl (Applied Biosystems, Foster City, CA, USA). The construction of the plasmid for Mito-RFP was previously described (Westermann and Neupert, 2000). Standard transformation techniques were applied (Gietz et al., 1992) and all strains were grown at 30°C in a selective minimal medium containing 2% of glucose (SD-Ura). Growth profiles were determined in 96-well microtiter plates with continuous shaking at 30°C in a Multiskan GO spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Overnight cultures of at least three different transformants were diluted to start new cultures for growth analysis. Growth was monitored until the stationary phase was reached. Growth curves are depicted with scaled OD units and as the mean values of the transformants, with error bars representing standard deviations. The growth profiles of the strains expressing HA- or EGFP-tagged wtDNFNA5 or mutDFNA5 were compared to that of a control strain transformed with either empty vector or a plasmid allowing for expression of EGFP. The differences in time required to reach half maximal optical densities (ΔT1/2) were calculated and used as standards for growth quantification. The difference in ΔT1/2 obtained for wtDFNA5 (ΔT1/2 wtDFNA5-Control) and mutDFNA5 (ΔT1/2 mutDFNA5-Control) in the BY4741 wild-type strain was used as reference and set as 100%.

Table 1 | Strain list.

Strain	Genotype	Reference/Source
BY 4741	Mat**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Brachmann et al., 1998
aac1Δ	BY4741 *YMR056c*::kanMX4	EUROSCARF
aac3Δ	BY4741 *YBR085w*::kanMX4	EUROSCARF
aif1Δ	BY4741 *YNR074c*::kanMX4	EUROSCARF
dnm1Δ	BY4741 *YLL001w*::kanMX4	EUROSCARF
fis1Δ	BY4741 *YIL065c*::kanMX4	EUROSCARF
mca1Δ	BY4741 *YOR197w*::kanMX4	EUROSCARF
mdv1Δ	BY4741 *YJL112w*::kanMX4	EUROSCARF
nma111Δ	BY4741 *YNL123w*::kanMX4	EUROSCARF
nuc1Δ	BY4741 *YJL208c*::kanMX4	EUROSCARF
por1Δ	BY4741 *YNL055c*::kanMX4	EUROSCARF
por2Δ	BY4741 *YIL114c*::kanMX4	EUROSCARF
rny1Δ	BY4741 *YPL123c*::kanMX4	EUROSCARF
snl1Δ	BY4741 *YIL016w*::kanMX4	EUROSCARF
tdh2Δ	BY4741 *YJR009c*::kanMX4	EUROSCARF
tdh3Δ	BY4741 *YGR192c*::kanMX4	EUROSCARF
tim18Δ	BY4741 *YOR297c*::kanMX4	EUROSCARF
ymr074cΔ	BY4741 *YMR074c*::kanMX4	EUROSCARF
ysp1Δ	BY4741 *YHR155w*::kanMX4	EUROSCARF

Human Embryonic Kidney 293T cells (HEK293T) were subcultured in 60 mm dishes at a density of 2×10^6 cells in Dulbecco's modified Eagle's medium containing 4500 mg/l glucose supplemented with 10% (v/v) fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (all products from Invitrogen, San Diego, CA, USA). The cells were incubated overnight at 37°C in a 5% CO_2 humidified environment. The plasmids used for wtDFNA5 and mutDFNA5 expression in mammalian HEK293T cells, after transfection with lipofectamine, were described before (Op de Beeck et al., 2011b). In addition, we used the pEGFP-N1 vector to construct plasmids for the expression of HCA-F2R and HCA-A3R mutants.

WESTERN BLOT ANALYSIS

Yeast samples were grown in 3 ml cultures on selective medium and were harvested at an $OD_{600\,nm}$ between 1.5 and 2.0. An equal

Table 2 | Primer pairs.

Construct	Forward primer	Reverse primer
wtDFNA5-HA	TATATATATAGAATTCATGTTTGCCAAAGCAACCAGGAATT	GACCGGTGGATCCCGTGAATGTTCTCTGCCTAAAGC
mutDFNA5-HA	TATATATATAGAATTCATGTTTGCCAAAGCAACCAGGAATT	GACCGGTGGATCCCGAGGTTGGGTCTTCAAGATCAG
wtDFNA5-EGFP	TATATATATAGAATTCATGTTTGCCAAAGCAACCAGGAATT	TATATATAGTCGACGCTTGTACAGCTCGTCCATGCC
mutDFNA5-EGFP	TATATATATAGAATTCATGTTTGCCAAAGCAACCAGGAATT	TATATATAGTCGACGCTTGTACAGCTCGTCCATGCC
DFNA5 domain A	TATATATATAGAATTCATGTTTGCCAAAGCAACCAGGAATT	CGGTGGATCCCGGTCCAGGTAGACAGAGTCAAT
DFNA5 domain B	AAGCTTCGAATTCTGGCCACCATGGACCCCCTGGTCTTTCGAGAG	GACCGGTGGATCCCGTGAATGTTCTCTGCCTAAAGC
HCA-F2R mutant*	GAGCTCAAGCTTCGAATTCTGATGCGTGCCAAAGCAACCAGG	CCTGGTTGCTTTGGCACGCATCAGAATTCGAAGCTTGAGCTCG
HCA-A3R mutant*	CGAGCTCAAGCTTCGAATTCTGATGTTTCGTAAAGCAACCAGG	CCTGGTTGCTTTACGAAACATCAGAATTCGAAGCTTGAGCTCG

Restriction sites used for cloning are underlined.

**Site directed mutagenesis primers.*

amount of cells were taken and lysed by boiling for 15 min in SDS sample buffer [50 mM Tris (pH 8.0), 10 mM β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol]. Proteins were separated by standard SDS-PAGE and blotted onto PVDF membranes (Immobilon-P transfer membranes, Millipore, MA, USA). For immunodetection we used the primary rabbit anti-HA or anti-EGFP antibodies (Santa Cruz, CA, USA) and a secondary horse radish peroxidase (HRP) conjugated goat anti-rabbit antibody (Santa Cruz, CA, USA). The endogenous yeast alcohol dehydrogenase Adh2 served as internal standard. Membranes were developed using the ECL detection kit (Thermo Scientific, IL, USA).

FLOW CYTOMETRIC ANALYSIS OF CELL DEATH, ROS ACCUMULATION AND CASPASE ACTIVITY

Tests for apoptotic and necrotic markers, using AnnexinV/Propidium Iodide (AV/PI) co-staining, as well as ROS accumulation, using the superoxide-driven conversion of non-fluorescent dihydroethidium (DHE) to fluorescent ethidium, were performed and quantified using BD Influx flow cytometry (BD Biosciences, New Jersey, NJ, USA) as described previously (Büttner et al., 2008). Yeast samples were harvested at different time points. Samples were collected at mid-exponential phase at an $OD_{600\,nm}$ between 3.5 and 4.0, just after cells had traversed the diauxic shift (PD) at an $OD_{600\,nm}$ between 6.0 and 7.0, and in stationary phase (ST) at an $OD_{600\,nm}$ of 8.5 or higher. Analysis of the BD influx flow cytometry data was performed using the software program FlowJo (Tree Star Inc., Ashland, MA, USA). Viability tests of the HCA-F2R and HCA-A3R mutants in HEK293T cells were performed on a FACScan flow cytometer (BD Biosciences, New Jersey, NJ, USA) after staining of the cells with PI. Cell viability was then determined as the ratio of cells showing no PI fluorescence to the total cell population.

A previously described protocol was used to measure the caspase activity (Madeo et al., 2002). Yeast cells grown on selective medium were harvested at an $OD_{600\,nm}$ of approximately 4.5. A staining solution containing 10 μM FITC-VAD-FMK in PBS (CaspACE, Promega, WI, USA) was added to an amount of cells corresponding to an $OD_{600\,nm}$ of 0.5 and incubated for 20 min at room temperature. After washing and resuspension in 200 μl PBS flow cytometric analysis was performed using a 530/40 bandpass filter.

FLUORESCENCE MICROSCOPY

Cells transformed with wtDFNA5 or mutDFNA5 fused to EGFP were grown till mid-exponential or post-diauxic phase as indicated and visualized using a Leica DM4000B fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany). Pictures were taken with a Leica DFC420C camera using the Leica Application Suite software. The percentages of post-diauxic cells with or without inclusions were determined by manual counting of at least 300 cells per sample.

Mitochondria were visualized by the expression of a mitochondria-targeted red fluorescent protein, Mito-RFP (Westermann and Neupert, 2000). To stain the vacuolar membrane, cells were in the post-diauxic phase and incubated with FM4-64 ([N-(3-triethylammoniumpropyl)-4-(p-diethylamino phenylhexa-trienyl) pyridinium dibromide]; Molecular Probes, Eugene, OR, USA) at room temperature for at least 30 min in a HEPES buffer containing 1% of glucose to facilitate the uptake of FM4-64. To visualize the nucleus, we performed a 4',6-diamidino-2-phenylindole (DAPI) staining. The cells were harvested in the post-diauxic phase and incubated for 20 min in a phosphate buffer (0.04 M K_2HPO_4, 0.01 M KH_2PO_4, 0.15 M NaCl, 0.1 g/100 ml NaN_3) containing 50% ethanol. After washing with PBS, DAPI was added (1 μg/μl) and samples were incubated at room temperature for 15 min.

STATISTICAL ANALYSIS

All experiments included biological replicates and the use of independent transformants. Statistical analysis was performed using unpaired *t*-tests or One-Way ANOVA.

RESULTS

MUTANT DFNA5 INDUCES APOPTOTIC AND NECROTIC CELL DEATH IN YEAST

To study the properties of human DFNA5 in a well-defined model, we expressed the cDNAs of wtDFNA5 and mutDFNA5 in the BY4741 wild-type strain. We used high-copy-number plasmids allowing expression of wtDFNA5 and mutDFNA5 as C-terminally HA-tagged proteins under the control of the strong constitutive *TPI1* promoter. For wtDFNA5, this resulted in good expression levels of the full-length protein, though we noticed that the protein was subject of proteolytic degradation as evidenced by the presence of discrete breakdown products upon

Western blot analysis (**Figure 1A**). A lower expression level was obtained for mutDFNA5 and, interestingly, no major proteolytic fragments were observed in this case, even not when higher concentrations of protein extracts were loaded or when the exposure time of the immunoblots was increased. Growth analysis of the transformants revealed that the expression of wtDFNA5 had only a moderate effect on growth, while expression of mutDFNA5 triggered a significant growth defect. The latter was characterized by a longer doubling time and a lower maximal optical density of the cultures (**Figure 1B**). Hence, as compared to a culture of the BY4741 strain transformed with the empty plasmid, a culture of cells expressing wtDFNA5 required on average only an additional 3 h (SD $\pm$ 0.24) to reach half of the maximal optical density (ΔT1/2), whereas for a culture of cells expressing mutDFNA5 this ΔT1/2 was extended to about 11.5 h (SD $\pm$ 0.32) (**Table 3**). Combined these data suggest that yeast cells tolerated the presence of wtDFNA5 fairly well but have problems to cope with mutDFNA5. Notably, since the mutant protein seemed to escape the protein breakdown, the cells apparently counter selected and reduced the expression of the mutant protein to prevent extreme harmful effects.

It is known that cytotoxic effects instigated by heterologous proteins are often a reflection of a failing protein quality control and clearance system, which then leads to enhanced oxidative stress and eventually increased cell death [reviewed in Winderickx et al., 2008]. To examine whether this is also the case for heterologous expression of DFNA5, we measured the level of reactive oxygen species (ROS) using a DHE staining on culture samples taken at different time points during growth (**Figures 1C,D**). In mid-exponential cultures, the ROS levels were only significantly increased in cells expressing mutDFNA5 when compared to the control. However, once the cultures traversed the diauxic shift and switched their metabolism to respiration, a marked increment of the ROS level was observed for both the cells expressing wtDFNA5 and those expressing mutDFNA5. In case of wtDFNA5, the level of ROS in the early post-diauxic phase was about three times higher than that of the control cells and it further increased in stationary phase. With mutDFNA5, the increment in ROS in the early post-diauxic phase was comparable to wtDFNA5, though by the time these cells reached the stationary phase the average ROS level was lower than that of cells expressing wtDFNA5.

We also performed a co-staining with AV/PI to detect cells showing signs of apoptotic and necrotic cell death (**Figure 1E**). This again revealed that only the expression of mutDFNA5 significantly enhanced cell death during the mid-exponential phase, while both expression of wtDFNA5 or mutDFNA5 enhanced cell death once the cultures were beyond the diauxic shift, albeit to a different extent. More in particular, we noticed that in the post-diauxic and stationary phase the expression of the wild-type and especially the mutant allele triggered an increase in the number of late apoptotic (AV^+/PI^+) and necrotic cells (AV^-/PI^+) and that in the stationary phase this was even associated with a significant decrease in early apoptotic (AV^+/PI^-) cells. That the increments of late apoptotic and necrotic cells are most pronounced upon expression of mutDFNA5 is consistent with the observed enhanced growth defect. Furthermore, these results are also in agreement with previously reported observations of enhanced apoptotic and necrotic cell death in mammalian cells transfected with mutDFNA5 (Van Laer et al., 2004; Op de Beeck et al., 2011b).

MUTANT DFNA5 ESCAPES PROTEIN QUALITY CONTROL DEPOSITION AND INTERACTS WITH MITOCHONDRIA

To analyse the intracellular localization, we expressed wtDFNA5 and mutDFNA5 as a C-terminally tagged EGFP fusion. Their relative expression levels were comparable to those of the HA-tagged counterparts. Furthermore, similar as for the HA-tagged versions, the wtDFNA5-EGFP fusion was subject to proteolytic degradation, while this was by far less pronounced for the mutDFNA5-EGFP fusion (**Figure 1A**). Despite of these similarities, we noticed that the fusion proteins induced slightly enhanced growth defects as judged from the ΔT1/2 values calculated based on the growth difference with control cultures expressing native EGFP (ΔT1/2 wtDFNA5-EGFP: 4.60 h $\pm$ 1.27; ΔT1/2 mutDFNA5-EGFP: 15.85 h $\pm$ 1.84; **Table 3**). Nonetheless, since also in this case mutDFNA5-EGFP instigated a much higher cytotoxicity than wtDFNA5-EGFP, we reasoned that further analysis would still provide important insight in the differential properties of the proteins.

Fluorescence microscopy confirmed the difference in expression level between wtDFNA5-EGFP and mutDFNA5-EGFP. It also showed that wtDFNA5-EGFP was evenly distributed over the cytoplasm in mid-exponential growth phase, although we noticed that about one fifth of the cells (22%) gradually accumulated fluorescent material in more dense inclusions (**Figure 2A**). This resulted in the formation of one or a few distinct deposits by the time these cells reached the post-diauxic phase. Similar as in transfected mammalian cells (Van Laer et al., 2004), the distribution of mutDFNA5-EGFP in mid-exponential phase cells appeared to be more granulated and possibly confined to intracellular structures, though it was difficult to assign a definite pattern due to the low expression level of the fusion protein. In the post-diauxic phase, inclusions were present in about one out of seven cells that expressed mutDFNA5-EGFP (14%). As compared to the deposits of wtDFNA5-EGFP, the inclusions formed by the mutant protein were usually less intense and occurring as small foci in the vicinity of the plasma membrane (**Figure 2A**).

It was previously shown that cells protect themselves by sequestering breakdown products and damaged or aggregated proteins in different protein quality control compartments, referred to as aggresomes or JUNQ and IPOD. JUNQ represents a juxta-nuclear quality control compartment that serves as a temporary storage site for misfolded proteins, keeping them in an ubiquitinated, soluble state for either refolding or degradation by the ubiquitin-proteasome system. IPOD, on the other hand, is a perivacuolar compartment for the deposit of insoluble, non-ubiquitinated substrates, such as amyloidic proteins, that possibly await clearance by means of autophagy (Bagola and Sommer, 2008; Kaganovich et al., 2008). To analyse in more detail the localization of the inclusions formed by wtDFNA5-EGFP and mutDFNA5-EGFP, we performed stainings with DAPI and FM4-64 to, respectively, visualize the nucleus and the vacuolar membrane. This demonstrated that the larger deposits of wtDFNA5-EGFP did not co-localize with the nucleus (**Figure 2B**). Instead,

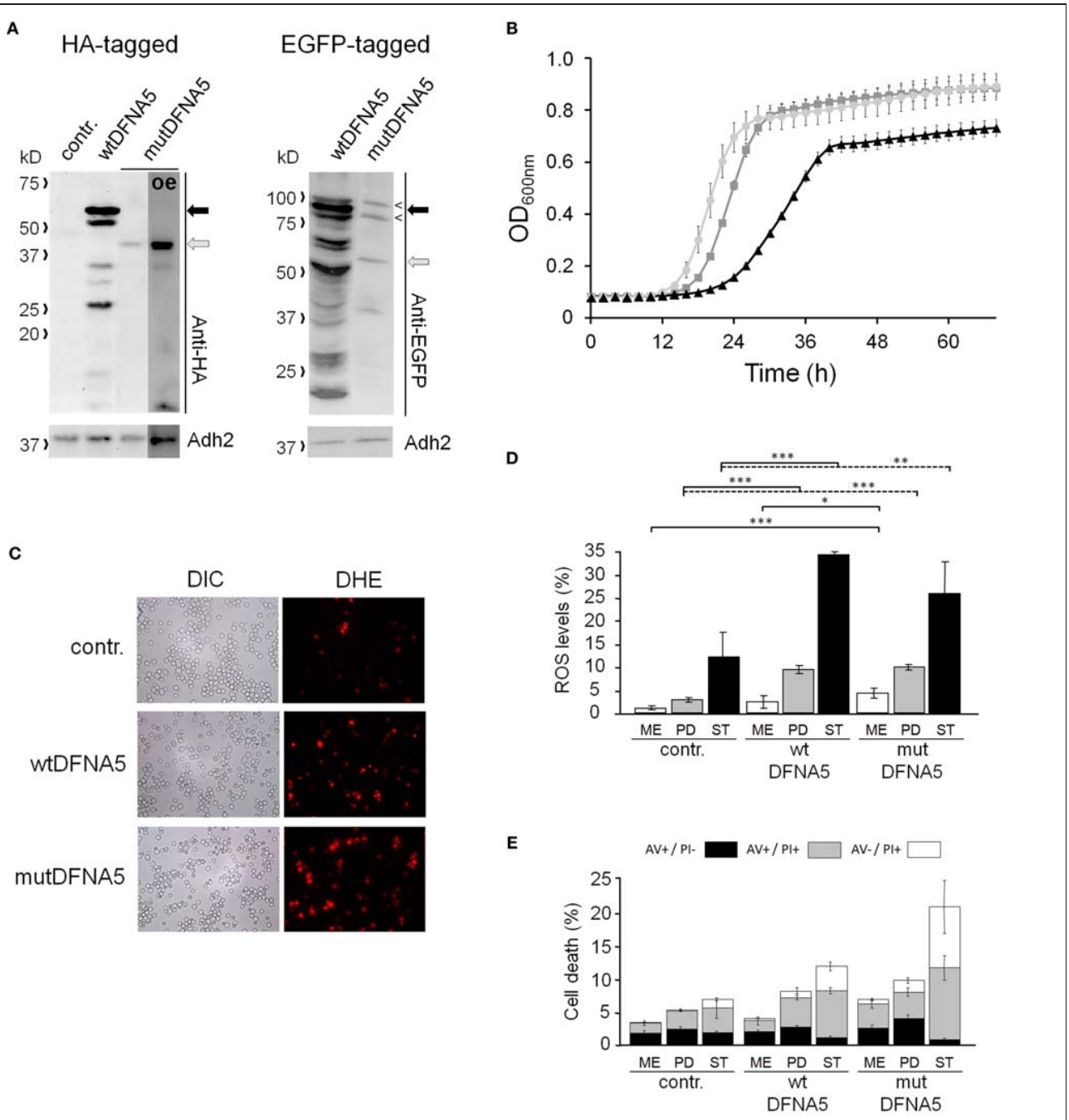

FIGURE 1 | Repercussions of wtDFNA5 and mutDFNA5 expression in BY4741 wild-type cells. (A) Western blot analysis of protein extracts of the BY4741 wild-type strain transformed with an empty plasmid (contr.) or constructs allowing for the expression of C-terminally HA-tagged and EGFP-tagged wtDFNA5 or mutDFNA5 as indicated. Immunodetection was performed using primary antibodies directed against the HA-tag, EGFP, or Adh2, which was used as loading control. The black arrow indicates full-length wtDFNA5 and the gray arrow full-length mutDFNA5. The small open arrowheads indicate a-specific bands detected by anti-EGFP. For mutDFNA5, the lane marked with [oe] represents an overexposure of the Western blot. **(B)** Growth of the BY4741 wild-type strain transformed with an empty plasmid (light gray circles) or a construct allowing for expression of C-terminally HA-tagged wtDFNA5 (dark gray squares) or mutDFNA5 (black triangles). **(C)** Visualization of ROS producing cells in control cultures (contr.) or cultures of cells expressing C-terminally HA-tagged wtDFNA5 or mutDFNA5 in post-diauxic phase. **(D)** Quantification of ROS accumulation using DHE staining in control cultures (contr.) or cultures of cells expressing C-terminally HA-tagged wtDFNA5 or mutDFNA5 when sampled at the mid-exponential growth phase (ME), at post-diauxic shift (PD), and in stationary phase (ST). **(E)** Quantification of the number of AV/PI positive cells in ME, PD, and ST phase of control cells or cells expressing HA-tagged wtDFNA5 or mutDFNA5. Significances were assayed using unpaired *t*-tests. For the AV/PI co-staining the following significances were obtained when compared to the control: for wtDFNA5 in ME: AV^-/PI^+ *; for wtDFNA5 in PD: AV^+/PI^+ ***, AV^-/PI^+ *; for wtDFNA5 in ST: AV^+/PI^- *, AV^+/PI^+ *, AV^-/PI^+ **; for mutDFNA5 in ME: AV^+/PI^- *, AV^+/PI^+ **, AV^-/PI^+ **; for mutDFNA5 in PD: AV^+/PI^- **, AV^+/PI^+ *, AV^-/PI^+ ***; for mutDFNA5 in ST AV^+/PI^- **, AV^+/PI^+ **, AV^-/PI^+ *. (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

Table 3 | Quantification of growth*.

Strain	Function	T1/2 Control	T1/2 wtDFNA5	T1/2 mutDFNA5	ΔT1/2 wtDFNA5-Control	ΔT1/2 mutDFNA5-Control	%ΔT1/2 mutDFNA5-wtDFNA5
BY4741 (HA-tagged)		19.83 ± 0.52	22.83 ± 0.26	31.38 ± 0.48	3.00 ± 0.24	11.54 ± 0.32	100.00 ± 1.42
BY4741 (EGFP-tagged)		20.30 ± 1.15	24.90 ± 2.61	35.88 ± 3.54	4.60 ± 1.27	15.58 ± 1.84	128.49 ± 8.60
*aac1*Δ	Mitochondrial ADP/ATP Carrier	31.30 ± 1.20	28.13 ± 5.36	31.08 ± 1.36	−3.18 ± 2.73	−0.22 ± 0.77	34.63 ± 11.17
*aac3*Δ	Mitochondrial ADP/ATP Carrier	24.70 ± 0.45	27.00 ± 0.82	29.40 ± 1.67	2.30 ± 0.45	4.70 ± 0.77	28.10 ± 3.37
*aif1*Δ	Mitochondrial Apoptosis-inducing factor	19.60 ± 0.22	23.13 ± 0.25	33.00 ± 0.82	3.53 ± 0.16	13.40 ± 0.42	115.61 ± 1.76
*dnm1*Δ	Dynamin-related GTPase, mitochondrial fission	20.20 ± 0.27	22.33 ± 1.61	31.50 ± 2.38	2.13 ± 0.94	11.30 ± 1.20	107.32 ± 6.07
*fis1*Δ	Mitochondrial membrane fission	26.70 ± 1.10	34.38 ± 1.80	48.67 ± 1.15	7.68 ± 1.02	21.97 ± 0.83	167.32 ± 5.26
*mca1*Δ	Putative cysteine protease, metacaspase	21.80 ± 0.27	24.30 ± 0.45	34.50 ± 2.27	2.50 ± 0.23	12.70 ± 1.14	119.41 ± 4.54
*mdv1*Δ	Dmn1 adaptor protein, mitochondrial fission	20.40 ± 1.34	24.38 ± 0.25	31.90 ± 2.01	3.98 ± 0.61	11.50 ± 1.08	88.10 ± 4.66
*nma111*Δ	Omi1/HtrA2 Ortholog, Serine protease	20.00 ± 0.00	23.67 ± 0.58	31.25 ± 2.22	3.67 ± 0.33	11.25 ± 1.11	88.78 ± 4.82
*nuc1*Δ	Mitochondrial nuclease, Endo G ortholog	22.38 ± 2.14	24.10 ± 0.55	33.67 ± 0.58	1.73 ± 1.10	11.29 ± 1.12	112.00 ± 6.54
*por1*Δ	Mitochondrial porin, VDAC homolog	20.50 ± 1.50	25.40 ± 0.65	40.00 ± 1.38	4.90 ± 0.91	19.50 ± 1.03	170.93 ± 5.53
*por2*Δ	Putative mitochondrial porin, VDAC homolog	21.20 ± 0.45	23.90 ± 0.42	31.30 ± 2.49	2.70 ± 0.27	10.10 ± 1.13	86.63 ± 4.31
*rny1*Δ	Ribonuclease from yeast	20.90 ± 0.55	22.80 ± 0.57	33.90 ± 2.13	1.90 ± 0.35	13.00 ± 0.98	129.95 ± 3.87
*snl1*Δ	Suppressor of nup116-C lethal, Bag-1 homolog	18.60 ± 0.89	20.38 ± 0.25	28.00 ± 0.00	1.78 ± 0.42	9.40 ± 0.40	89.27 ± 2.33
*tdh2*Δ	Triose-phosphate dehydrogenase	20.13 ± 0.25	23.00 ± 0.50	31.00 ± 1.54	2.88 ± 0.31	10.88 ± 0.70	93.66 ± 3.07
*tdh3*Δ	Triose-phosphate dehydrogenase	22.00 ± 0.00	23.88 ± 0.25	35.50 ± 1.00	1.88 ± 0.13	13.50 ± 0.50	136.10 ± 2.13
*tim18*Δ	Translocase of the inner mitochondrial membrane	21.40 ± 0.42	25.50 ± 0.50	33.50 ± 0.58	4.10 ± 0.34	12.10 ± 0.34	93.66 ± 1.96
*ymr074c*Δ	Protein with homology to human PDCD5	20.75 ± 0.50	23.70 ± 0.57	29.00 ± 3.32	2.95 ± 0.36	8.25 ± 1.50	62.05 ± 6.03
*ysp*Δ	Yeast suicide protein	20.00 ± 0.00	23.40 ± 1.39	34.10 ± 1.95	3.40 ± 0.62	14.10 ± 0.87	125.27 ± 4.18

T1/2: time to reach half maximal optical density (hours).

ΔT1/2: difference in time to reach half maximal optical density (hours).

**Values are expressed as mean ± standard deviation.*

these deposits were found at the periphery of the vacuole and thus are likely to correspond to IPOD (**Figure 2C**). The small foci formed by mutDFNA5-EGFP, neither co-localized with the nucleus, nor with the vacuolar membrane but, interestingly, seemed to partially overlap with DAPI-stained mitochondrial DNA. This led us to visualize the mitochondrial network. The strains were therefore co-transformed with a plasmid enabling the expression of a mitochondrial red fluorescent marker protein, Mito-RFP (Westermann and Neupert, 2000). Further analysis revealed that, indeed, the small foci of mutDFNA5-EGFP often co-localized with punctuated fragmented mitochondria and this in contrast to the larger deposits of wtDFNA5-EGFP (**Figure 2D**).

MUTANT DFNA5 INDUCES CELL DEATH INDEPENDENTLY OF CASPASE

Next, we systematically assessed the repercussion of wtDFNA5 and mutDFNA5 expression in strains harboring deletions of key players of the programmed cell death machinery. We monitored the expression of the HA-tagged DFNA5 proteins, compared the growth profiles and measured the levels of ROS and cell death during mid-exponential growth. One of the strains analysed was the mutant lacking the yeast caspase, Mca1, which allowed us to establish whether DFNA5-induced cell death involves the previously described caspase-dependent or caspase-independent processes (Madeo et al., 2009). As compared to the corresponding

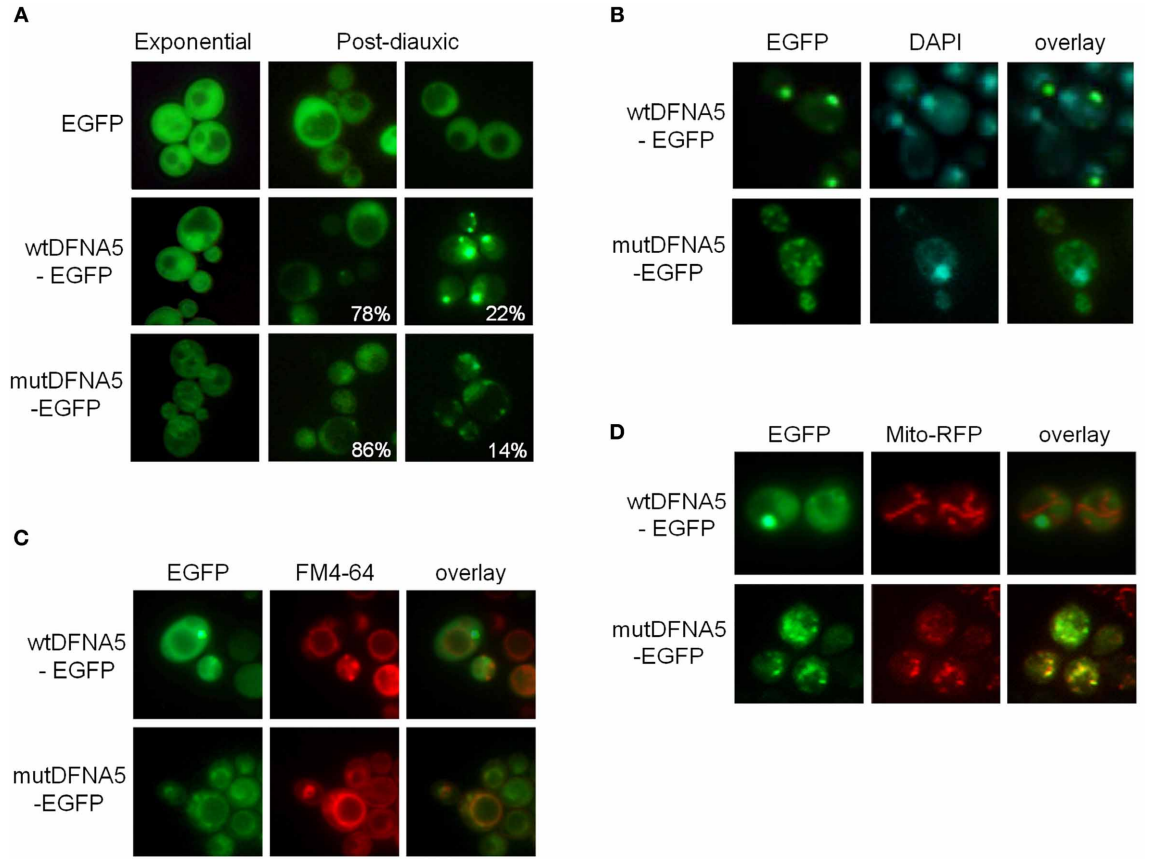

FIGURE 2 | Wild-type and mutant DFNA5 form inclusions in yeast. (A) Fluorescence microscopic visualization and intracellular localization of wtDFNA5-EGFP and mutDFNA5-EGFP fusion proteins in the BY4741 wild-type yeast strain sampled at the mid-exponential growth phase or in the post-diauxic growth phase as indicated. Percentages refer to the number of cells displaying a dispersed cytoplasmic localization or with inclusions. Cells expressing only EGFP served as control. **(B,C)** Pictures of post-diauxic wild-type cells with inclusions formed by wtDFNA5-EGFP or mutDFNA5-EGFP and stained with DAPI (panel **B**) to visualize the nucleus or with FM4-64 (panel **C**) to visualize the vacuolar membrane. **(D)** Pictures of post-diauxic wild-type cells expressing wtDFNA5-EGFP or mutDFNA5-EGFP and a mitochondrial red fluorescent marker protein, Mito-RFP.

wild-type strains, the *mca1*Δ strains displayed similar expression profiles of wtDFNA5 and mutDFNA5 (**Figure 3A**) and albeit the mutant strains grew more slowly, they maintained similar DFNA5-dependent growth defects as determined by calculating the ΔT1/2 values (**Table 3** and **Figure 3B**). In addition, the deletion of *MCA1* did not prevent accumulation of ROS upon expression of mutDFNA5, nor did it prevent the mutDFNA5-instigated induction of apoptotic and necrotic cell death markers (**Figures 3C,D**). Consistently, treatment of BY4741 wild-type cells with the FITC-labeled pancaspase inhibitor z-VAD-FMK, which binds to the active site of caspases in yeast (Madeo et al., 2002), did not provide evidence for enhanced caspase activity upon the expression of wtDFNA5 or mutDFNA5 (**Figure 3E**). When combined, these observations suggest that mutDFNA5-induced cell death occurs mainly independent of the caspase Mca1. Likewise, we could exclude several of the other known players of the yeast apoptotic machinery to have a major impact on the DFNA5-dependent phenotypes. These included the mitochondrial cell death effector, Aif1, the mitochondrial endonuclease G, Nuc1, the ortholog of the mammalian Omi/HtrA2 serine protease, Nma111, as well as the yeast suicide protein, Ysp2 (**Table 3** and data not shown).

MUTANT DFNA5 INDUCES CELL DEATH THROUGH MITOCHONDRIAL FUNCTIONS

In contrast to the mutant strains mentioned above, we found increased DFNA5-induced cytotoxicity in the strains lacking either the mitochondrial outer membrane protein, Fis1 or the voltage-dependent anion channel protein, Por1. The Fis1 protein is involved in mitochondrial fission that attracts the dynamin-related GTPase, Dnm1 through the adaptors Mdv1 and Caf4. The complex then forms a contractile ring that promotes outer membrane division. Interesting is that with the *fis1*Δ strains, both the cultures expressing wtDFNA5 or mutDFNA5 displayed a comparable lower maximal optical density (**Figure 4A**). This is similar to what we observed for cultures of wild-type cells transformed with mutDFNA5 and it probably reflects the disturbance of mitochondrial dynamics. The role of Fis1 and mitochondrial fission in programmed cell death is still not fully clarified and seems to depend on the type of the cell death stimulus (Braun and Westermann,

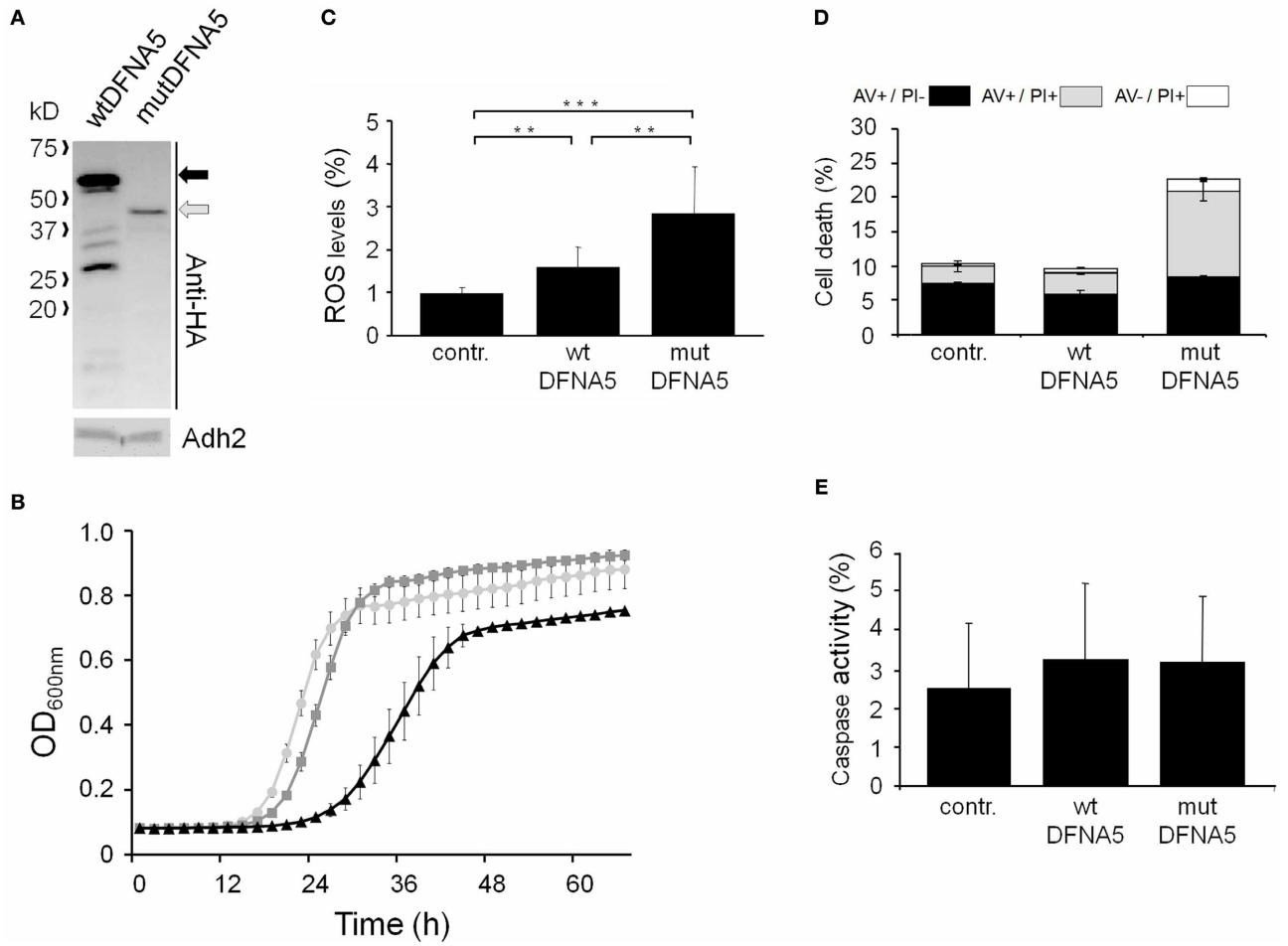

FIGURE 3 | mutDFNA5-induced cell death does not involve the yeast caspase. (A) Western blot analysis of protein extracts of *mca1*Δ cells transformed with constructs allowing for the expression of C-terminally HA-tagged wtDFNA5 or mutDFNA5 as indicated. Immunodetection was performed using primary antibodies directed against the HA-tag or Adh2, which was used as loading control. The black arrow indicates full-length wtDFNA5 and the gray arrow full-length mutDFNA5. **(B)** Growth profiles of *mca1*Δ cells expressing HA-tagged wtDFNA5 (dark gray squares) or mutDFNA5 (black triangles) or transformed with an empty plasmid (light gray circles). **(C,D)** Quantification of ROS accumulation using DHE staining (panel **C**) or AV/PI positive cells (panel **D**) in mid-exponential cultures of *mca1*Δ cells transformed with an empty plasmid (contr.) or with constructs allowing for expression of C-terminally HA-tagged wtDFNA5 or mutDFNA5. **(E)** Assessment of the caspase activity using FITC-tagged z-VAD-FMK in BY4741 wild-type cells expressing C-terminally HA-tagged wtDFNA5 or mutDFNA5 or transformed with an empty plasmid (contr.). Significances were assayed using unpaired *t*-tests. For the AV/PI co-staining the following significances were obtained when making the comparison with the control: wtDFNA5: AV^-/PI^+ *; mutDFNA5: AV^+/PI^+ ***, AV^-/PI^+ ***. (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

2011). For instance, for ethanol-induced apoptosis, Fis1 was shown to mediate mitochondrial fragmentation and cell death independently of Dnm1 and Mdv1 (Kitagaki et al., 2007), whereas for acetic acid-induced apoptosis, Fis1 was reported to protect cells by inhibition of Dnm1- and Mdv1-mediated mitochondrial fission and cell death (Fannjiang et al., 2004). Concerning the DFNA5-induced cell death, Fis1 obviously exerted a protective function, but this appeared to be largely independent of Dnm1 and Mdv1 because neither the deletion of *DNM1*, nor the deletion of *MDV1* affected the DFNA5-mediated growth phenotype (**Table 3**). As was to be expected, the levels of ROS were generally higher in the *fis1*Δ strains than in the wild-type strains with a minor increment in case of wtDFNA5 expression, but a clear augmentation in case of mutDFNA5 expression (**Figure 4B**). Likewise, the amount of dying cells in mid-exponential cultures were in general higher in the *fis1*Δ strains as compared to the wild-type strains, especially the number of early apoptotic cells, and while there was no significant increase in the total number of cells showing signs of cell death between the control and cultures expressing wtDFNA5 or mutDFNA5, the latter two still showed a trend toward enhanced late apoptosis and necrosis (**Figure 4C**). Analysis of *fis1*Δ cells with combined expression of Mito-RFP and EGFP-fusions showed that most cells contained fragmented and aggregated mitochondria, which did not overlap with the deposits formed by wtDFNA5-EGFP but clearly co-localized with the foci of mutDFNA5-EGFP (**Figure 4D**). Similar as the loss of Fis1, also the absence of the channel protein Por1 appeared to sensitize cells for DFNA5-mediated cell death. However, with

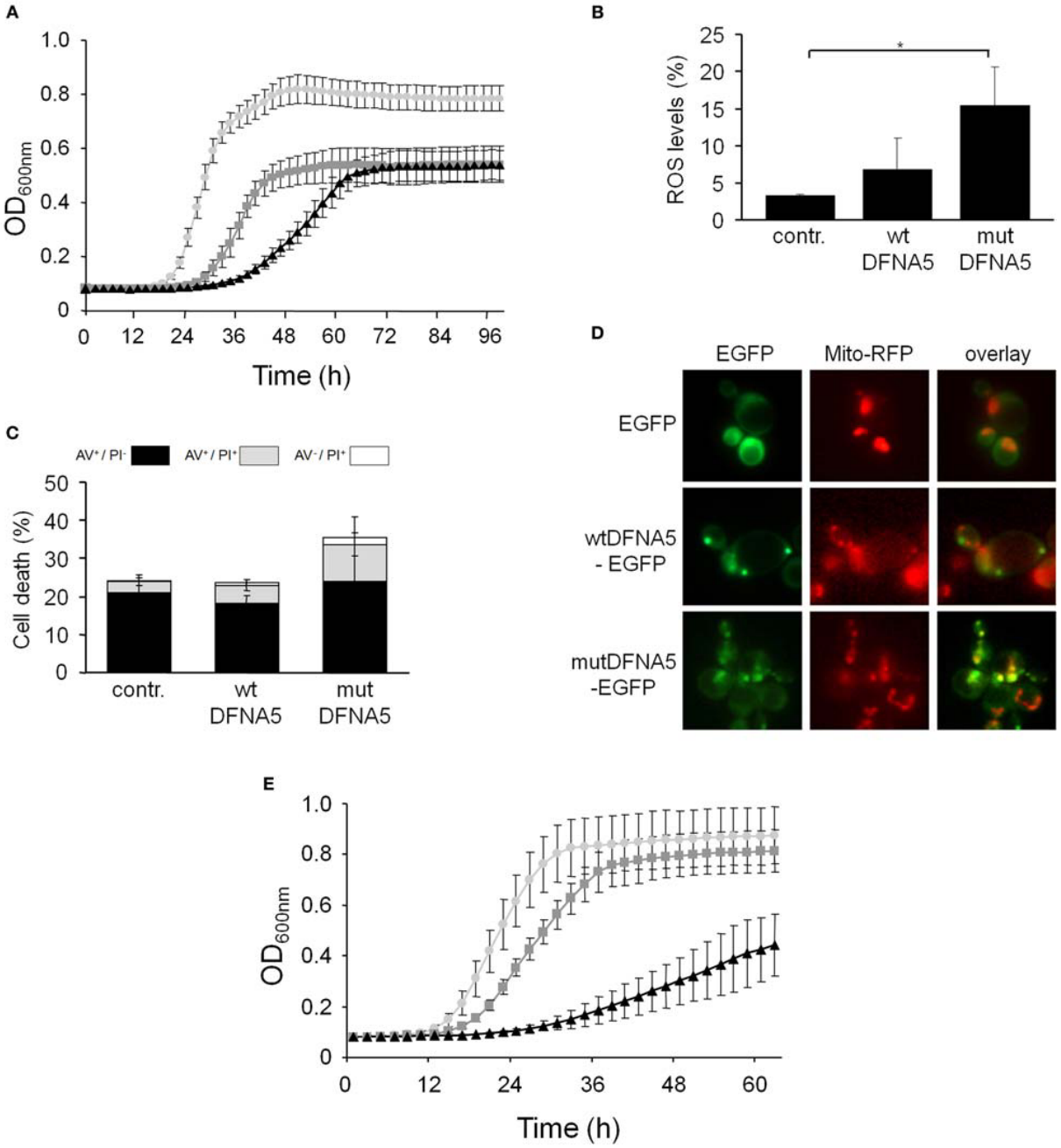

FIGURE 4 | DFNA5 cytotoxicity is enhanced in cells lacking mitochondrial functions. (A) Growth profiles of *fis1*Δ cells expressing C-terminally HA-tagged wtDFNA5 (dark gray squares) or mutDFNA5 (black triangles) or transformed with an empty plasmid (light gray circles). **(B,C)** Quantification of ROS accumulation using DHE staining (panel **B**) or AV/PI positive cells (panel **C**) in mid-exponential *fis1*Δ cultures without (contr.) or with expression of HA-tagged wtDFNA5 or mutDFNA5. **(D)** Fluorescence microscopy pictures of *fis1*Δ cells expressing EGFP or displaying inclusions of wtDFNA5-EGFP or mutDFNA5-EGFP and co-transformed with Mito-RFP to visualize mitochondria. **(E)** Growth profiles of *por1*Δ cells expressing HA-tagged wtDFNA5 (dark gray squares) or mutDFNA5 (black triangles) or transformed with an empty plasmid (light gray circles). Significances were assayed using unpaired *t*-tests. For the AV/PI co-staining of the *fis1*Δ cultures the following significant *p*-values were obtained when making the comparison with the control and mutDFNA5: AV^+/PI^+ **. (* = $p < 0.05$; ** = $p < 0.001$).

this *por1*Δ deletion strain it was difficult to correctly assess the repercussions on ROS accumulation or cell death due to the very slow growth of the cells transformed with mutDFNA5 (**Figure 4E**).

In two other mitochondrial mutants, i.e., the *aac1*Δ and *aac3*Δ deletion strains (**Figures 5A,B**), the growth differences between the control cultures and the cultures expressing wtDFNA5 or mutDFNA5 were almost annihilated, indicative that the lack of

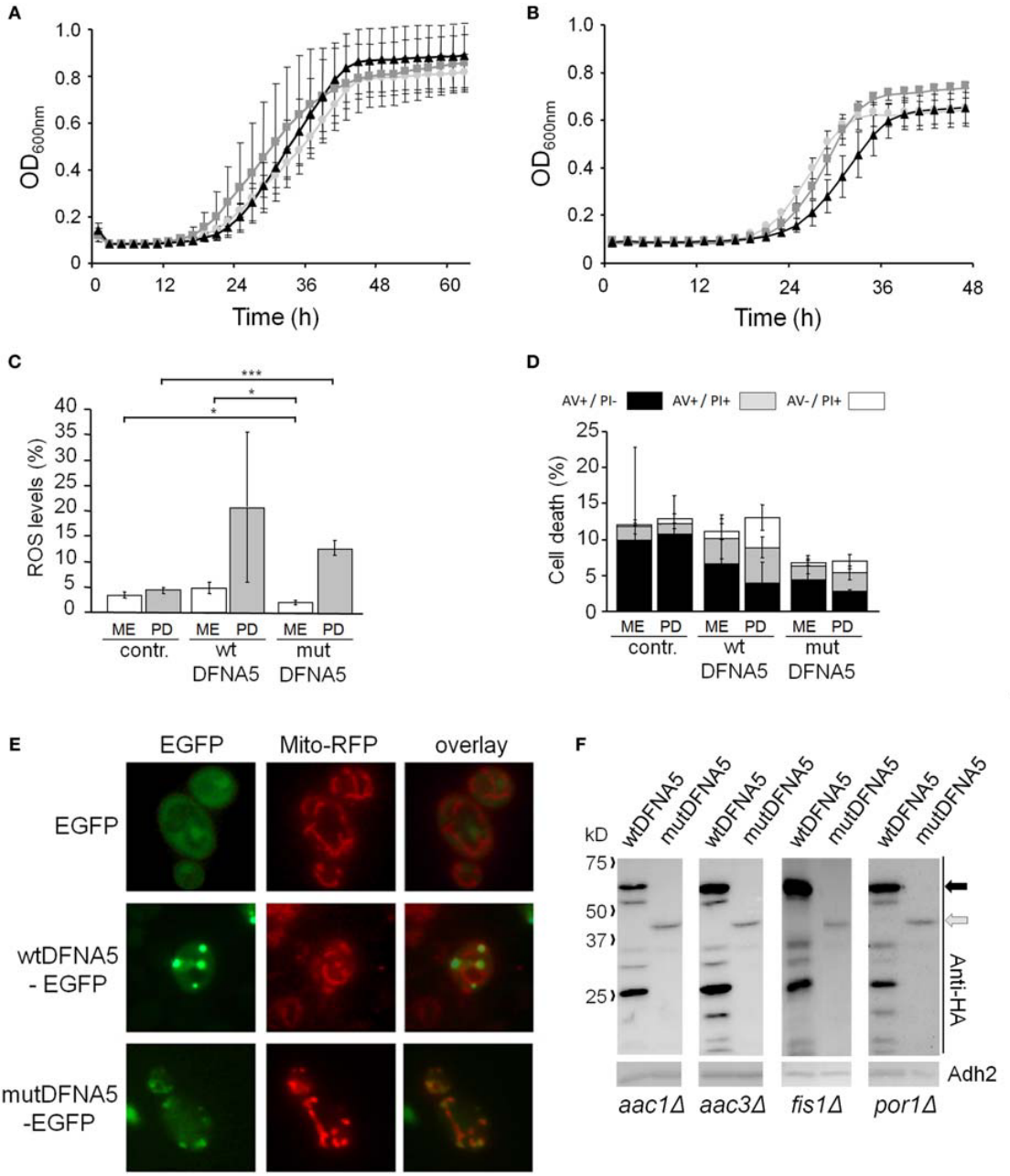

FIGURE 5 | mutDFNA5 cytotoxicity depends on the mitochondrial ADP/ATP carriers. (A,B) Growth profiles of **(A)** *aac1*Δ cells or **(B)** *aac3*Δ cells expressing C-terminally HA-tagged wtDFNA5 (dark gray squares) or mutDFNA5 (black triangles) or transformed with an empty plasmid (light gray circles). **(C,D)** Quantification of ROS accumulation using DHE staining (panel **C**) or AV/PI positive cells (panel **D**) in mid-exponential (ME) and post-diauxic (PD) phase in *aac3*Δ cultures without (contr.) or with expression of HA-tagged wtDFNA5 or mutDFNA5. **(E)** Fluorescence microscopy pictures of *aac3*Δ cells expressing EGFP or displaying inclusions of wtDFNA5-EGFP or mutDFNA5-EGFP and co-transformed with Mito-RFP to visualize mitochondria. **(F)** Western blot analysis of protein extracts of the *aac1*Δ, *aac3*Δ, *fis1*Δ, *por1*Δ mutant strains transformed with constructs allowing for the expression of C-terminally HA-tagged wtDFNA5 or mutDFNA5 as indicated. Immunodetection was performed using primary antibodies directed against the HA-tag or Adh2, which was used as loading control. Significances were assayed using unpaired *t*-tests. For the AV/PI co-staining the following significances were obtained when compared to the control: for wtDFNA5 in PD: AV^+/PI^+ *, AV^-/PI^+ *. (* = $p < 0.05$; *** = $p < 0.001$).

Aac1 or Aac3 abrogated the mutDFNA5-associated cytotoxicity. *AAC1* and *AAC3* encode for two of the three ADP/ATP carriers of the inner mitochondrial membrane. Previously reported studies implicated these proteins as effectors of acetic acid-induced apoptosis, a role which apparently does not depend on their ADP/ATP translocase activity but rather on their impact on the mitochondrial outer membrane permeabilization and mitochondrial degradation (Pereira et al., 2007, 2010). As documented

for the *aac3*Δ mutant, the deletion of the ADP/ATP carrier did not prevent the accumulation of ROS during the post-diauxic phase in cells expressing wtDFNA5 or mutDFNA5 (**Figure 5C**). However, the lack of Aac3 clearly interfered with the appearance of cell death markers. In the *aac3*Δ control cultures, the levels of dying cells were markedly higher as compared to control culture of wild-type cells, which is consistent with the fact that the *aac3*Δ mutant grows slower. The cultures with cells expressing wtDFNA5 or mutDFNA5 still displayed altered ratios between early or late apoptotic and necrotic cells, but the total number of cells with signs of cell death did not alter in the different growth phases. Furthermore, the total number of dying cells was comparable for the control culture and the culture of cells expressing wtDFNA5 and it was consistently lower for the culture of cells expressing mutDFNA5 (**Figure 5D**). Similar data were obtained for the *aac1*Δ mutant (data not shown). These observations confirm that mutDFNA5 requires the ADP/ATP carriers to instigate cytotoxicity and cell death. Furthermore, while ROS production has been described as an event common to most of the yeast apoptosis scenarios, our data demonstrate that in the ADP/ATP carrier mutants the correlation between ROS accumulation and viability does not hold. As such, our data are completely in line with previously reported results obtained with a triple *aac1-3*Δ mutant for acetic acid-induced apoptosis (Pereira et al., 2007).

Analysis of *aac3*Δ cells co-transformed with Mito-RFP and the EGFP-fusions revealed that these cells harbor a well developed mitochondrial tubular network. Even in cells expressing mutDFNA5-EGFP such a tubular network was present, but there were still punctuated mitochondria co-localizing with the foci of the EGFP fusion (**Figure 5E**). This led us to conclude that the absence of the ADP/ATP carriers did not prevent mutDFNA5 to interfere with mitochondrial fission and fusion dynamics or the clearance of fragmented mitochondria, which both are aspects that remain to be studied in more detail.

Finally, it should be noted that we did not observe significant differences in expression or degradation of the HA-tagged wtDFNA5 or mutDFNA5 proteins between the wild-type strain and the different mutant strains (**Figure 5F**). This indicates that the observed changes in DFNA5-instigated cytotoxicity in the mutant strains are related to their deleted functions and not to alterations in DFNA5 expression.

DFNA5 TOXICITY IS CONFINED TO ITS FIRST GLOBULAR DOMAIN

We recently proposed that wtDFNA5 is composed of two globular domains, which are separated by a hinge region (**Figure 6A**). In that study, we also demonstrated that the first domain induces apoptotic cell death in transfected HEK293T cells, which led to a model where the second domain can fold back to mask and regulate the apoptotic activity of the first domain (Op de Beeck et al., 2011b). Here, we expressed the two domains separately in yeast. As shown, the expression of the first domain, designated domain A and corresponding to the amino acid residues 1–256, triggered a very pronounced growth defect that by far surpassed the defect observed for mutDFNA5 (**Figure 6B**). Expression of the second domain, referred to as domain B and corresponding to residues 282–496, did not affect growth and the growth curve almost perfectly overlapped with the one obtained for the empty vector control.

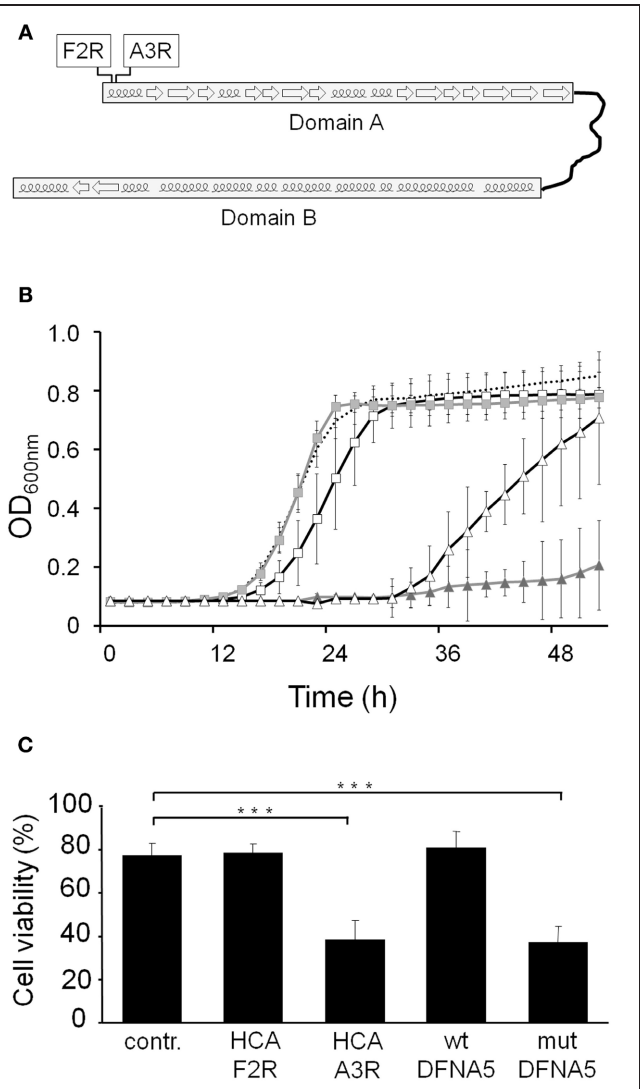

FIGURE 6 | DFNA5 cytotoxicity is confined to its first globular domain. **(A)** Schematic representation of wtDFNA5 with the two globular domains connected by the hinge region and the indication of the mutants HCA-F2R and HCA-A3R (boxed) in the first domain. **(B)** Growth profiles of BY4741 cells expressing the first (gray triangles) or the second (gray squares) globular domain of wtDFNA5 or expressing point mutants in the first globular domain, i.e., mutants HCA-F2R (open squares) or HCA-A3R (open triangles). The dashed line is given as reference and depicts the growth of BY4741 cells transformed with an empty plasmid. **(C)** Cell viability of HEK293T cells transiently expressing the HCA-F2R and HCA-A3R mutants, wtDFNA5 and mutDFNA5 as indicated. Significances were assayed using One-Way ANOVA (*** = $p < 0.001$). Cells expressing EGFP served as control (contr.).

DFNA5 belongs to the gasdermin protein family, named after the founder protein GSDMA, which is involved in gastric cancer and also harbors pro-apoptotic activities (Saeki et al., 2007). Sequence alignment of the different gasdermin family members show a high degree of conservation, especially in the first domain, which based on hydrophobic cluster analysis contains

short α-helical folds interspaced by β-sheets (Op de Beeck et al., 2011b). To document the importance of these structures for the apoptotic-inducing activity of the domain, we used PCR-based site directed mutagenesis and phenotypically tested two of the mutants generated in domain A (**Figure 6A**). The first mutant, designated HCA-F2R, contained the substitution of a highly conserved hydrophobic phenylalanine into a basic arginine, thereby disrupting the first α-helical fold. When expressed in yeast, this mutant only triggered a small growth defect (ΔT1/2: 3.33 h ± 0.93) and thus lost most of the apoptotic-inducing activity (**Figure 6B**). In the second mutant, i.e., HCA-A3R, a non-conserved alanine residue was changed into arginine. Although this mutation also affected the first α-helical fold, its repercussions on the apoptotic-inducing activity were less pronounced as evidenced by the observation that the expression of this mutant in yeast resulted in an intermediate growth defect (ΔT1/2: 20.50 h ± 5.96; **Figure 6B**).

Next to the experiments in yeast, we also assessed the cell viability of human HEK293T cells transiently expressing the generated DFNA5 mutants. The expression of the HCA-A3R construct led to a significant decrease of cell viability (mean viability: 38.28% ± 9.37), whereas expression of HCA-F2R did not (mean viability: 78.60% ± 4.03), as it gave a similar cell viability as that observed for cells transfected with an empty EGFP vector (mean viability: 77.59% ± 5.58; **Figure 6C**). In fact, the cell viability of cells expressing HCA-A3R is highly comparable to those expressing mutDFNA5, while cell viability of cells expressing HCA-F2R is comparable to cells expressing wtDFNA5. As such, the results obtained in HEK293T cells confirm those obtained in yeast.

DISCUSSION

In this study we analysed the repercussion of heterologous expression of human wtDFNA5 or mutDFNA5 in *Saccharomyces cerevisiae*. Our data clearly demonstrate that mutDFNA5 causes a significant growth defect, which is associated with an increased number of late apoptotic and necrotic cells especially when the culture entered the stationary phase, and this in contrast to wtDFNA5. These findings confirm previous reported results showing that mutDFNA5 induces apoptotic and necrotic cell death when expressed in mammalian cells (Van Laer et al., 2004; Op de Beeck et al., 2011b).

Detailed analysis of the expression of wtDFNA5 and mutDFNA5 in the yeast system revealed that both proteins form inclusions. For wtDFNA5, these inclusions most likely correspond to IPOD as they are found at the periphery of the vacuole. The IPOD is a protein quality control compartment where proteins are deposited that presumably await autophagic clearance (Bagola and Sommer, 2008; Kaganovich et al., 2008). This suggests that wtDFNA5 is subjected to the normal cellular repertoire of protein quality control and clearance mechanisms. MutDFNA5 seems to escape these quality control systems. It does not form large IPOD-like deposits but rather smaller and more numerous foci, which are less intense and occurring mostly in the vicinity of the plasma membrane. Previous studies already suggested an association of mutDFNA5 with the plasma membrane and/or a membrane protein in mammalian cells, but the exact location remained unclear (Van Laer et al., 2004; Op de Beeck et al., 2011a). We now show that in yeast the foci formed by mutDFNA5 often co-localize with fragmented mitochondria, suggesting that the mutant proteins may interact either with the mitochondrial membrane or one of the mitochondrial membrane proteins, which in turn may lead to mitochondrial impairment. The latter is further evidenced by the fact that mutDFNA5 is even more toxic in the *fis1*Δ and *por1*Δ mutants, while it lost its specific toxicity-inducing capacity in mutant strains lacking the ADP/ATP carriers Aac1 and Aac3. It is remarkable that the same mitochondrial proteins have previously been identified as key players with similar contributions for acetic acid-induced apoptosis in yeast (Fannjiang et al., 2004; Pereira et al., 2007). This underscores that mutDFNA5-instigated cytotoxicity and acetic acid-induced apoptosis build on common molecular mechanisms. Note that we did not analyse the third ADP/ATP carrier Aac2 in our studies for the simple reason that its deletion is lethal in the BY4741 background (Chen, 2004).

The studies on acetic acid-induced apoptosis demonstrated a protective function of Fis1 that relates to inhibition of Dnm1-mediated mitochondrial fission and possible additional pro-apoptotic Dnm1 functions (Fannjiang et al., 2004). Also for mutDFNA5-induced cell death we found that Fis1 fulfils a protective role but this apparently does not involve Dnm1 or its adaptor Mdv1. Indeed, our observation that neither the deletion of *DNM1* nor of *MDV1* prevented mutDFNA5-induced cell death excludes these fission proteins as downstream effectors. That Fis1 could have a specific function not shared by the other fission factors Dnm1 and Mdv1 was already noted before in studies dealing with ethanol-induced apoptosis. These studies suggested that Fis1 has a specific role for the maintenance of mitochondrial fragmentation in response to ethanol (Kitagaki et al., 2007). However, most recent studies revealed that *fis1*Δ mutants accumulate secondary loss-of-function mutations in the *WHI2* gene (Cheng et al., 2008; Mendl et al., 2011), which encodes a protein involved in cell cycle regulation (Radcliffe et al., 1997), the general stress response, actin dynamics and Ras-cAMP-PKA signaling (Kaida et al., 2002; Leadsham et al., 2009) as well as the selective degradation of dysfunctional mitochondria via autophagy, a process known as mitophagy (Muller and Reichert, 2011). In fact, the studies on cell death and mitophagy showed that the enhanced sensitivity of the *fis1*Δ mutants toward cell death stimuli is solely due to the loss of the Whi2 function and not to the lack of Fis1 (Cheng et al., 2008; Mendl et al., 2011). Also the *fis1*Δ mutant strain of the yeast deletion collection that was used in our study appears to contain such a secondary loss-of-function mutation in *WHI2* (Cheng et al., 2008). Hence, it is feasible that the enhanced mutDFNA5-instigated cytotoxicity in the *fis1*Δ mutant strain relates to the *WHI2* mutation and the consequent diminished stress resistance and lower rate of mitophagy, rather than to a deficiency in mitochondrial fission. At least, it would explain the observed accumulation of fragmented and aggregated mitochondria co-localizing with the foci of mutDFNA5-EGFP in the *fis1*Δ mutant, and as such provide an additional confirmation that mutDFNA5 triggers cell death through mitochondrial damage.

Another aspect of Fis1 is that the protein has similar biophysical properties as the mammalian Bcl2 and Bcl-xL and although these anti-apoptotic proteins cannot replace the mitochondrial

fission function of Fis1, they do substitute for Fis1 in cell viability assays (Fannjiang et al., 2004). Bcl2 and Bcl-xL have important roles as regulators of mitochondrial membrane permeabilization, since they inhibit non-specific pore formation by the adenine nucleotide translocator, ANT, the mammalian ortholog of the Aac1/2/3 ADP/ATP carriers (Brenner et al., 2000; Belzacq et al., 2003). Previous studies in yeast identified Fis1 as a potential regulator together with the mitochondrial permeability transition pore components Aac1/3 and the VDAC protein Por1 for acetic acid-induced cell death (Fannjiang et al., 2004; Pereira et al., 2007, 2010). Our data now demonstrate that mutDFNA5 cytotoxicity is enhanced in the absence of Por1, while this toxicity is basically abrogated in the absence of Aac1 or Aac3. Whether this means that mutDFNA5 directly targets the ADP/ATP carriers to alter mitochondrial membrane permeability remains to be clarified. In humans, mutations in the *ANT1* gene are associated with progressive external ophthalmoplegia (Sharer, 2005) and to our knowledge there are no reports that link *DFNA5* to this disorder or, conversely, that link *ANT1* to autosomal dominant deafness.

DFNA5 belongs to the gasdermin family. Although the members of this family appear to have different molecular functions, they share conserved structural features, such as the presence of a globular domain in their N-terminal half (Saeki et al., 2007; Tamura et al., 2007; Op de Beeck et al., 2011b). Intriguingly, this domain harbors the DFNA5 capacity to induce cell death as confirmed in previous studies (Op de Beeck et al., 2011b) and ours. It is not known whether this capacity to induce cell death is a common physiological property of all gasdermin family members, but at least for one other member, i.e., GSDMA, this seems to be the case since the protein was reported to induce apoptosis in a gastric epithelial cell line (Saeki et al., 2007). The apoptosis-inducing globular domain was proposed to be shielded in wtDFNA5 by a second C-terminal regulatory domain. In mutDFNA5 a large part of this regulatory domain is missing and therefore the apoptosis-inducing domain is presumably more exposed (Op de Beeck et al., 2011a,b). Structurally, the apoptosis-inducing domain is composed of short α-helical folds interspaced by β-sheets. Here, we show that mutations disrupting the first α-helical fold strongly reduce the cell death-inducing capacity of the N-terminal domain in yeast and human HEK293T cells. This demonstrates the feasibility to use the yeast system to further dissect the structural requirements of DFNA5 associated with its apoptosis-inducing property.

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Mitochondrion-mediated cell death: dissecting yeast apoptosis for a better understanding of neurodegeneration

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Mitochondrial damage and dysfunction are common hallmarks for neurodegenerative disorders, including Alzheimer, Parkinson, Huntington diseases, and the motor neuron disorder amyotrophic lateral sclerosis. Damaged mitochondria pivotally contribute to neurotoxicity and neuronal cell death in these disorders, *e.g.*, due to their inability to provide the high energy requirements for neurons, their generation of reactive oxygen species (ROS), and their induction of mitochondrion-mediated cell death pathways. Therefore, in-depth analyses of the underlying molecular pathways, including cellular mechanisms controlling the maintenance of mitochondrial function, is a prerequisite for a better understanding of neurodegenerative disorders. The yeast *Saccharomyces cerevisiae* is an established model for deciphering mitochondrial quality control mechanisms and the distinct mitochondrial roles during apoptosis and programmed cell death. Cell death upon expression of various human neurotoxic proteins has been characterized in yeast, revealing neurotoxic protein-specific differences. This review summarizes how mitochondria are affected in these neurotoxic yeast models, and how they are involved in the execution and prevention of cell death. I will discuss to which extent this mimics the situation in other neurotoxic model systems, and how this may contribute to a better understanding of the mitochondrial roles in the human disorders.

Keywords: mitochondria, mitochondrial dysfunction, mitochondrial quality control, neurodegeneration, neurotoxicity, cell death, *Saccharomyces cerevisiae*

INTRODUCTION

Mitochondria are important organelles; they produce most of the ATP via oxidative phosphorylation, they are involved in lipid and phospholipid metabolism, in the biosynthesis of essential intermediates, including heme and iron-sulfur clusters, and they contribute to various cellular stress responses, including programmed cell death (Nunnari and Suomalainen, 2012). Functional mitochondria are essential for neurons, which have an extreme high demand for energy in their cell bodies and synapses (Nunnari and Suomalainen, 2012). Therefore, it is evident that neurons are extremely vulnerable against mitochondrial damage.

Mitochondrial damage is a hallmark for Alzheimer, Parkinson, Huntington diseases, and amyotrophic lateral sclerosis (Martin, 2011; Correia et al., 2012; Cozzolino et al., 2012). Affected neurons suffer from ATP depletion, loss of respiratory capacity, elevated levels of reactive oxygen species (ROS), the induction of mitochondrion-specific cell death pathways, and immotile mitochondria, which fail to localize to the sites with increased energy demands, such as synapses (Martin, 2011; Correia et al., 2012; Cozzolino et al., 2012) (**Figure 1**). Mitochondrial damage in neurons can be described by (1) mutations in the mitochondrial DNA (mtDNA), (2) the loss of the mitochondrial membrane potential, (3) the loss of mitochondrial protein import and protein biosynthesis, (4) reduced activities of enzymes of the mitochondrial respiratory chain and the TCA cycle, (5) increased leakage of electrons from the respiratory chain generating ROS, (6) the loss of mitochondrial motility, (7) the destruction of the mitochondrial network, and (8) the rupture of the mitochondrial outer and inner membranes, leading (9) to the release of mitochondrial pro-death factors, including cytochrome *c* (Cyt. *c*), apoptosis-inducing factor, or endonuclease G.

Nature developed many mechanisms to prevent and repair mitochondrial damage or to remove damaged mitochondria (**Figure 1**): (1) the dynamic fusion and fission of mitochondria enables the maintenance of intact mtDNA (Westermann, 2010; Hori et al., 2011), (2) the phospholipid turnover between the ER and mitochondria allows for the rejuvenation of mitochondrial membranes (Fujimoto and Hayashi, 2011), (3) retrograde signaling senses mitochondrial damage, eliciting a nuclear transcriptional response to counteract mitochondrial damage (Liu and Butow, 2006; Pellegrino et al., 2012), (4) mitochondrial biogenesis leads to the replenishment of damaged mitochondria and its components (Michel et al., 2012), (5) mitochondrial protein quality control is guaranteed by chaperones and the proteolytic machinery, including the proteasome-dependent mitochondrion-associated degradation (MAD) of mitochondrial outer membrane proteins (Heo et al., 2010; Taylor and Rutter, 2011), the proteolytic degradation of proteins of the intermembrane space, the inner membrane, and the matrix (Fischer et al., 2012; Rugarli and Langer, 2012), and (6) removal of damaged mitochondria occurs via mitochondrion-specific autophagy (mitophagy) (Kanki and Klionsky, 2010). Thus, the detailed analysis of mitochondrial damage, and the mechanisms counteracting mitochondrial damage and its devastating consequences

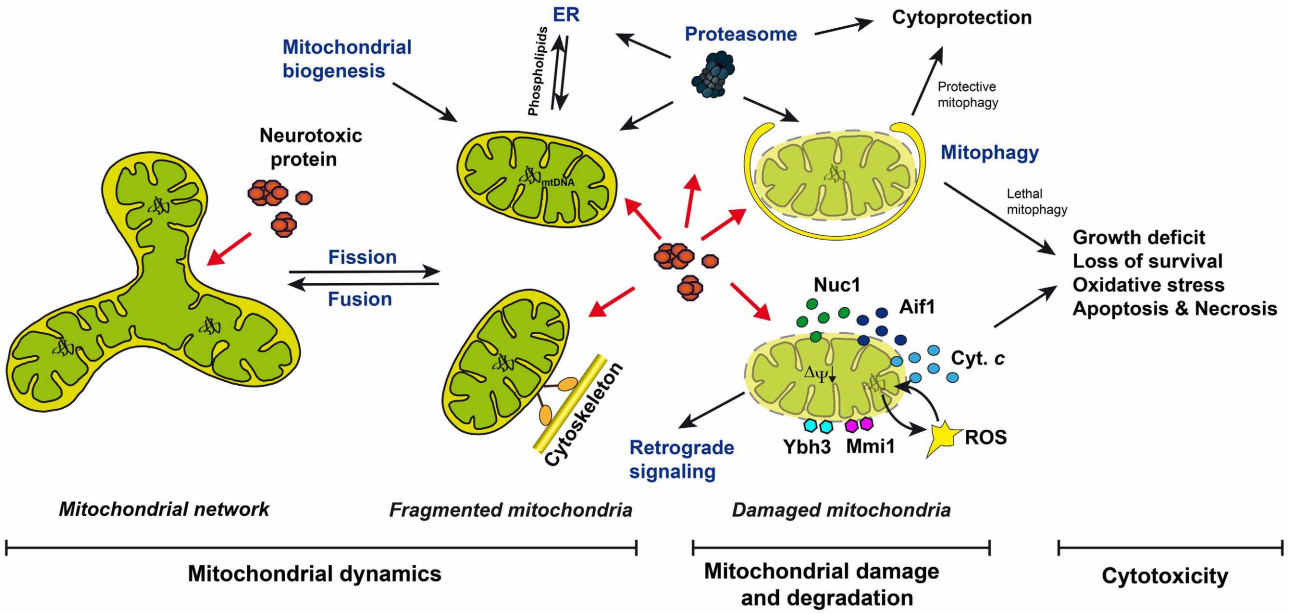

FIGURE 1 | Mitochondrial dynamics, damage and degradation, and mitochondrion-dependent cell death upon neurotoxic protein expression in yeast. Mitochondria are part of a network promoted by fusion or are fragmented into individual organelles by fission. They are transported along the cytoskeleton. Damaged mitochondria are targeted by cytoplasmic proteins, including Ybh3 and Mmi1, produce ROS or release proteins into the cytosol, including cytochrome *c*, Aif1, and Nuc1, triggering apoptosis and necrosis. Mitophagy and proteasome-dependent pathways remove damaged mitochondria, and mitochondrial biogenesis and retrograde signaling are involved in the replenishment and repair of the mitochondrial pool, respectively. Neurotoxic proteins trigger mitochondrial damage and cell death: they could interfere with mitochondrial fusion, fission, and motility, or could interrupt with mitochondrion degradation triggering "lethal mitophagy", or could directly affect mitochondrial function.

for neurons, is of high importance for a better understanding of neurodegenerative disorders.

Yeast is an established model for dissecting conserved mechanisms of programmed cell death including apoptosis and necrosis (Carmona-Gutierrez et al., 2010). In yeast, cytotoxicity can easily be measured by complementary methods (Braun et al., 2010). Growth assays measure growth rates on agar plates or in liquid cultures, clonogenic survival assays measure the ability of yeast cells to form new colonies, and necrosis and apoptosis can be discriminated by analyzing cells for morphological markers of these subroutines of cell death. The importance of mitochondria in distinct cell death scenarios is well described in yeast (Eisenberg et al., 2007; Pereira et al., 2008; Guaragnella et al., 2012) (**Figure 1**). The mitochondrial permeabilization and the consequent release of the mitochondrial cell death proteins Cyt. *c*, yeast apoptosis-inducing factor (Aif1), or yeast endonuclease G (Nuc1) was observed (Ludovico et al., 2002; Wissing et al., 2004; Büttner et al., 2007; Pereira et al., 2007). ROS are produced from the mitochondrial respiratory chain, including the internal NADH dehydrogenase (Ndi1) and complex III (Braun et al., 2006, 2011; Li et al., 2006). Cytoplasmic proteins are recruited to the mitochondrial outer membrane, such as the BH3-only protein Ybh3 and the microtubule-associated protein Mmi1 (Rinnerthaler et al., 2006; Büttner et al., 2011). Mitochondrial quality control mechanisms and their influence on cell survival and aging have been intensively examined in yeast (Westermann, 2010; Braun and Westermann, 2011; Müller and Reichert, 2011; Fischer et al., 2012; Nunnari and Suomalainen, 2012; Rugarli and Langer, 2012). Indeed, our knowledge about mitochondrial dynamics, including mitochondrial fusion, fission, motility, degradation, and protein quality control is strongly influenced by data originally obtained in yeast, and later validated in other organisms, including humans. In the recent years, many yeast models have been established to analyze the influence of human neurotoxic protein expression on yeast cell survival, including models for Alzheimer, Parkinson, Huntington, and motor neuron disorders (Gitler, 2008; Miller-Fleming et al., 2008; Winderickx et al., 2008; Bharadwaj et al., 2010; Braun et al., 2010; Khurana and Lindquist, 2010; Bastow et al., 2011; De Vos et al., 2011; Mason and Giorgini, 2011).

Here, I summarize how mitochondria are damaged in these neurotoxic yeast cell death models, which role they play in the execution of cell death, and which mitochondrial quality control mechanisms potentially influence cytotoxicity. Further, I will critically discuss the similarities and discrepancies between the neurotoxic yeast models, and the animal and cell culture disease models.

NEUROTOXIC YEAST MODELS

ALZHEIMER DISEASE (AD) AND FRONTOTEMPORAL LOBAR DEGENERATION (FTLD-tau)

AD is the most prevalent form of age-related dementia (Querfurth and Laferla, 2010). It is characterized by a progressive deterioration, concomitant with behavior impairments and deficits in language and visuospatial skills (Querfurth and Laferla, 2010). Synaptic loss and neuronal decline can be observed in

affected brain regions, including the hippocampus and the cortex (Querfurth and Laferla, 2010). The accumulation of extracellular senile plaques and intracellular aggregates, composed of the β-amyloid peptide, and the intracellular accumulation of neurofibrillary tangles comprising hyperphosphorylated variants of the microtubule-associated protein tau contribute to the progression of AD (Laferla et al., 2007; Benilova et al., 2012; Mandelkow and Mandelkow, 2012).

FTLD is the second most common form of early onset dementia after AD (Sieben et al., 2012). It is characterized by a selective atrophy of the frontal and anterior temporal lobes of the brain, leading to disturbances of behavior and personality (Sieben et al., 2012). Intracellular accumulation of the hyperphosphorylated protein tau is a hallmark of one subtype of FTLD, called FTLD-tau (Sieben et al., 2012). Mutations in *MAPT*, the gene encoding tau, trigger neuronal degeneration and FTLD-tau (Sieben et al., 2012). In order to elucidate conserved mechanisms of AD/FTLD-relevant cytotoxicity, yeast models expressing AD-associated β-amyloid, and AD/FTLD-associated wild-type and mutant tau were established (Bharadwaj et al., 2010; De Vos et al., 2011).

AD-associated β-amyloid triggers cytotoxicity and mitochondrial damage upon localization to the secretory pathway

Several yeast AD models for dissecting the cytotoxic role of intracellular β-amyloid have been established (Bharadwaj et al., 2010; Treusch et al., 2011; D'Angelo et al., 2012). Whereas cytosolic β-amyloid and β-amyloid fusion proteins did not exert a marked cytotoxicity on yeast, two recent studies demonstrated that directing β-amyloid to the secretory pathway resulted in significant cytotoxicity, as measured by growth assays (Treusch et al., 2011; D'Angelo et al., 2012) (**Table 1**). In these models β-amyloid oligomerization could be observed, and aggregation and growth deficits were increased expressing AD-associated mutant β-amyloid (Treusch et al., 2011; D'Angelo et al., 2012). Although β-amyloid-triggered cytotoxicity was elicited in the secretory pathway, there are some hints that mitochondria were damaged and involved in the execution of cytotoxicity (D'Angelo et al., 2012). β-amyloid-expressing cells demonstrated decreased growth rates on obligatory respiratory growth media, and the oxygen consumption was markedly reduced in these cells under these conditions. Since the decrease in oxygen consumption preceded the observed growth deficits, these data suggest that mitochondrial impairment is causative for β-amyloid-triggered cytotoxicity. In a genome-wide overexpression screen to identify enhancers and suppressors of β-amyloid-triggered cytotoxicity (growth assays), *PET111* and *SLS1* encoding two mitochondrion-associated translation regulators were found as enhancers, and *RTG3*, encoding a transcriptional activator of the retrograde response, a response to counteract mitochondrial damage (Liu and Butow, 2006), was identified as suppressor (Treusch et al., 2011). Further studies will have to show, in which way mitochondrial protein translation and the retrograde response are involved in modulating β-amyloid-triggered cytotoxicity. It will also be of interest to investigate how β-amyloid expression can result in the potential mitochondrial damage in yeast, and whether it induces apoptotic and/or necrotic cell death. Although part of β-amyloid, which was directed to the secretory pathway, was later found to be cytosolic (D'Angelo et al., 2012), it remains an open question whether β-amyloid directly or indirectly affects mitochondrial function, and therewith influence cell survival.

Oxidative stress and mitochondrial dysfunction enhance aggregation of AD/FTLD-associated wild-type and mutant tau in yeast

Expression of FTLD-associated wild-type and mutant (P301L) tau did not trigger cytotoxicity in yeast, although it increased growth deficits upon co-expression with PD-associated α-synuclein (Zabrocki et al., 2005) (**Table 1**). Human tau formed sarkosyl-insoluble aggregates, which were highly phosphorylated by the yeast tau kinases Mds1 and Pho85 (Vandebroek et al., 2005, 2006; Vanhelmont et al., 2010). Treatment with ferrous sulfate, which increases ROS production, resulted in a significant increase in pathological tau aggregates in yeast cells expressing tau (Vanhelmont et al., 2010). This phenomenon was increased with FTLD-associated mutant (P301L) tau compared to wild-type tau, and it was independent of the phosphorylation status of tau, suggesting that ROS-increased tau aggregation acted mainly in parallel to tau phosphorylation (Vanhelmont et al., 2010). Increased pathological tau aggregates were also observed in yeast cells lacking the mitochondrial antioxidant enzyme superoxide dismutase 2 (*sod2*Δ), and in yeast cells lacking mtDNA (*rim1*Δ) (Vanhelmont et al., 2010). These data suggest that mitochondrially localized ROS and mitochondrial dysfunction contribute to tau pathology in yeast (Vanhelmont et al., 2010; De Vos et al., 2011).

Yeast AD models recapitulate key features observed in animal AD models and in AD patients

Mitochondrial damage and dysfunction are characteristics of both familiar and sporadic AD (Correia et al., 2012). Decreased abundances and activities of the pyruvate dehydrogenase complex, the TCA cycle enzymes, and mitochondrial respiratory chain complexes were observed in AD patients, animal and cell culture AD models (Correia et al., 2012). These data point to decreased mitochondrial activities and a consequent loss in energy supply (Correia et al., 2012). Yeast cells expressing β-amyloid demonstrated decreased oxygen consumption and decreased growth rates on obligatory respiratory growth media (D'Angelo et al., 2012), mimicking the energy hypometabolism observed in AD patients and other model systems. In AD patients and higher AD models, high levels of lipid and protein oxidation, as well as high incidences of mtDNA mutations hint to increased levels of oxidative stress in affected neurons (Correia et al., 2012). Oxidative stress has been suggested to facilitate pathological aggregation of β-amyloid and tau, which are then believed to further damage mitochondria in a vicious cycle (Swerdlow et al., 2010; Leuner et al., 2012). Although it remains undetermined whether β-amyloid or tau expression in yeast triggers ROS production, chemically induced oxidative stress in yeast cells expressing tau markedly increased its conversion into pathological tau aggregates (Vanhelmont et al., 2010). Thus, high levels of oxidative stress, *e.g.*, due to damaged mitochondria, may contribute to increased levels of pathological tau, accelerating cell death (De Vos et al., 2011).

Table 1 | Cytotoxicity, mitochondrial damage and mitochondrial quality control in yeast models for neurodegeneration.

Yeast model	Disease protein	Cytotoxicity	Mitochondrial damage	Mitochondrial quality control	Remarks	References
AD	β-Amyloid	Yes	Respiratory impairment	Beneficial retrograde response?	–	Treusch et al., 2011; D'Angelo et al., 2012
AD/FTLD-tau	Tau	No	n.d.	n.d.	Mitochondrial dysfunction and mitochondrially produced ROS contribute to tau aggregation	Vanhelmont et al., 2010; De Vos et al., 2011
	Tau/α-synuclein	Yes	n.d.	n.d.	Synergistic cytotoxicity (growth impairment) upon co-expression of tau and α-synuclein	Zabrocki et al., 2005
PD	α-Synuclein	Yes	Mitochondrially produced ROS, mitochondrial fragmentation, mitochondrial swelling, cytochrome *c* release, loss of mitochondrial membrane potential	Lethal mitophagy, beneficial retrograde response? beneficial mitochondrial biogenesis?	No direct interaction between α-synuclein and mitochondria; impaired ER homeostasis may contribute to mitochondrial damage	Willingham et al., 2003; Flower et al., 2005; Büttner et al., 2008; Gitler et al., 2008; Lee et al., 2008; Yeger-Lotem et al., 2009; Su et al., 2010; Sampaio-Marques et al., 2012
	Lrrk2	Yes	n.d.	n.d.	Abnormal autophagic vacuoles	Xiong et al., 2010
	Ypk9 (ATP13A2)	No	n.d.	n.d.	Rescues α-synuclein- and manganese-triggered cytotoxicity; upon deletion mitochondrion-dependent hypersensitivity against manganese treatment	Gitler et al., 2009; Chesi et al., 2012
	Hsp31 (DJ-1)	n.d.	n.d.	n.d.	Upon deletion increased ROS levels, and hypersensitivity against oxidative stress	Skoneczna et al., 2007
HD	Huntingtin	Yes	Mitochondrially produced ROS, respiratory impairment, mitochondrial fragmentation and swelling, loss of mitochondrial membrane potential, decreased mitochondrial protein synthesis, accumulation of intermediates of the kynurenine pathway	Beneficial mitochondrial biogenesis, beneficial retrograde response?	Direct interaction of Huntingtin with mitochondria; impaired ER homeostasis may contribute to mitochondrial damage	Willingham et al., 2003; Giorgini et al., 2005, 2008; Sokolov et al., 2006; Solans et al., 2006; Wang et al., 2009; Ocampo et al., 2010; Mason and Giorgini, 2011; Tauber et al., 2011

(continued)

Table 1 | continued

Yeast model	Disease protein	Cytotoxicity	Mitochondrial damage	Mitochondrial quality control	Remarks	References
ALS	SOD1	n.d.	Respiratory impairment	n.d.	Increased localization of ALS-associated SOD1 in the mitochondrial intermembrane space	Gunther et al., 2004; Klöppel et al., 2010; Bastow et al., 2011
	TDP-43	Yes	Mitochondrially produced ROS, respiratory capacity determines cytotoxicity	n.d.	Peri-mitochondrial TDP-43-containing aggregate-like foci	Johnson et al., 2008; Braun et al., 2011
	FUS/TLS	Yes	n.d.	n.d.	Deletion of genes encoding mitochondrion-localized proteins increase FUS/TLS-triggered cytotoxicity	Sun et al., 2011

n.d.: not determined.

Since β-amyloid was found to directly interact with mitochondria in higher AD models, these data suggest that at least part of the mitochondrial damage might be elicited by a detrimental interaction between the disease peptide and the organelle (Correia et al., 2012). In fact, in yeast β-amyloid was also identified in the cytosol (D'Angelo et al., 2012). Whether a direct interaction between β-amyloid and mitochondria occurs remains unknown. In animal and cell culture AD/FTLD models expressing AD-associated β-amyloid or FTLD-associated tau, mitochondrial dynamics was demonstrated to be aberrantly altered: abnormal mitochondrial morphology, and altered mitochondrial fission and fusion, as well as decreased mitochondrial motility and increased mitophagy were observed (Correia et al., 2012; Schulz et al., 2012). Since the pathways regulating mitochondrial dynamics and mitochondrial quality control are conserved from yeast to humans (Westermann, 2010; Fischer et al., 2012; Rugarli and Langer, 2012), yeast may help to elucidate how these pathways influence cytotoxicity upon expression of β-amyloid or tau.

PARKINSON DISEASE (PD)

PD is the most prevalent age-related movement disorder characterized by a progressive loss of dopaminergic neurons in the substantia nigra pars compacta (Lees et al., 2009). This results in a loss of the neurotransmitter dopamine in the striatum and consequently impairs with normal motor function leading to resting tremor, bradykinesia and rigidity (Lees et al., 2009). In most familiar and sporadic cases, PD is associated with Lewy bodies, *i.e.*, intracellular cytoplasmic aggregates composed of the protein α-synuclein (Uversky, 2007). Consistently, missense mutations in the *SNCA* gene, resulting in the expression of α-synuclein variants (A30P, A53T, E46K), as well as duplication and triplication of *SNCA*, leading to elevated α-synuclein levels, are causative for PD in some familiar forms of the disorder (Uversky, 2007). Mutations in other genes, including genes encoding the leucine-rich repeat kinase 2 (LRRK2), the E3 ubiquitin ligase parkin, the chaperone DJ-1, the mitochondrial PTEN-induced putative kinase 1 (PINK1), and the lysosomal ATPase ATP13A2 also lead to PD (Dehay and Bezard, 2011). Numerous disease models either overexpressing or deleting these disease-associated genes have been established, in order to describe common and distinct pathways relevant for PD progression (Dehay and Bezard, 2011).

Overexpression of α-synuclein in yeast results in cytotoxicity, and mitochondrion-dependent programmed cell death

Yeast cells overexpressing wild-type and disease-associated α-synuclein demonstrated growth deficits and age-dependent loss of clonogenic cell survival (Willingham et al., 2003; Flower et al., 2005; Witt and Flower, 2006; Büttner et al., 2008; Lee et al., 2008) (**Table 1**). α-Synuclein-triggered cytotoxicity in yeast was characterized by increased levels of ROS, and by the emergence of morphological markers of both apoptosis and necrosis (Flower et al., 2005; Büttner et al., 2008; Su et al., 2010). Mitochondria are critically damaged upon α-synuclein expression: (1) The mitochondrial network was fragmented (Sampaio-Marques et al., 2012), (2) mitochondria were found to be abnormally swollen (Su et al., 2010), (3) Cyt. *c* was released from mitochondria into the cytosol (Flower et al., 2005), (4) α-synuclein-expressing

cells treated with the proteasome inhibitor lactacystin demonstrated loss of mitochondrial membrane potential (Lee et al., 2008), and (5) mRNA profiling revealed that 60% of the downregulated genes encode proteins localized to mitochondria (Yeger-Lotem et al., 2009). Mitochondrial damage pivotally contributes to α-synuclein-triggered cytotoxicity, because α-synuclein expression in ρ^0 cells, which lack mtDNA and are devoid of respiratory competent mitochondria, significantly relieved the loss of cell survival, reduced the number of apoptotic and necrotic cells and markedly decreased ROS levels (Büttner et al., 2008). Mitochondrially produced ROS are important in α-synuclein-triggered cytotoxicity: (1) Deletion of *SOD2* encoding the mitochondrial antioxidant enzyme superoxide dismutase 2 markedly increased α-synuclein-triggered growth deficits (Willingham et al., 2003), (2) α-synuclein-expressing yeast cells were hypersensitive against oxidative stress (clonogenic cell survival assay upon hydrogen peroxide treatment) (Flower et al., 2005), and (3) α-synuclein-triggered ROS accumulation could efficiently be suppressed with the antioxidant glutathione (Flower et al., 2005). In another study treatment of α-synuclein-expressing cells with the antioxidants *N*-acetylcysteine, riboflavin, and melatonin did not show marked suppression of cytotoxicity (plasma membrane integrity and growth assays) (Su et al., 2010), suggesting that ROS rather accelerate α-synuclein-triggered cytotoxicity but are not essential for it.

Indirect mechanisms result in mitochondrial damage upon α-synuclein expression in yeast

α-Synuclein did not localize to yeast mitochondria, neither in cultures with intermediate nor in cultures with high expression levels of this protein (Gitler et al., 2008; Su et al., 2010). These data suggest that α-synuclein does not directly induce mitochondrial damage by mislocalizing to this organelle, as was discussed earlier (Witt and Flower, 2006), but that mitochondria are impaired in more indirect ways (Su et al., 2010). In fact, high expression levels of α-synuclein triggered defects in ER homeostasis in yeast (Gitler et al., 2008). This could have detrimental effects on the phospholipid turnover from the ER to and from the mitochondrial membranes (Fujimoto and Hayashi, 2011), thereby damaging mitochondrial membranes (Su et al., 2010). Impaired ER trafficking could also impair mitophagy, resulting in the accumulation of damaged mitochondria (Su et al., 2010). Further studies will be needed to shed light into cellular mechanisms affecting yeast mitochondria during α-synuclein expression.

Mitophagy determines α-synuclein-triggered cytotoxicity in yeast

Using an approach combining data from mRNA profiling of α-synuclein-expressing cultures with data from genetic suppressor screens searching for modifiers of α-synuclein-triggered cytotoxicity (growth assays) predicted the target of rapamycin (TOR) pathway, as a modulator of α-synuclein-triggered cytotoxicity (Yeger-Lotem et al., 2009). Indeed, addition of the TOR-inhibitor rapamycin markedly enhanced the growth deficits elicited by α-synuclein (Yeger-Lotem et al., 2009). Since inactivation of the TOR pathway induces autophagy-related pathways, these data gave first hints, that enhancing autophagy could be harmful for cultures expressing α-synuclein. Consistently, both rates of autophagy and mitochondrion-specific autophagy (mitophagy) were significantly increased upon α-synuclein expression (Sampaio-Marques et al., 2012). Pharmacological inhibition of autophagy (and mitophagy) by treatment with chloroquine markedly extended chronological life span of yeast cells expressing α-synuclein (Sampaio-Marques et al., 2012). Notably, inhibition of mitophagy alone was sufficient to relieve α-synuclein-triggered cytotoxicity (Sampaio-Marques et al., 2012): yeast cultures lacking the mitophagy-specific genes *ATG11* and *ATG32* demonstrated loss of mitophagy upon expression of α-synuclein, concomitant to markedly increased chronological life spans, significantly reduced incidences of morphological and metabolic markers of cell death, markedly decreased ROS levels, and the restoration of the mitochondrial network. These data suggest that mitophagy, a *per se* protective pathway to remove damaged mitochondria, can exert lethal functions in yeast upon high levels of α-synuclein.

Inhibition of the mitochondrial retrograde response by overexpressing its negative regulator Mks1 enhanced α-synuclein-triggered growth deficits (Yeger-Lotem et al., 2009). In contrast, overexpression of *HAP4*, encoding a transcriptional activator of mitochondrial biogenesis genes, markedly suppressed α-synuclein-triggered growth deficits (Yeger-Lotem et al., 2009). Thus, the retrograde response and mitochondrial biogenesis potentially play protective roles in cultures upon high levels of α-synuclein, whereas mitophagy potentially exert a lethal function. Further studies are needed to discriminate the distinct roles of these different mitochondrial quality control mechanisms in modulating α-synuclein-triggered cytotoxicity.

Dissecting mitochondrial roles in other yeast PD models remains a future task

Yeast cultures expressing fragments of the PD-associated GTPase and protein kinase Lrrk2 demonstrated cytotoxicity as manifested as growth deficits (Xiong et al., 2010). Expression of the GTPase domain was sufficient to induce Lrrk2-triggered cytotoxicity (growth assays) (Xiong et al., 2010) (**Table 1**). PD-associated mutant variants with altered GTPase activity demonstrated markedly increased cytotoxicity when compared with cultures expressing wild-type GTPase fragments, underlining the notion that altered GTPase activity of Lrrk2 is an important factor determining neuronal cell loss during PD progression (Gloeckner et al., 2006; Xiong et al., 2010). Remarkably, expression of Lrrk2 GTPase variants resulted in a significant increase in autophagic vacuoles in yeast, and deletion of *GCN4*, encoding a transcriptional activator of autophagy genes, led to a marked decrease in Lrrk2-triggered cytotoxicity (growth assays) (Xiong et al., 2010). These data suggest that, like in yeast cells expressing α-synuclein, autophagy potentially plays detrimental roles in Lrrk2-induced cytotoxicity. However, whether mitophagy and mitochondrial damage are important in modulating Lrrk2-triggered cytotoxicity in yeast remain to be explored.

Expression of the vacuolar protein Ypk9 (yeast PARK9), the yeast homolog of the PD-associated lysosomal ATPase ATP13A2, suppressed α-synuclein-triggered cytotoxicity (growth assays) in yeast (Gitler et al., 2009) (**Table 1**). Ypk9 expression increased the cellular tolerance against elevated levels of manganese, which

is an environmental risk factor for PD (Gitler et al., 2009). In contrast, deletion of *YPK9* resulted in cells demonstrating hypersensitivity against manganese treatment (growth assays) (Gitler et al., 2009; Chesi et al., 2012). This hypersensitivity was executed, at least in part, by functional mitochondria, as evidenced by the suppression of manganese-triggered cytotoxicity in yeast strains lacking genes encoding mitochondrial proteins, such as the GTPase Mgm1 and mitochondrial ribosomal proteins (Chesi et al., 2012). Further studies will have to show, whether Ypk9 expression prevents pivotal mitochondrial damage and aberrant mitophagy upon expression of α-synuclein.

The *S. cerevisiae* genome contains four homologs (*HSP31*, *HSP32*, *HSP33*, and *SNO4* (*HSP34*)) of the PD-associated gene DJ-1 (Skoneczna et al., 2007). Deletion of *HSP31* led to increased levels of ROS and to hypersensitivity against oxidative stress independent of the respiratory competence of the yeast strains (clonogenic survival assays) (Skoneczna et al., 2007). The cellular levels of Hsp31 were increased upon stress, dependent on the oxidative stress response transcription factor Yap1 (Skoneczna et al., 2007). These data suggest that Hsp31 is an oxidative stress-induced chaperone that ameliorates cytotoxicity elicited by elevated levels of ROS. Although Hsp31 expression did not relieve Lrrk2-triggered cytotoxicity in yeast (growth assays) (Xiong et al., 2010), it is of interest whether Hsp31 is able to modulate cytotoxicity elicited by α-synuclein expression.

Yeast PD models may be used to elucidate the role of diverse mitochondrial quality control mechanisms in modulating cytotoxicity and cell death

Mitochondrial dysfunction is a common mechanism underlying the pathogenesis of both sporadic and familiar forms of PD (Correia et al., 2012). In PD patients and animal PD models, activities of complex I of the mitochondrial respiratory chain were significantly decreased (Correia et al., 2012). The importance of complex I deficiency can be estimated by the fact, that pharmaceutical treatment of animal models and humans with complex I inhibitors, including MPTP and rotenone, was sufficient for the loss of dopaminergic neurons, and consequently elicited clinical symptoms typical for PD (Correia et al., 2012). *S. cerevisiae* cannot be used as model for analyzing complex I dysfunction, because it lacks a complex I ortholog (Li et al., 2006). Instead three enzymes, one internal NADH dehydrogenase (Ndi1), and two external NADH dehydrogenases (Nde1 and Nde2), accomplish the function of mammalian complex I (Li et al., 2006). Notably, yeast Ndi1 can fully complement complex I deficiency in mammalian cells, and therefore Ndi1 expression has been suggested to cure complex I dysfunction in PD patients (Marella et al., 2009).

Consistent to the mentioned complex I deficiency, mtDNA encoded defects, increased levels of ROS, and a decrease in the mitochondrial membrane potential could be observed in PD patients, animal and cell culture PD models (Correia et al., 2012). The observed increased susceptibility for mitochondrial permeabilization points to mitochondrion-specific cell death (Correia et al., 2012). In yeast cells expressing α-synuclein, increased levels of mitochondrially produced ROS (Flower et al., 2005; Büttner et al., 2008; Su et al., 2010), decreased mitochondrial membrane potential (Lee et al., 2008), and release of Cyt. *c* into the cytosol were observed (Flower et al., 2005). These data suggest that aberrant mitochondrial respiratory chain activities lead to ROS production and mitochondrial permeabilization in yeast, i.e., similar events that occur in PD patients.

Increased mitochondrial degradation via mitophagy was observed in PD patients and animal PD models (De Castro et al., 2011). Notably, the PD genes encoding PINK1 and parkin promote mitophagy of damaged mitochondria (Geisler et al., 2010; Springer and Kahle, 2011; Jin and Youle, 2012). PINK1 is a mitochondrial kinase, which serves as a sensor of damaged and depolarized mitochondria. In healthy mitochondria, PINK1 is imported into the organelles, whereas in depolarized mitochondria it rapidly accumulates at the mitochondrial outer membrane with its kinase domain facing to the cytosol. Here, it recruits the E3 ligase parkin, which ubiquitylates the mitochondrial fusion proteins mitofusin 1 and 2, initiating mitochondrial fragmentation and mitophagy. Since PD-associated mutations of PINK1 and parkin interrupted this pathway, these data suggest that in PD the accumulation of damaged mitochondria due to an impairment of mitophagy contributes to PD pathogenesis (Geisler et al., 2010). This model is underlined by the fact that PD-associated mutations in the PD gene encoding ATP13A2 also resulted in lysosomal dysfunction, which also impairs autophagic pathways (Dehay et al., 2012).

Whereas in mammalian PD models, mitophagy appears to play a beneficial role, the data observed in yeast PD models suggest for a lethal function of mitophagy: mitophagy is lethal during cell death upon expression of α-synuclein in yeast (Sampaio-Marques et al., 2012), and the expression of Lrrk2 fragments in yeast triggered a marked increase in autophagic vacuoles hinting to a disturbance of autophagic processes (Xiong et al., 2010). Future studies will be needed to address the obvious discrepancy between the data obtained in mammalian and yeast PD models. It is tempting to speculate that, in the first instance, mitophagy plays as *per se* cytoprotective role by removing damaged mitochondria, which can be very harmful for cell survival. However, it is thinkable that upon overload of this pathway, the process of mitophagy may get inefficient and cytotoxic. In an alternative scenario, the efficiency of mitophagy may be too high, and as consequence too many healthy mitochondria are removed from the cell. Yeast cells could tolerate a severe loss of healthy mitochondria, because they can easily switch from respiration to fermentation. However, since neurons essentially depend on high rates of respiration, they would severely suffer from the loss of healthy mitochondria.

HUNTINGTON DISEASE (HD)

HD is an autosomal dominant neurodegenerative disorder characterized by a progressive loss of neurons in the striatum and the cortex with a consequent decline of cognitive and motor functions (Ross and Tabrizi, 2011). HD is caused by an abnormal polyglutamine (polyQ) expansion in the protein huntingtin due to an aberrant CAG codon expansion in the exon 1 of the gene encoding huntingtin (Ross and Tabrizi, 2011). This results in an aggregation-prone protein eventually triggering cytotoxicity and neuronal cell loss (Ross and Tabrizi, 2011). Increasing the length of the polyQ expansion accelerates aggregation of huntingtin and strictly correlates with the increase in cytotoxicity and

the decrease in disease onset (Ross and Tabrizi, 2011). In order to dissect underlying mechanisms, various disease HD models have been established, comprising transgenic HD mouse, mammalian cell culture, and yeast (Bossy-Wetzel et al., 2008; Mason and Giorgini, 2011; Ross and Tabrizi, 2011; Correia et al., 2012).

Expression of disease-associated huntingtin in yeast results in cytotoxicity, cell death, and critical mitochondrial damage

Yeast HD models expressing GFP-fusion constructs of exon 1 of human huntingtin comprising various lengths of polyQ expansion were generated (Meriin et al., 2002; Duennwald et al., 2006; Solans et al., 2006; Ocampo et al., 2010). PolyQ constructs encoding 103 glutamine residues (103Q) efficiently triggered growth deficits and apoptotic cell death (Sokolov et al., 2006; Solans et al., 2006; Ocampo et al., 2010) (**Table 1**). In contrast, polyQ constructs encoding 25 glutamine residues (25Q) remained nontoxic, and the constructs with intermediate polyQ lengths showed intermediate cytotoxicity (Sokolov et al., 2006; Solans et al., 2006; Ocampo et al., 2010). Upon 103Q expression, the mitochondrial network disrupted, the mitochondrial membrane potential dissipated, the mitochondrial protein synthesis efficiency decreased, correlated with markedly reduced respiratory rates and defective energetic coupling (Solans et al., 2006; Ocampo et al., 2010). The defects in respiratory capacity were a result of impaired activities and decreased steady-state levels of the respiratory chain complexes II and III (Solans et al., 2006). As consequence of the impairment of the respiratory chain increased ROS levels were observed (Sokolov et al., 2006; Solans et al., 2006). Treatment with the antioxidants α-tocopherol and resveratrol relieved the level of mitochondrial fragmentation and reduced respiratory impairment, but failed to decrease cytotoxicity (growth assays) or increase cell survival (Sokolov et al., 2006; Solans et al., 2006). These data suggest that ROS are not essential for cell death but may accelerate cytotoxicity in a vicious cycle by damaging the iron-sulfur clusters of the respiratory chain, which are very susceptible for oxidative damage, culminating in the production of increased ROS levels (Solans et al., 2006). Mitochondria isolated from yeast cultures expressing polyQ (103Q) demonstrated altered osmotic properties and facilitated mitochondrial permeabilization (Ocampo et al., 2010), which is characteristic for mitochondrion-dependent cell death in yeast and mammalian cells (Galluzzi et al., 2009; Guaragnella et al., 2012). Thus, critical mitochondrial damage upon polyQ expression in yeast induces mitochondrion-dependent cell death.

Critical mitochondrial damage in yeast HD models is due to direct and indirect mechanisms

Some polyQ aggregates directly interacted with mitochondria (Solans et al., 2006) and polyQ domains localized to the mitochondrial outer membrane (Ocampo et al., 2010). PolyQ aggregates sequestered the mitochondrial proteins Ilv5, which is involved in mtDNA maintenance, Atp2, which is a component of complex V of the mitochondrial respiratory chain, and Ptk1, a mitochondrial protein kinase (Wang et al., 2009). These data suggest that at least part of polyQ-triggered cytotoxicity is due to a direct damage of mitochondria (Mason and Giorgini, 2011). In addition, polyQ-triggered cytotoxicity could impair mitochondria in more indirect pathways. For instance, polyQ expression resulted in destabilization of the actin cytoskeleton (Solans et al., 2006), which is in yeast essential for mitochondrial motility and the maintenance of the mitochondrial network, and therefore is pivotally linked with mitochondrial function (Frederick and Shaw, 2007). PolyQ expression also drastically impaired the ubiquitin-dependent ER-associated protein degradation pathway (ERAD) by sequestering the pivotal ERAD components Cdc48 and Npl4 (Duennwald and Lindquist, 2008). This resulted in the induction of the unfolded protein response and ER stress (Duennwald and Lindquist, 2008). Since ER and mitochondria are physically and functionally linked, *e.g.*, the ER is pivotally involved in the turnover of phospholipids to and from the mitochondrial membranes (Fujimoto and Hayashi, 2011), ERAD dysfunction due to polyQ expression may indirectly result in the damage of mitochondrial membranes (Braun and Zischka, 2008). Recently, a new ubiquitin-dependent protein quality control mechanism on the mitochondrial outer membrane was identified (Heo et al., 2010; Taylor and Rutter, 2011). This so-called mitochondrion-associated protein degradation pathway (MAD) shares the components Cdc48 and Npl4 with the ERAD pathway (Heo et al., 2010; Taylor and Rutter, 2011). Therefore, it is tempting to speculate that polyQ expression also impairs MAD, culminating in a more direct damage of the mitochondrial outer membrane.

Stimulation of mitochondrial biogenesis and repair pathways can cure critical mitochondrial damage and cytotoxicity in yeast HD models

Overexpression of *HAP4*, encoding the catalytic subunit of the transcriptional activator complex triggering mitochondrial biogenesis, reduced growth deficits upon polyQ expression, ameliorated the cellular respiratory defects, restored mitochondrial membrane potential, and increased mitochondrial protein translation efficiency (Ocampo et al., 2010). Expression of polyQ in three different knock-out strains, which relieved polyQ-triggered cytotoxicity (growth assays), resulted in the induction of the gene encoding D-lactate dehydrogenase (Dld3) (Tauber et al., 2011). In contrast, expression of polyQ in a yeast strain deleted for the peroxisomal citrate synthase (Cit2) dramatically increased polyQ-triggered cytotoxicity (growth assays) (Willingham et al., 2003). Both Dld3 and Cit2 are induced upon mitochondrial damage and are part of the retrograde response, which is a cellular defense mechanism to counteract mitochondrial damage (Liu and Butow, 2006). Therefore, the mitochondrial retrograde response and mitochondrial biogenesis could play protective roles in relieving mitochondrial damage and polyQ-triggered cytotoxicity.

Mitochondrion-associated metabolites contribute to cytotoxicity in yeast HD models

A genome-wide loss-of-function suppressor screen, in which gene deletions were identified, that suppress cytotoxicity of polyQ (growth assays), resulted in the identification of another mitochondrion-associated pathway, modulating polyQ-triggered cytotoxicity (Giorgini et al., 2005). Deletion of the *BNA4* gene, encoding the mitochondrial enzyme kynurenine

3-monooxygenase, involved in the kynurenine pathway of tryptophan degradation and NAD^+ synthesis, resulted in the most potent suppression of polyQ-triggered cytotoxicity (growth assays) (Giorgini et al., 2005). Whereas *bna4*Δ cells lacked the intermediates 3-hydroxykynurenine and quinolinic acid of the kynurenine pathway, wild-type cells expressing polyQ accumulated these metabolites and induced expression of enzymes of the kynurenine pathway (Giorgini et al., 2005, 2008; Tauber et al., 2011). Treatment of polyQ-expressing yeast cells with an inhibitor of Bna4 reduced the levels of the metabolites and reduced polyQ-triggered cytotoxicity (growth assays) and ROS production (Giorgini et al., 2005). Consistently, 15 of the 28 identified yeast knock-out strains, which suppressed polyQ-triggered cytotoxicity (growth assays), demonstrated significantly decreased levels in 3-hydroxykynurenine and quinolinic acid (Giorgini et al., 2008). These data indicate that accumulation of these metabolites contributes to polyQ-triggered cytotoxicity (Mason and Giorgini, 2011). However, how accumulation of these metabolites triggers mitochondrial damage remains unknown.

Yeast HD models recapitulate mitochondrial respiratory and metabolic defects

Expression of disease-associated huntingtin is associated with critical mitochondrial dysfunction in HD patients, in transgenic murine and mammalian cell culture HD models (Bossy-Wetzel et al., 2008; Ross and Tabrizi, 2011; Correia et al., 2012). Bioenergetic defects, impaired activity of the respiratory chain complexes II and III, depolarization of mitochondrial membrane potential, fragmentation of the mitochondrial network, high levels of oxidative stress, and accumulation of the intermediates 3-hydroxykynurenine and quinolinic acid of the kynurenine pathway are characteristic for the progression of HD (Reynolds and Pearson, 1989; Bossy-Wetzel et al., 2008; Ross and Tabrizi, 2011; Correia et al., 2012). Since these hallmarks could also be identified in yeast HD models, yeast appears to be an appropriate model organism to elucidate pathological mechanisms on mitochondria upon polyQ-triggered cytotoxicity. Further key findings in yeast are that the stimulation of mitochondrial biogenesis and repair pathways and genetic and pharmacological manipulation of the kynurenine pathway can markedly relieve cytotoxicity upon polyQ expression. Indeed, transcriptional regulation of the kynurenine pathway and its role in modulating cytotoxicity upon polyQ expression is very similar in yeast and in transgenic HD mouse models (Giorgini et al., 2008; Campesan et al., 2011; Zwilling et al., 2011). Therefore, yeast may facilitate the discovery of pathological pathways, which can be exploited for new therapeutic approaches for HD treatment.

In yeast HD models, mitochondrion-dependent molecular cell death could be discriminated between Cyt. *c*-, apoptosis-inducing factor-, and endonuclease G-dependent or alternative scenarios. Further, yeast HD models could be applied for elucidating the role of mitochondrial fusion, fission, morphology and motility, and for describing the maintenance of mtDNA. Mitochondrial permabilization concomitant with mitochondrion-dependent cell death and damage of mtDNA are hallmarks of HD (Bossy-Wetzel et al., 2008; Correia et al., 2012). Inhibiting mitochondrial fission and promoting mitochondrial fusion has recently been described to relieve polyQ-triggered cytotoxicity in mammalian cells (Song et al., 2011). Therefore, a more detailed analysis in yeast HD models could be helpful for better understanding the roles of these conserved pathways in HD.

Autophagy is involved in the degradation of huntingtin aggregates (Qin et al., 2003), and mitophagy has been described to occur in immortalized cell lines from HD patients (Mormone et al., 2006). Different huntingtin domains demonstrate homology to the yeast autophagy proteins Atg23, Vac8, and Atg11, raising the question whether huntingtin itself is involved in autophagy/mitophagy regulation (Steffan, 2010). Therefore, analyzing the role of mitophagy in modulating polyQ-triggered cytotoxicity in yeast is also of high interest.

AMYOTROPHIC LATERAL SCLEROSIS (ALS)

ALS is a frequent degenerative motor neuron disease characterized by adult-onset loss of the lower and upper motor neuron systems, resulting in muscle weakness and wasting (Andersen and Al-Chalabi, 2011). Mutations in 13 different genes are causative for hereditary forms of ALS (Andersen and Al-Chalabi, 2011; Wu et al., 2012), and even more genes are putatively associated with ALS (Andersen and Al-Chalabi, 2011). The most common ALS-associated genes are *SOD1*, *TARDBP*, and *FUS*, encoding the ROS-scavenger enzyme superoxide dismutase 1 (SOD1), and the RNA-binding proteins TDP-43 and FUS/TLS (Andersen and Al-Chalabi, 2011). ALS-associated variants of these proteins demonstrate mislocalization and/or a high tendency for aggregation (Andersen and Al-Chalabi, 2011; Da Cruz and Cleveland, 2011). Yeast models expressing ALS-associated wild-type and mutant SOD1, TDP-43, and FUS/TLS have been established to further analyze the detrimental roles of these proteins on cell survival (Bastow et al., 2011).

ALS-associated mutant Sod1 variants demonstrate increased mitochondrial localization and impair mitochondrial respiratory chain activity

Yeast cells lacking *SOD1*, the ortholog of human *SOD1*, are sensitive against oxidative stress (growth assays), highlighting the role of Sod1 as superoxide scavenger (Bastow et al., 2011). Sod1 is also involved in the assembly of iron-sulfur clusters, which are important for enzymes of the respiratory chain, the TCA cycle, and amino acid biosynthetic pathways (Strain et al., 1998; Wallace et al., 2004; Sehati et al., 2011). Sod1 demonstrates dual localization between the cytosol, where the vast majority of Sod1 is localized, and the mitochondrial intermembrane space (Klöppel et al., 2010). Surprisingly, expression of ALS-associated mutant (G93A) Sod1 in yeast resulted in an increased mitochondrial localization and an increased tolerance against mitochondrially produced ROS (Klöppel et al., 2010) (**Table 1**). Notably, a decrease in electron transport in the mitochondrial respiratory chain was observed upon expression of ALS-associated mutant (G93A) Sod1 (Gunther et al., 2004). Therefore, it is tempting to speculate that increased mitochondrial localization of Sod1 disturbs the mitochondrial respiratory chain, and that this mechanism contributes to cell loss (Bastow et al., 2011). Both activation and mitochondrial localization of Sod1 depend on the copper chaperone Ccs1, which demonstrates

high sequence identity with human SOD1 (Leitch et al., 2009; Groß et al., 2011; Klöppel et al., 2011). ALS-associated mutant Sod1 variants were rapidly degraded in yeast cells lacking *CCS1* (Carroll et al., 2006), underlining the assumption that their mitochondrial localization is important for their detrimental action.

Expression of TDP-43 and FUS/TLS are cytotoxic in yeast, and mitochondrial respiratory capacity plays a pivotal role in modulating TDP-43-triggered cytotoxicity

Expression of wild-type and disease-associated mutant variants of TDP-43 and FUS/TLS triggered cytoplasmic protein aggregation leading to growth deficits, loss of clonogenic survival, and apoptosis and necrosis in yeast (Johnson et al., 2008, 2009; Elden et al., 2010; Armakola et al., 2011; Braun et al., 2011; Couthouis et al., 2011; Fushimi et al., 2011; Ju et al., 2011; Kryndushkin et al., 2011; Robinson, 2011; Sun et al., 2011) (**Table 1**). TDP-43-expressing yeast cells showed an age-dependent loss of clonogenic survival concomitant to ROS accumulation (Johnson et al., 2008; Braun et al., 2011). Mitochondria play a pivotal role in executing TDP-43-triggered cell death (clonogenic survival assays): (1) Cytotoxicity was significantly decreased in cells lacking mtDNA and functional mitochondria, (2) impairment of the respiratory chain relieved cytotoxicity with a stringent correlation between cytotoxicity and the degree of respiratory capacity or mtDNA stability, and (3) increasing the respiratory capacity enhanced TDP-43-triggered ROS production and cell death (Braun et al., 2011). Mitochondrion-dependent cell death was independent of the mitochondrial cell death proteins apoptosis-inducing factor, endonuclease G, and Cyt. *c*, suggesting that an alternative mitochondrion-dependent cell death pathway may be active (Braun et al., 2011).

Whether FUS/TLS-triggered cytotoxicity is also modulated by mitochondrial function is still an open question. However, in a genome-wide screen to identify modulators of FUS/TLS-triggered cytotoxicity (growth assays) upon overexpression or deletion, several mitochondrion-associated candidate genes were identified (Sun et al., 2011). Yeast strains deleted for genes encoding components of the mitochondrial protein translation machinery, the mitochondrial respiratory chain, and the TCA cycle increased FUS/TLS-triggered cytotoxicity (growth assays) (Sun et al., 2011). These data suggest that impaired mitochondria upon deletion of these genes are more vulnerable against FUS/TLS expression, and raise the possibility that mitochondrial damage contributes to the execution of FUS/TLS-triggered cytotoxicity.

Yeast ALS models may support and complement hypotheses obtained in higher model systems

Structural mitochondrial abnormalities as seen in electron microscopy and markers of oxidative stress were observed in ALS patients, and in mouse models expressing ALS-associated mutant variants of SOD1 and TDP-43 (Martin, 2011; Cozzolino et al., 2012). In ALS patients with SOD1 etiology Cyt. *c* release correlated with apoptosis, and in mouse ALS models for SOD1-associated ALS, mitochondrial permeabilization correlated with necrosis (Martin, 2011). These data hint to the induction of mitochondrion-specific cell death during ALS. In yeast, expression of TDP-43 triggered oxidative stress, and apoptosis and necrosis, and mitochondrial respiration was found to be an important modulator of cytotoxicity (clonogenic survival assays) (Braun et al., 2011). Thus, the determination and critical comparison of cell death pathways in yeast cells expressing ALS-associated wild-type and mutant SOD1, TDP-43, and FUS/TLS may help to elucidate common and distinct mechanisms underlying cytotoxicity and mitochondrion-dependent cell death with potential relevance for ALS.

Abnormally aggregated mitochondria, as seen in mouse models for TDP-43 and FUS/TLS-mediated ALS, suggest for deficits in mitochondrial trafficking in motor neurons, a phenomenon which has already been proven in mouse models expressing ALS-associated mutant SOD1 (Martin, 2011; Cozzolino et al., 2012). Expression of ALS-associated mutant SOD1, TDP-43, and FUS/TLS triggered mitochondrial fragmentation in mouse motor neurons, suggesting a further role of the mitochondrial fusion/fission balance in modulating pathogenesis (Magrane et al., 2009, 2012; Xu et al., 2010, 2011; Cozzolino et al., 2012; Tradewell et al., 2012). The mechanisms of mitochondrial fusion/fission are conserved from humans to yeast (Westermann, 2010). Therefore, yeast ALS models may help to dissect components of the mitochondrial fusion/fission machinery, which pivotally contribute to the modulation of cytotoxicity. In contrast, for analyzing the role of mitochondrial trafficking in ALS pathology, yeast ALS models might be of limited use, because the mitochondrial trafficking in yeast depends on the actin cytoskeleton, whereas neuronal mitochondria are transported along the microtubule cytoskeleton (Frederick and Shaw, 2007).

ALS-associated mutant SOD1 demonstrated increased mitochondrial association, which is correlated with elevated ROS production and pathology (Martin, 2011; Cozzolino et al., 2012). In contrast, mitochondrial aggregates in mouse models expressing TDP-43 and FUS/TLS did not contain the disease-causing proteins, suggesting that the detrimental effects of these proteins for mitochondria are rather indirect (Xu et al., 2010; Cozzolino et al., 2012). Consistent to the observations in mouse models, ALS-associated mutant (G93A) SOD1 demonstrated increased localization in the mitochondrial intermembrane space in yeast (Klöppel et al., 2010), with potential harmful effects for mitochondrial respiratory chain efficiency (Gunther et al., 2004). Due to the extensive knowledge on the regulation of mitochondrial import of SOD1 in yeast (Leitch et al., 2009; Groß et al., 2011; Klöppel et al., 2011), yeast might be a good model to elucidate molecular components and pathways involved in SOD1 pathology. In yeast, TDP-43 aggregates demonstrated a peri-mitochondrial localization, although a strong interaction between TDP-43 and mitochondria could not be observed (Braun et al., 2011). These data suggest that in yeast, as in humans, indirect effects may contribute to the pivotal mitochondrial damage. The elucidation of these mechanisms of mitochondrial damage will be one interesting future task.

OTHER NEURODEGENERATIVE DISORDERS

Polyalanine (polyA) disorders are associated with trinucleotide repeat expansions in genes involved in developmental processes, including central nervous system development (*ZIC2*), neural development (*SOX3*), and cerebral development and patterning

(*ARX*) (Messaed and Rouleau, 2009). The trinucleotide repeat expansions encode abnormal polyA expansions causing protein aggregation and neuronal loss, and therewith triggering neurodegenerative diseases, such as holoprosencephaly, X-linked hypopituitarism, and X-linked mental retardation (Messaed and Rouleau, 2009). Recently, a yeast model for polyA-triggered cytotoxicity has been established (Konopka et al., 2011). With increasing length of the polyA stretches, cytotoxicity markedly increased, as measured by growth deficits and decreased plasma membrane integrities (Konopka et al., 2011). Whether mitochondria are affected and pivotally involved in polyA-triggered cytotoxicity in yeast remains unknown. However, mitochondrial dysfunction, the formation of the mitochondrial permeability transition pore, Cyt. *c* release, and the translocation of pro-apoptotic BAX to mitochondria have been observed in mammalian polyA models (Toriumi et al., 2008, 2009; Bhattacharjee et al., 2012). Therefore, it might be a fruitful approach to dissect the role of mitochondria in polyA-triggered cytotoxicity in yeast.

Prion disorders are fatal neurodegenerative conditions characterized by the conversion of the soluble cellular protein PrP^{C} into its insoluble and infectious prion form PrP^{Sc} (Imran and Mahmood, 2011). For instance in Creutzfeld-Jakob disease, PrP^{Sc} accumulation results in neuronal loss causing rapid progressive dementia and cerebellar dysfunction (Imran and Mahmood, 2011). Yeast models have been established expressing mammalian PrP^{C} (Li and Harris, 2005; Bounhar et al., 2006; Halfmann et al., 2010; Josse et al., 2012). PrP^{C} expression was not cytotoxic for yeast cells (Li and Harris, 2005; Bounhar et al., 2006). In contrast, PrP^{C} expression suppressed yeast cytotoxicity triggered by the expression of the mammalian pro-apoptotic BAX, as measured by growth assays, clonogenic survival assays, and assays measuring metabolic activities (Li and Harris, 2005; Bounhar et al., 2006). Consistently, PrP^{C} expression prevented BAX-induced cell death in primary neuronal cultures (Bounhar et al., 2001). Since BAX triggers mitochondrion-dependent cell death in mammalian cells and in yeast (Khoury and Greenwood, 2008), these data suggest that PrP^{C} plays a protective role in preventing mitochondria from executing cell death (Li and Harris, 2005; Bounhar et al., 2006).

CONCLUSIONS AND OUTLOOK

Mitochondrial damage and dysfunction are hallmarks of AD, PD, HD, and ALS (Martin, 2011; Correia et al., 2012; Cozzolino et al., 2012). Yeast models for the elucidation of mechanisms of cytotoxicity and cell death upon expression of AD-, PD-, HD-, and ALS-associated proteins have been established (Gitler, 2008; Miller-Fleming et al., 2008; Winderickx et al., 2008; Bharadwaj et al., 2010; Braun et al., 2010; Khurana and Lindquist, 2010; Bastow et al., 2011; De Vos et al., 2011; Mason and Giorgini, 2011). In these models mitochondrial (dys)function contributes to cytotoxicity. The description of mitochondrial damage, the elucidation of mitochondrion-specific cell death pathways, and the dissection of the roles of mitochondrial quality control mechanisms, including mitochondrial dynamics and degradation, has just initiated in these yeast models. Therefore, neurotoxic yeast models will elucidate novel paradigms of mitochondrial pathobiology, which can be easily validated in other model organisms for neurodegenerative disorders.

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Untangling the roles of anti-apoptosis in regulating programmed cell death using humanized yeast cells

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Genetically programmed cell death (PCD) mechanisms, including apoptosis, are important for the survival of metazoans since it allows, among things, the removal of damaged cells that interfere with normal function. Cell death due to PCD is observed in normal processes such as aging and in a number of pathophysiologies including hypoxia (common causes of heart attacks and strokes) and subsequent tissue reperfusion. Conversely, the loss of normal apoptotic responses is associated with the development of tumors. So far, limited success in preventing unwanted PCD has been reported with current therapeutic approaches despite the fact that inhibitors of key apoptotic inducers such as caspases have been developed. Alternative approaches have focused on mimicking anti-apoptotic processes observed in cells displaying increased resistance to apoptotic stimuli. Hormesis and pre-conditioning are commonly observed cellular strategies where sub-lethal levels of pro-apoptotic stimuli lead to increased resistance to higher or lethal levels of stress. Increased expression of anti-apoptotic sequences is a common mechanism mediating these protective effects. The relevance of the latter observation is exemplified by the observation that transgenic mice overexpressing anti-apoptotic genes show significant reductions in tissue damage following ischemia. Thus strategies aimed at increasing the levels of anti-apoptotic proteins, using gene therapy or cell penetrating recombinant proteins are being evaluated as novel therapeutics to decrease cell death following acute periods of cell death inducing stress. In spite of its functional and therapeutic importance, more is known regarding the processes involved in apoptosis than anti-apoptosis. The genetically tractable yeast *Saccharomyces cerevisiae* has emerged as an exceptional model to study multiple aspects of PCD including the mitochondrial mediated apoptosis observed in metazoans. To increase our knowledge of the process of anti-apoptosis, we screened a human heart cDNA expression library in yeast cells undergoing PCD due to the conditional expression of a mammalian pro-apoptotic Bax cDNA. Analysis of the multiple Bax suppressors identified revealed several previously known as well as a large number of clones representing potential novel anti-apoptotic sequences. The focus of this review is to report on recent achievements in the use of humanized yeast in genetic screens to identify novel stress-induced PCD suppressors, supporting the use of yeast as a unicellular model organism to elucidate anti-apoptotic and cell survival mechanisms.

Keywords: heart failure, apoptosis, programmed cell death, anti-apoptotic genes, Bax, genetic screen, pre-condition, hormesis

INTRODUCTION

Getting rid of unwanted or potentially damaging cells is critical for the normal functioning of metazoans (Wyllie, 2010; Portt et al., 2011; Ulukaya et al., 2011). More recently, genetically encoded mechanisms have been discovered to be of equal importance in regulating cell death and cell survival in all eukaryotes including single cell microbes such as the yeast *Saccharomyces cerevisiae* (Carmona-Gutierrez and Madeo, 2009; Shemarova, 2010; Kaczanowski et al., 2011). This has led to dramatic changes in how programmed cell death (PCD) is perceived and it has opened up a number of important avenues of research that allows genetic approaches to the study of death inducing and cell survival strategies. It has long been established that the functional expression of human genes in yeast has facilitated the study of individual members of complex gene family in an isolated but functional cell system. The use of humanized yeast cells for the study of apoptosis started many years ago when key regulators of mammalian apoptosis were found to retain their pro- and anti-apoptotic functions when expressed in yeast (Manon et al., 1997; Ligr et al., 1998; Lisa-Santamaria et al., 2009). Today humanized yeast cells

are commonly used as a means of identifying and characterizing novel apoptotic regulators and processes (Greenwood and Ludovico, 2010; Silva et al., 2011a). As a prelude to a detailed discussion of the use of humanized yeast, it is necessary to introduce a few related topics. A general discussion will begin with the fact that although a multitude of specialized sub-forms have been described there are three main types of PCDs that receive the most attention (Hotchkiss et al., 2009; Orrenius et al., 2011; Portt et al., 2011; Galluzzi et al., 2012b).

Type I PCD or apoptosis has long been recognized as containing two distinct types called the extrinsic and intrinsic forms (Kroemer et al., 2009; Wyllie, 2010). The extrinsic form is largely due to the activation of cell surface death receptors such as TNFα and is more studied in the context of the immune cells. The intrinsic form is centered on the mitochondria and is activated by a variety of stresses including a number of chemicals (pesticides, cancer therapeutics), physical agents (high osmolarity, change in temperature or pH), and intracellular stresses such as DNA damage and accumulation of misfolded proteins especially in the endoplasmic reticulum (ER; Carmona-Gutierrez et al., 2010; Orrenius et al., 2011). These stresses lead to the activation of intracellular pathways and processes that cause alterations in mitochondrial membrane permeability and the release of pro-apoptogenic factors including cytochrome c, AIF, and Endo G. The effects of cytochrome c are mediated by its ability to form an active apoptosome complex with the Apoptosis Protease Activating Factor 1 (APAF-1) that serves to activate procaspase 9. This in turn leads to cleavage mediated activation of executioner caspases such as caspase 3. There are number of other interrelated pathways associated with the intrinsic pathway which involves a large variety of pro-apoptotic proteins. Many of these stresses can be shown to induce cell death when overexpressed and to reduce stress mediated cell death when their genes are knocked out or down regulated by siRNA based strategies. The reader is referred to a number of recent reviews for more detailed accounts of these processes (Orrenius et al., 2011; Shamas-Din et al., 2011; Ulukaya et al., 2011; Galluzzi et al., 2012b).

AUTOPHAGY

Type II PCD or autophagic cell death may not be more complex, but at the moment, it certainly is a lot more confusing (Denton et al., 2012; Galluzzi et al., 2012a,b; Shen et al., 2012). This is because autophagy (Greek, self-eating) is in itself a cellular process that serves to protect the cell from stress (Moreau et al., 2009). Although there are multiple different forms, the most understood form is called macro-autophagy (He and Klionsky, 2009). Here the autophagic machinery is activated in response to nutritional stress where it earmarks cellular constituents that are expendable (i.e., material required for growth). These are then broken down and serve as building blocks for the synthesis of molecules and the expression of genes that can serve to prevent premature cell death. The autophagic machinery gets activated in the absence of nutrient by a well studied complex process involving a variety of regulatory proteins including TOR (Loewith and Hall, 2011). The ability to genetically identify a large number of autophagic (*ATG*) genes in yeast was instrumental in developing our understanding of autophagy (He and Klionsky, 2009). The availability of mutants lacking ATG genes due to knock out or knock down have shown that the process of autophagy is critical for cellular survival in response to a variety of stresses including amino acid and glucose starvation as well chemical inducers of apoptosis (Pan et al., 2009). Other forms of autophagy that carry out specialized functions such as the selective removal of certain cellular constituents including the specific removal of damaged mitochondria by mitophagy may also be critical for cellular survival under some conditions (Kissova and Camougrand, 2010). On the other hand low level removal of damaged cellular material by constitutive autophagy is thought to have housekeeping functions that are required to maintain proper order in a cell (Gottlieb et al., 2009; Marino et al., 2011).

In contrast to the large body of knowledge about the protective effects and processes associated with the pro-survival functions of autophagy (see also below) even the bare essential framework for autophagic cell death is lacking (Chen and Klionsky, 2011). Autophagic cell death was originally defined as a form of cell death that is associated with autophagosomes that is likely non-apoptotic (caspase, Bax independent; Marino et al., 2011; Meschini et al., 2011). Autophagosomes are intracellular vesicles that are the site of the autophagic cargo destined for degradation that consist of the membrane engulfed cargo fused to lysosomes. The connection between autophagy and autophagic cell death still remains obscure. In fact there is quite a strong and growing conviction by many that autophagic cell death would be better labeled as cell death associated with autophagosomes (Denton et al., 2012; Shen et al., 2012). Thus autophagy is often labeled as an "innocent bystander" in the process of an ongoing death (Rami, 2009). It is argued that conditions that serve to initiate cell death, will lead to stress mediated activation of autophagy as a sort of cellular defense mechanism (Schleicher et al., 2010). The increased autophagy is thus a way for the cell to try and mitigate and resist undergoing inappropriate cell death (Meschini et al., 2011). The up-regulation of such cellular survival processes have been identified in response to numerous stresses (Fulda et al., 2010; Portt et al., 2011). The inability to prevent cell deaths that are thought to be occurring by autophagy, by inhibiting the autophagic process is regarded as strong evidence that autophagic cell death is likely over diagnosed (Galluzzi et al., 2012b; Shen et al., 2012). Strong guidelines or recommendations by a number of leaders in the field have been proposed to try and demystify the process (Galluzzi et al., 2012b). It nevertheless remains technically challenging to identify cell death by autophagy. This is partially due to the fact that small chemical inhibitors of autophagy used are non-specific. The ability to develop specific chemical inhibitors or genes encoding negative regulators, as was recently done for necrosis, would be required to clearly identify and characterize autophagic death (Wu et al., 2012).

Nevertheless, autophagic cell death has been acknowledged to exist, even by the most ardent anti-autophagic death crowd, under certain circumstances, and in some organisms (Shen et al., 2012). For example apoptotic inducing stimuli leads to autophagic cell death in mouse embryonic fibroblasts (MEFs) cells that are unable to carry out apoptosis due to knock outs of both pro-apoptotic Bcl-2 family members, Bax and Bak (Shimizu et al., 2004). Others have argued that autophagic cell death may simply be a sort of death of last resort that occurs after prolonged autophagy where

the cell simply becomes exhausted and has no more resources to combat prolonged stresses (Rami, 2009). This could occur by a process of autophagosome lysis and subsequent death by the released catalytically active enzymes in a process analogous to lysosomal rupture that occurs in some forms of ER stress or necrosis (Orrenius et al., 2011). The application of proteomic approaches to the study of autophagy may be useful in further delineating the role of autophagy in cell death (Dengjel et al., 2012).

NECROSIS

Type III PCD is also known as necrosis. This cell death differs from apoptosis in a number of readily detectable key features including several biochemical, cellular, and morphological differences (Berghe et al., 2010; Eisenberg et al., 2010; Fulda et al., 2010; Galluzzi et al., 2012b). The early loss of membrane integrity observed in necrotic cells coupled to the fact that membrane externalization occurs in apoptosis allows the use to use fluorescently labeled nuclear vital dyes and differentially labeled protein capable of binding inner membranes (annexin V) to discriminate between these forms of death. Also key among the differences is the blebbing of apoptotic cells followed by the engulfment of the debris by immune cells. Necrotic cells on other hand remain within the tissue and lead to inflammation as they decay. Necrosis is the form of cell death that occurs in response to severe catastrophic stress that leads to deleterious and irreversible cellular injury. More recent studies suggest that there is a genetically programmed form of necrosis, called necroptosis, which can be triggered by severe stresses including some forms of DNA damage (Weinlich et al., 2011; Wu et al., 2012). A biochemical pathway involving the death type receptor (i.e., TNF-α) mediated formation of a necrosome type complex that leads to activation of Receptor Interacting Protein Kinase 1 and 3 (RIPK-1 and 3) can lead to both apoptotic and necrotic cell death (Green et al., 2011). Compounds capable of inhibiting the RIPKs as well as potential intrinsically expressed proteins such as the prepro region of cathepsin D have been identified as inhibitors of the process of necroptosis (Carmona-Gutierrez et al., 2011a). These and other studies suggest that clinical strategies aimed at preventing some forms of necrosis may be possible (Christofferson and Yuan, 2010).

OTHER FORMS OF PCD

Alternative forms of apoptosis have been known for a long time (Orrenius et al., 2011). There are non-traditional forms of PCD that diverge due to a number of differences such as being caspase or Bax independent (Kroemer et al., 2009). Other forms include infections with organisms such as *Salmonella* that initiate a caspase 1 dependent cell death called pyroptosis. A caspase 12 dependent process appears to be involved in certain forms of cell death such as the pro-inflammatory condition that is seen in sepsis. Anoikis refers to the PCD that occurs in response to cell detachment from its neighbors. Increased resistance to this form of cell death may lead to the ability to grow in an anchorage independent manner that is often seen in cancer cells (Sakamoto and Kyprianou, 2010). Many of these PCDs appear to have characteristics of apoptosis such as similar morphological changes indicating that many may simply represent variants on apoptosis. Thus despite differences in triggering the many forms of PCDs, the mitochondria still plays a central role in mediating many of these varied forms of cell death. Even the PCD that occurs in aging, which shows a great deal of dependence on glucose metabolism, appears to be dependent on the mitochondria (Laun et al., 2008). Understanding how a cell integrates all information to come up with the appropriate cell death response will require more knowledge about the regulation of cell death.

CROSS-TALK

All the different forms of PCD are not always stand-alone processes. There is a great deal of reported cross-talk between the different forms of cell death (Amelio et al., 2011; Giansanti et al., 2011; Orrenius et al., 2011; Shen and Codogno, 2012). On the large scale the three types of PCDs can be shown to influence one another such that the process of autophagy can serve to inhibit apoptosis and necrosis while cells unable to undergo apoptosis will undergo autophagy or necrotic cell death (Zhivotovsky and Orrenius, 2010; Shen and Codogno, 2012). The most highly reported of such an example is the early experiment using MEFs cultured from embryos having a double knock out (DKO) of the two genes encoding the pro-apoptotic Bcl-2 proteins Bax and Bak (Shimizu et al., 2004). The DKO embryos are lethal which likely reflects the importance of these two proteins in regulating developmental apoptosis. When challenged with apoptotic stimuli, DKO MEFs appear to undergo a cell death that has the hallmarks not of apoptosis but of autophagic cell death. On the other hand, there are a number of other cases where the inhibition of caspase can effectively prevent apoptotic cell death without sparring the cell's life since it induces necrosis (Rami et al., 2008). Alternatively autophagy may be co-activated with apoptosis as a defense mechanism that can serve to prevent premature execution of apoptosis or necrosis (Amelio et al., 2011; Shen and Codogno, 2012). These and multiple other forms of cross-talk have been observed in a number of different ways including the fact that many individual proteins play roles in more than one cell death modality. For example, the BH3 containing Bcl-2 family member Beclin 1 is a key regulator of autophagy that can be inhibited by Bcl-2 (Kang et al., 2011). Thus Bcl-2 mediated inhibition of Beclin and of Bax allows it to have direct roles in preventing autophagy and apoptosis induction. On the other hand caspase mediated cleavage of Beclin can serve as a mechanism by which the activation of apoptotic machinery can inhibit autophagy (Djavaheri-Mergny et al., 2010). The regulation does not seem to be reciprocal since the overexpression does not appear to inhibit the anti-apoptotic effects of Bcl-2 (Kang et al., 2011). Other well-known examples of proteins with dual functions include FLIP and the autophagic specific gene ATG5 (Giansanti et al., 2011). FLIP is a well-known inhibitor of the extrinsic apoptotic pathway that can also interact with ATG3 and serve as a negative regulator of autophagy (Lee et al., 2009). Less is known about possible cross-talk in unicellular eukaryotes such as yeast. So although the three functional PCDs exist in yeast, there will be a number of strategic differences by which cross-talk between the pathways is used. For example, the *ATG6* gene serves the same autophagic regulatory function as Beclin 1 but the yeast ortholog does not appear to have a BH3 domain.

REGULATION OF PROGRAMMED CELL DEATH

INDUCTION

The most commonly examined form of cell death is the intrinsic form of apoptosis that, as mentioned above, involves the stress mediated release of pro-apoptogenic factors from the mitochondria. This pathway mediates the effects of many of the stresses a cell encounters including a wide range of chemicals and physical agents as well as a myriad of others including intracellular stresses such as DNA damage, ER stress as well as the stimuli that occurs in a large number of pathophysiological conditions (see below). The proportion of cells that undergo apoptosis in a given population of cells is directly related to the intensity of the stress encountered (**Figure 1**; Orrenius et al., 2011; Qi et al., 2012). The intensity of the stress is directly related to a combination of both the dose encountered and the time of exposure to any given stress. For example, keeping the time of exposure constant, we can decrease the percentage of viable cells in a population by increasing the concentration of the stress inducing agent. Thus the average cell population under normal type conditions will respond in a dose dependent manner to a stress and give a standard profile of intensity of stress vs. percentage viability that can be experimentally determined (**Figure 1**). This profile can shifted to the right or to left by respectively increasing or reducing the expression of a number of pro-apoptotic genes (**Figure 1**). Thus the normal response to a given stress can be changed by altering the apoptotic machinery. If the stress is too intense, accidental death will occur since the cells will undergo necrosis.

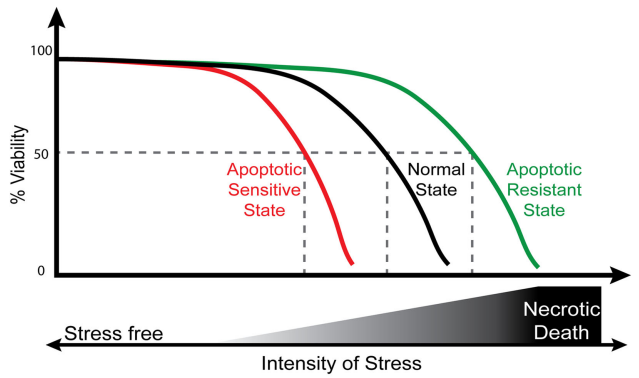

FIGURE 1 | Stress is a dose dependent mediator of cell death. A theoretical and graphical depiction of the effects of increasing the intensity of stress on cellular viability. In a stress free environment cellular viability is maintained close to 100% with only a basal level of cell death. In the normal state (depicted in black), the percentage of cell viability begins to decreases when a specific threshold of stress intensity is encountered. Intensity of stress reflects a combination of time of exposure to the stress and to its intensity. Cell death inducing stresses include a variety of different physical or chemical agents or pathophysiological stresses as discussed in the text. Once the threshold level of stress is encountered, the % viability further decreases in dose dependent manner with respect to the increase in the intensity of stress. In an apoptotic sensitive state (depicted in red), the minimal threshold of the intensity of stress that is required in order to observe a decrease in viability is reduced. Such an apoptotic sensitive state can be observed in cells that are lacking mechanism that are involved in apoptotic resistance such as the loss of an anti-apoptotic resistant gene or a mutation leading to defects in the induction of autophagy. Alternatively, overexpression of a pro-apoptotic gene can lead to a similar phenotype. The opposite phenotype, that is the state of apoptotic resistance (depicted in green), occurs in cells that are lacking some pro-apoptotic genes, that are overexpressing an anti-apoptotic gene or that have increased activation of other pro-survival processes such as autophagy. The net effect of altering a cell's sensitivity to apoptosis can be observed in the different intensity of stress that is required to give rise to half maximal viability (50% viability, depicted by dashed lines). Thus the threshold of sensitivity to death inducing stress is variable and is established by complex regulatory processes that involve both pro- and anti-apoptotic processes. It should be noted that the processes that trigger apoptosis and that serve to induce cell death are not altered by the apoptotic resistant or sensitive states.

The mechanism by which stress leads to the activation of apoptotic response involves the increases in the levels of two well characterized intermediates, activated Bax, and Reactive Oxygen Species (ROS). Stress also results in an increases in the levels of a number of other pro-apoptotic second messengers including calcium, cAMP, the nucleotide dUTP, and the sphingolipid ceramide (Ozbayraktar and Ulgen, 2009; Vertessy and Toth, 2009; Circu and Aw, 2010; Ganesan and Colombini, 2010; Pourova et al., 2010; Insel et al., 2011; Li et al., 2011; Orrenius et al., 2011; Wilson et al., 2012). Increases in the level of these pro-apoptotic second messengers appear to be tightly coordinated and to be synergistic in their effects (Portt et al., 2011; Ray et al., 2012). For example, increasing activated Bax leads to increased ROS and ceramide while increases in ROS and ceramide can serve to activate Bax (Kumar and Jugdutt, 2003; Lecour et al., 2006; Ganesan et al., 2010). Functionally, one can manipulate a decrease in the levels of ROS or ceramide and this can serve to prevent Bax mediated cell death (see also below; Moon et al., 2002; Yang et al., 2006; Separovic et al., 2007). This suggests that stress mediated cell death involves the cooperative response of numerous cellular pathways and that this cross-talk may serve as redundant, additive, or even synergistic processes to induce cell death (Portt et al., 2011). Specific recent examples of additive effects include Bax mediated permeabilization of the outer mitochondrial membrane serving to increase the release of ROS from the mitochondria. Increased levels of sphingolipids such as ceramide in the mitochondrial membrane may in turn serve to facilitate the insertion of Bax into the mitochondria (Ganesan et al., 2010; Chipuk et al., 2012).

NEGATIVE REGULATORS OF PCD

In order to avoid triggering inappropriate PCD, cells have developed a large repertoire of pro-survival tools and strategies that play critical roles in the balance between life and death (Owsianowski et al., 2008; Busca et al., 2009; Fulda et al., 2010; Ashida et al., 2011; Portt et al., 2011; Rodrigues et al., 2012). Anti-apoptotic responses are not simply the inverse of apoptosis instead it is more like a regulatory network that serves to prevent cell death when it is not warranted. As we shall describe in the following few sections, this network also includes and cross-talks extensively with the pro-survival component of autophagy. Of further importance is the fact that this network may be pre-activated in order to create a state of increased resistance to future death inducing stimuli. Examples of the importance of the modulatory nature of apoptotic responses are well illustrated by a recent paper by Shlezinger et al. (2011). They examined the role that apoptosis and anti-apoptosis may play in the ability of the necrotrophic fungus *Botrytis cinerea* to infect plants. Using genetically altered organisms, they were able

to show that plants send out signals to try and induce apoptosis in the invader while an increase in the expression of anti-apoptotic sequences in the fungus increased its ability to infect the plant. Thus the ability to modulate responses to apoptotic stimuli can be a useful tool in organisms with complex life cycles (Rodrigues et al., 2012). Here we will focus on the major regulatory processes that serve to negatively regulate PCD.

ANTI-APOPTOTIC GENES

Anti-apoptotic genes can be defined as sequences that confer an apoptotic resistant state to a cell while its knock out or a knock down of its normal expression levels leads to a apoptotic sensitive state (**Figure 1**). Exceptions may exist especially if the anti-apoptotic gene functions in a parallel pathway to another similar genes or processes. The most common anti-apoptotic gene, Bcl-2, functions at least in part by antagonizing the pro-apoptotic Bcl-2 protein Bax (Khoury and Greenwood, 2008). Other very well characterized anti-apoptotic proteins include the IAPs (Inhibitors of Apoptosis), HSPs (Heat Shock Proteins), and ROS scavengers. IAPs function to inhibit caspase activation; HSPs act as chaperones that help in stabilizing protein structure while ROS scavengers serve in inactivating damage inducing ROS (Beere, 2004; Busca et al., 2009; Circu and Aw, 2010). The functional analysis of new anti-apoptotic genes often serves to reinforce the importance of some apoptotic pathways or they may serve to uncover novel ones. Thus the importance of ROS and misfolded/denatured proteins in the induction of apoptosis from multiple stresses is reinforced by the classical observation that overexpression of genes encoding ROS scavengers and chaperonin type HSPs serve to decrease cell death (Garrido et al., 2003; Kim et al., 2006; Guaragnella et al., 2008). Uncovering unexpected cell survival sequences such as the Metalloprotease 15 (MMP15) suggests a novel role for extracellular matrix or alternative functions to well-known proteins (Abraham et al., 2005). The ability to increase cell survival by increasing the expression of the gene encoding dUTPase supports the emerging consensus that nucleotides such as dUTP serve as stress-induced pro-apoptotic second messengers (Williams et al., 2011; Wilson et al., 2012). On the other hand, the cytoprotective effects of the ceramide utilizing enzyme sphingomyelin synthase is consistent with the long standing role of ceramide as a stress-induced pro-apoptotic second messenger (Yang et al., 2006; Separovic et al., 2007, 2008). The diversity in the function of anti-apoptotic sequences exemplifies the diverse roles played by these sequences.

Sequences capable of specifically preventing other forms of PCD without effecting apoptotic cell death have been reported. This is especially true for the ability of genes capable of preventing necrotic cell death (Weinlich et al., 2011). For example death receptor mediated activation of receptor interacting protein kinases-1 and 3 (RIPK-1 and 3) is one of the early steps in the activation of necroptosis. Proteins like FLIP are capable of inhibiting RIPKs and preventing necroptosis and are thus functionally similar to anti-apoptotic sequences (Green et al., 2011). Other examples of such necroptotic regulators, is the ability of *Pep4* (encoding the yeast ortholog of the mammalian cathepsin D) to decrease aging induced necrotic death (Carmona-Gutierrez et al., 2011a).

On the other hand there are a number of genes that can be considered as conditional anti-apoptotic genes. For example, there are genes that serve to repair DNA damage in response to stresses like UV light and thus can prevent cell death in response to DNA damage. Most of these sequences are unable to prevent PCD in response to most other stresses. So these are pro-survival genes that represent stimulus dependent pro-survival anti-apoptotic genes. Finally, there are a large number of genes that have been identified that can prevent autophagy (Kroemer et al., 2009; Galluzzi et al., 2012b). These genes are essential for autophagy and they function in the process of inducing autophagy, so their removal does not in fact prevent autophagic cell death (He and Klionsky, 2009). The ability to prevent autophagic cell death is limited to a number of non-specific pharmacological agents that mostly serve to prevent acidification of lysosomes. These are thought to function by preventing similar processes in autophagolysosomes.

AUTOPHAGY AND OTHER PROTECTIVE PROCESSES

As mentioned above, basal autophagy serves as a housekeeping process that can get rid of damaged cellular constituents that accumulate while running the basic processes of cellular life. When activated in response to stress, it can increase the removal of damaged material and serve as a mechanism to prevent premature or inappropriate cell death (Shen and Codogno, 2012). Thus the removal of damaged mitochondria can decrease stress mediated increases in ROS levels while the removal of ER clogged with denatured proteins can prevent unwanted ER stress mediated PCD (Gottlieb et al., 2009). Thus a number of other more specialized sub-forms of autophagy such as mitophagy may also be operational in preventing PCD (Kissova and Camougrand, 2010; Marino et al., 2011).

Other cellular processes, analogous to autophagy, can also have specific pro-survival functions in response to specific types of stresses and are thus not always considered in general discussions of cellular survival (Schonthal, 2009; Taylor and Rutter, 2011). A common example is the ER stress response (Schonthal, 2009). This pathway is activated in response to stresses such as the accumulation of misfolded proteins in the ER. Thus defects in this pathway may account for pathological situations like Parkinson's disease (Nagley et al., 2010). Activating the ER stress response can serve to prevent PCD is response to ER stress but it will be unable to prevent general forms of PCD. Similar to ERAD, a stress inducible mitochondrial quality control system (Mitochondrial-associated degradation, MAD) has recently been described (Heo et al., 2010). This process involves Cdc48p and a newly identified Cdc48p interacting protein called VSM1. This process is thought to be able to clean up damaged mitochondria and prevent cell death (Taylor and Rutter, 2011).

PRO-SURVIVAL INTRACELLULAR SECOND MESSENGERS

Just as the regulation of cell death is a balance between pro- and anti-apoptotic machineries, it is of interest that cells produce both anti- and pro- survival intracellular second messengers. ROS represents the classical second messenger regulator of apoptosis since it can switch from being anti- to pro- apoptotic depending on its levels (Ray et al., 2012). Similarly, cAMP also has the ability to be both pro- and anti-apoptotic but that may be more dependent on cell type (Insel et al., 2011). One of the most interesting but less studied intracellular secondary messenger is spermidine.

Spermidine along with putrescine and spermine make up a family of polyamine compounds that are ubiquitous and present in high amounts in cells from bacteria, plants, and other eukaryotes including man and yeast (Igarashi and Kashiwagi, 2010; Minois et al., 2011). Although their exact function remains largely enigmatic they are involved in a number of different cellular processes including apoptosis (Igarashi and Kashiwagi, 2010; Minois et al., 2011). In spite of the existence of a few reports demonstrating a pro-apoptotic effect of polyamines, their ability to serve as anti-apoptotic agents appears to be more common (Igarashi and Kashiwagi, 2010; Gill and Tuteja, 2011). The potential importance of spermidine as a pro-survival and protective effector has been increased by the recent report showing that it has protective effects in a number of different aging models including yeast, worm, fly, and mouse (Eisenberg et al., 2009). Of interest, the effects of spermidine appear to function by activating autophagy. The ability to activate the protective effects of autophagy has been reported for a number of compounds such as the anti-aging compound resveratrol. This is widely attributed to be the anti-aging mediator that is responsible for the reported beneficial effects of red wine (Rockenfeller and Madeo, 2010). This up-regulating of autophagy may be a common phenomenon as it is seen in the apoptotic resistance associated with many pathophysiological conditions such as cancer (see also below) and may also account for the pro-survival effects of a wide range of other conditions (Periyasamy-Thandavan et al., 2009; Wilkinson and Ryan, 2010). Clues as to the mechanisms involved in regulating the levels of protective spermidine come from further studies of Madeo's group who show the ability of cathepsin D to prevent aging mediated necroptosis is associated with an increase in polyamines (Carmona-Gutierrez et al., 2011a).

HORMESIS AND PRE-CONDITIONING: ACTIVATION OF THE INTRINSIC ANTI-APOPTOTIC PROGRAMS

Adaptation to stresses that do not lead to cell death induces a transient condition where the cell shows an enhanced resistance to later exposure of otherwise lethal stresses (Balakumar et al., 2008; Le Bourg, 2009; Lehotsky et al., 2009; Calabrese et al., 2011a). A series of experiments using yeast cells reported by Davies et al. (1995) represent a simple but elegant demonstration of the processes of pre-conditioning. They were able to show that a 2-h exposure to 3.2 mM H_2O_2 was sufficient to kill greater than 99.9% of yeast cells. Cells that were pre-treated with a sub-lethal dose of H_2O_2, say 0.1 mM H_2O_2 for 45 min, showed enhanced viability with 5% of cells surviving in response to a later exposure 2 h exposure to 3.2 mM H_2O_2. This response was dose dependent with increasing concentration of H_2O_2 used as pretreatment with 0.4 mM leading to a greater than 30% viability following the 2-h exposure to 3.2 mM H_2O_2. This and numerous other studies have shown that there is a window of opportunity in which the protection occurs and that the protection that is elicited in response to a sub-lethal stress may lead to cross-protection and confer resistance to the lethal effects of a different stress (Berry and Gasch, 2008; Calabrese et al., 2011a). A similar process is seen in all cell types including mammalian and bacterial cells. The diagram in **Figure 1** illustrates in a simple schematic form, the differences that exist between the responses of a cell in a "Normal State" compared to an adapted cell that now has entered an "Apoptotic Resistant State." The adapted cell shows an enhanced resistance to the cell death inducing effects of stress and this is demonstrated by the observation that they require a greater dose of stress in order to kill off 50% of the population of cells (**Figure 1**). This process of adaptation induced by mild stress that serves to increase resistance to stronger stress is often referred to as a form of hormesis. As we will discuss later on, hormesis is also likely to play an important role in our evolving concept of the role ROS stress plays in the cell death that occurs in processes such as aging.

ROLES FOR ROS, ANTI-APOPTOTIC GENES, AND AUTOPHAGY

An increase in the levels of ROS, albeit not as intense as observed for cells undergoing apoptosis, plays a role in inducing the process by which cellular adaptation occurs in response to mild stress seen in hormesis and pre-conditioning (Martins et al., 2011; Ristow and Schmeisser, 2011; Ristow and Zarse, 2011). As in apoptosis, the mitochondria appear to be the major source of ROS for hormesis (Pan, 2011). In contrast when ROS is present at physiologically relevant levels such as observed during normal or mildly stressed conditions, ROS functions as an intracellular second messenger (Ray et al., 2012). The moderate increase in ROS levels can have a number of effects on proteins for example, it can react with cysteine residues and alter structure and the function of several proteins (Ray et al., 2012). Thus ROS has been shown to be able to alter a number of cellular responses by affecting a variety of different pathways such as MAP and PI3 kinase cascades. The role of ROS in mediating hormetic effects have been reported in all organisms including bacteria. For example, the hormetic effect of sub-lethal low levels of bactericidal antibiotic has been reported to be mediated by increased levels of ROS (Belenky and Collins, 2011).

The free radical theory of aging is a long standing hypothesis that has been used to explain aging (Harman, 1956). At its simplest, the theory posits that free radicals accumulate during the aging process and this accounts for the increased damage that accumulates at the cellular, tissue, and organismal level (Ristow and Zarse, 2011). First proposed in the 1950s the hypothesis gained a lot of momentum as it made a lot of inherent sense and it was consistent with many observations. It is so widely accepted, even by the general public, that anti-oxidants such as vitamin C and E are commonly accepted anti-aging components of most anti-aging creams. The theory of increased free radicals was also commonly adopted as a mechanism to explain the damage that occurs in response to numerous pathophysiological stresses including many that lead to cell death (Kumar et al., 2002; Misra et al., 2009). Thus anti-oxidants were examined for their potential to prevent cellular death in multiple diseases including numerous forms of cardiomyopathies. Numerous discrepancies uncovered over the years have served to question the validity of the model. Of importance to this skepticism was the effect of calorie restriction (CR) on aging. CR, without malnutrition, had been widely reported to slow down aging and lead to increased longevity in all species examined including yeast, flies, worms, and mammals (Ristow and Zarse, 2011). An excess production of ROS has not been able to fully explain the effects of CR. Instead, CR appears to act like a mild stress leading to moderate increases in ROS levels and subsequent anti-aging effects that are mediated by hormesis (Martins et al.,

2011; Ristow and Schmeisser, 2011; Ristow and Zarse, 2011). The similarities between CR and hormesis include up-regulating the same pro-survival strategies including increases in the expression of anti-apoptotic sequences and activation of autophagy (Calabrese et al., 2011a; Martins et al., 2011). In support of this, are the widespread observations that the anti-aging effects mediated by other processes such as the removal of specific genes and the effects of compounds like resveratrol and spermidine function, at least in part, by increasing stress response and autophagy (Calabrese et al., 2011a). For example the loss of the *CTA1* catalase encoding gene in yeast serves to extend chronological lifespan by increasing the levels of ROS in a manner analogous to CR (Mesquita et al., 2010).

Other stress inducible pro-apoptotic second messengers such as ceramide may also have dual functions in regulating cell death responses (Lecour et al., 2005). Of interest, sub-lethal increases in ROS may also account for the increased autophagy that is observed in many scenarios involving mildly stressed cells. This suggests that there is a mechanism for the direct cross-talk between different forms of pro-survival processes. Other evidence for such cross-talk comes from the recent observations that some anti-apoptotic genes may function by activating autophagy (Gurusamy et al., 2009; Qian et al., 2009). An alternative scenario to explain the later observation is based on the fact that the overexpression of a number of genes is mildly toxic and they serve to stress the cell (Liu et al., 1992). For example, it is well-known that cells used in the biotechnology industry that are forced by genetic manipulations to overexpress different gene products are often stressed to the point where they undergo apoptotic like cell death (Krampe and Al-Rubeai, 2010; Mokdad-Gargouri et al., 2012). It could thus be envisioned that some anti-apoptotic genes prevent cell death due the fact that their overexpression leads to mild stress and subsequent induction of the pro-survival machinery including activation of autophagy and hormesis. Other scenarios that serve to induce a protective pre-condition or hormetic phenotype include the loss by knockout of some genes as well as number of different compounds including many toxic chemicals administered at sub-lethal levels (Kharade et al., 2005; Wang et al., 2009; Mesquita et al., 2010; Martins et al., 2011; Orrenius et al., 2011). This indicates that there are multitudes of ways of eliciting a protective phenotype. This serves to increase the repertoire of strategies that can be clinically used to combat unwanted cell death (Fleming et al., 2011).

ONE GENE CAN RECAPITULATE THE ENTIRE PROCESS OF HORMESIS

Analysis of the expression of numerous genes, and more recently the analysis of global gene expression profiles, reveal that cellular adaptation (pre-conditioning and hormesis) is associated with alterations in the expression of many genes including the increased expression of a number of anti-apoptotic genes (Wu et al., 2004; Coles et al., 2005; Balakumar et al., 2008; Fulda, 2009b). In spite of the fact that that the expression of so many different genes are observed, a stress resistant phenotype can be recapitulated by the simple overexpression of a multitude of different but individual anti-apoptotic genes (Gil-Gómez and Brady, 1998; Yenari et al., 2005; Khan et al., 2006; Nakka et al., 2008). The fact that a single gene is sufficient for this phenotype suggests that there is a strong redundancy in the numerous anti-apoptotic processes that are invoked in the process of adaptation. Increased autophagy, probably macro-autophagy, is also a response to mild stress. Similarly to what is observed with the increased expression of anti-apoptotic genes, increased autophagy on its own is capable of increasing pro-survival phenotypes. This can be demonstrated by using specific macro-autophagy activating drugs like rapamycin (Galluzzi et al., 2012a). Such cells show increased resistance to numerous stresses including many that would be sufficient to induce apoptosis. One of the questions that remain is why there is so much apparent redundancy and co-activation of anti-apoptotic and pro-survival responses.

In eukaryotic microbes, pre-conditioning is a process that has numerous similarities to hormesis. Classical pre-conditioning can be demonstrated by temporally decreasing blood supply to a tissue leading to mild ischemia like conditions (Balakumar et al., 2008; Porter et al., 2012). As in hormesis, the mildly stressed tissue can be subsequently shown to have acquired an increased resistance to more intense levels of stress including longer periods of ischemia. The normal form of pre-conditioning called late onset pre-conditioning involves an increase in the expression of anti-apoptotic genes and is likely to be similar if not identical to what occurs during hormesis (as described above). There is also an early form of cellular protection that occurs soon after the pre-conditioning stimuli. This process is much quicker than the late onset form of pre-conditioning and usually involves post-translational mechanisms that serve to activate pro-survival proteins and cascades such as MAP kinases (Hausenloy et al., 2005; Balakumar et al., 2008). The question as to whether such an early form of hormesis exists in yeast has not been fully addressed.

DISEASES ASSOCIATED WITH ALTERED PCD

CONDITIONS WITH INCREASED RESISTANCE TO APOPTOSIS

There a plethora of diseases and pathophysiologies that are associated with defective apoptotic responses (Krakstad and Chekenya, 2010; Whelan et al., 2010; Zhivotovsky and Orrenius, 2010; Ashida et al., 2011; Lavu et al., 2011; Liu et al., 2011; Orrenius et al., 2011; Strasser et al., 2011; Ulukaya et al., 2011; Oerlemans et al., 2012; Porter et al., 2012). Some such as cancer and some virally infected cells have decreased apoptotic responses that lead to the accumulation of unwanted cells. These conditionally apoptotic resistant cells have been useful in identifying genes whose up-regulation can confer anti-apoptotic phenotypes (Busca et al., 2009; Fulda, 2009b). In effect, the most common anti-apoptotic gene, Bcl-2, was first identified as a gene that is up-regulated in cancer cells (Vaux et al., 1988). Altered Bcl-2 levels are now known to occur in a large proportion of cancers. Developing combinational chemotherapeutic strategies aimed at killing cancer cells combined with inhibitors of anti-apoptotic genes or of their protein product are being developed as useful clinical processes (Strasser et al., 2011). Cancer cells develop in stressful micro-environments that can include low nutrient and oxygen levels (Fulda, 2010). Strategies aimed at preventing new blood vessel formation (angiogenesis) are based on preventing the establishment of these tumor cells (Galluzzi et al., 2010b). These types of stresses faced by cancer cells is thought to lead to pre-condition or hormesis like responses that serve to increase apoptotic resistance (Fulda, 2009b; Fulda et al., 2010). Thus there is also an increase in other cell survival strategies in

these cells including an increase in autophagy that accounts for some of the anti-apoptotic responses (Liu et al., 2011; Meschini et al., 2011). These observations have led to the current interest in the development of autophagy inhibitors to be used as chemotherapeutic adjuvants (Wilkinson and Ryan, 2010; Meschini et al., 2011).

CONDITIONS SHOWING ENHANCED APOPTOSIS

On the other hand, there are a large number of other pathophysiological conditions where an increase in cell death occurs (Fulda et al., 2010; Whelan et al., 2010; Ulukaya et al., 2011). These are seen in response to stresses such as ischemia/reperfusion that occur due to heart attacks and strokes, in autoimmune diseases like Multiple Sclerosis (MS), and in diseases associated with defects of protein folding and quality control leading to the accumulation of misfolded proteins such as occurs in Alzheimer's disease. In unicellular eukaryotes such as yeast, exposure to excess mating pheromone in the absence of an appropriate mating partner or the heterologous expression of disease causing human genes like α-synuclein can be seen as analogous to pathological situations that lead to premature death (Buttner et al., 2006; Franssens et al., 2010; Khurana and Lindquist, 2010). In more practical situations, inappropriate, or unwanted cell death occurs in industrially used cells including yeast and cultured mammalian cells that undergo apoptosis due to the stresses imposed by the forced overproduction of biotechnologically important compounds and therapeutics (Krampe and Al-Rubeai, 2010; Mokdad-Gargouri et al., 2012). In many pathologies, there are very limited therapies given that the underlying causes of many of these diseases are not known. Increased apoptosis is not usually the cause since it occurs as a result of the stress a cell encounters due to the pathophysiological condition. Nevertheless, the ability to prevent PCD following the onset of the disease process could be of tremendous clinical value. This is apparent for the PCD that occurs after acute conditions like a heart attack following an ischemic/reperfusion event (Oerlemans et al., 2012). The most common form of a heart attack involves a blockage of a blood vessel leading to ischemic event that leads to nutrients and oxygen deprivation stress of the cells downstream of the obstruction (Ong and Gustafsson, 2012; **Figure 2**). The first priority is to unblock the blood vessel and allow tissue reperfusion (Porter et al., 2012). The level of stress is graded with the most severe stress occurring in the cells that are most deprived of blood (**Figure 2**). In addition, there is a great deal of stress that occurs in the cells due to the effects of reperfusion (Ong and Gustafsson, 2012). Necrosis is likely to occur in the part of the tissue that is severely deprived, while apoptosis is triggered in some other areas having less stress (Ong and Gustafsson, 2012). Thus there is a zone of necrotic death that likely occurs soon after a heart attack and these cells are likely beyond therapeutic rescue (**Figure 2**). Conversely, cell death due to PCD (apoptotic and possibly autophagic death), will occur over the next few days (Ong and Gustafsson, 2012). Thus there is a therapeutic window in which the prevention of PCD would be a great benefit in limiting the infarct size, increasing survivability and decreasing morbidity, following ischemia/reperfusion events. Thus the process of post-conditioning can be used to prevent some of the post-infarct induced PCD (Balakumar et al., 2008; Lehotsky et al., 2009). Post-conditioning refers to the ability to stimulate a hormetic like response in cells using chemical agents that can induce pre-conditioning even after the apoptosis inducing event has occurred (Balakumar et al., 2008; Lehotsky et al., 2009). Further, there is a great deal of evidence that serves to convince that therapeutic interference in the process of PCD post-infarct would serve to limit cellular demise (Oerlemans et al., 2012). Of importance here is the observation that transgenic animals that overexpress anti-apoptotic genes in a cardiac or brain specific manner, have significantly decreased zones of death following ischemic events (**Figure 2**; Yenari et al., 2005; Khan et al., 2006; Nakka et al., 2008). These effects are widespread since they are observed with a number of different anti-apoptotic genes and it demonstrates the potential clinical usefulness of anti-apoptosis (Rami et al., 2008; Fulda, 2009a; Krakstad and Chekenya, 2010; Dietz, 2011; Lavu et al., 2011).

CLINICAL APPROACHES AND DEVELOPMENT OF APOPTOTIC REGULATING THEARAPEUTICS

Numerous strategies have been developed in order to try and overcome the apoptotic resistance encountered in cancer cells (Strasser et al., 2011; Porter et al., 2012). Most chemotherapeutics as well as radiation therapies appear to function by targeting the rapidly dividing phenotype associated with these cells in order to induce a specific apoptotic response. As mentioned above, strategies such as the inhibition of autophagy, are being developed to render cancer cells more sensitive to apoptotic inducing stimuli. In the case of cells that are more resistant to apoptosis due to an infection by an infective agent, investigations are underway to understand the life cycle of these organisms to target the factors that increase apoptotic resistance (Galluzzi et al., 2010a; Ashida et al., 2011; Rodrigues et al., 2012).

On the other hand, given the success achieved by overexpressing anti-apoptotic genes in transgenic animals to prevent ischemic damage, there is intense investigation to try and develop strategies that can serve to inhibit apoptosis (Rami et al., 2008; Yacoubian and Standaert, 2009; Dietz, 2011; Oerlemans et al., 2012). Thus the development of small molecule inhibitors of proteins involved in mediating apoptotic death was examined by many (Oerlemans et al., 2012). Given their central role in apoptosis, caspases were identified as ideal targets given that their inhibition is predicted to serve to prevent at least some forms of PCD. Caspase inhibitors with a good deal of specificity and effectiveness were developed and shown to be effective *in vitro* cell cultures (Oerlemans et al., 2012). Many of the inhibitors were indeed effective at blocking caspase and caspase mediated cell death. In contrast these were of limited effectiveness at preventing cell death *in vivo* (Oerlemans et al., 2012). It appears that cells that are stimulated with appropriate stress will undergo alternate modes of PCD if their apoptotic machinery is impaired. This situation is commonly observed and is reminiscent of the autophagic cell death that occurs in apoptotically stimulated cells that are unable to undergo apoptosis due to a double KO of the pro-apoptotic Bcl-2 family members Bax and Bak (Shimizu et al., 2004).

The limited effectiveness encountered with these caspase inhibitors suggests that targeting a single protein may not be able to inhibit apoptosis. Thus a great deal of effort is being made

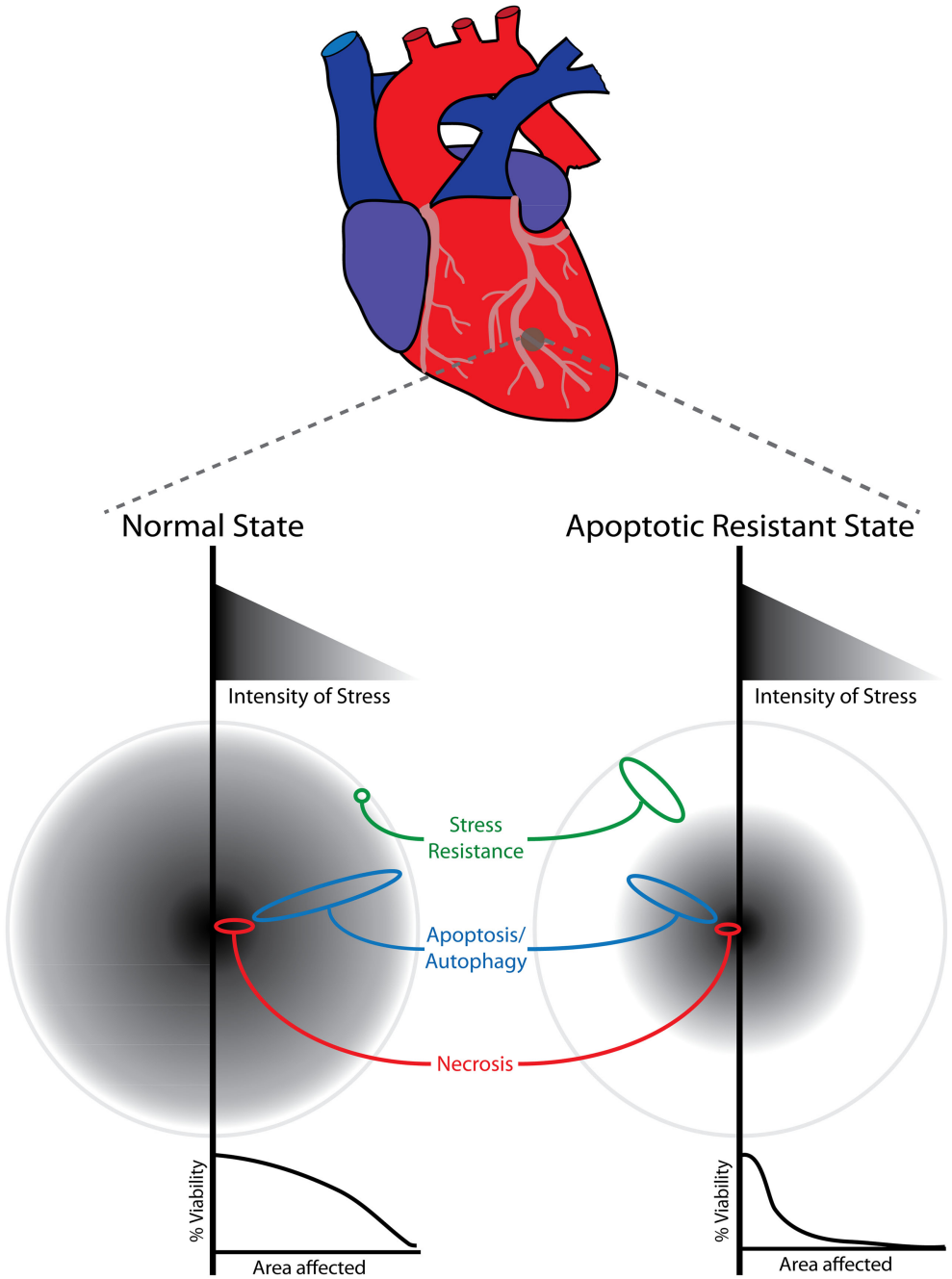

FIGURE 2 | Schematic depiction of the stress generated and subsequent cell death mediated by cardiac stress events. Zones of cardiac cell death can be induced experimentally including the generation of myocardial infarction (MI) by ligation of left descending artery or by direct cardiac ischemia reperfusion (Tarnavski et al., 2004). In the normal state (left side), a gradient of stress intensity occurs in the deprived tissue where the most severely deprived cells undergo necrosis while other stressed cells undergo cell death that appears to include apoptosis and possibly autophagic death. Loss of viability is more pronounced as we move closer to the site of the most intense stress. Transgenic animals that overexpress an anti-apoptotic gene in a cardiac specific manner represent an apoptotic resistant state (right side). Identical levels of stress generated in the hearts of these animals leads to significant reduction in the zone of dead cells.

into understanding the processes that occur during apoptosis and anti-apoptosis in order to develop strategies aimed at tapping into the natural anti-apoptotic network (Balakumar et al., 2008; Lehotsky et al., 2009; Lochner et al., 2009; Yacoubian and Standaert, 2009; Boll et al., 2011). This concept is in line with the fact that agents causing up-regulation of endogenous defense mechanisms by hormesis or by pre-conditioning are effective at preventing or decreasing cell death (Balakumar et al., 2008; Rami et al., 2008; Calabrese et al., 2011b; Martins et al., 2011; Porter et al., 2012). Thus, anti-apoptotic genes, which are part of the *in vivo* regulatory

responses to counteract stress mediated cell death, are readily effective when overexpressed *in vivo* (via transgenics; Gil-Gómez and Brady, 1998; Yenari et al., 2005; Khan et al., 2006). Strategies are currently being developed to increase the levels of anti-apoptotic genes in tissues. Approaches include delivery of expressible gene constructs by gene therapy or the use of lipophilically modified recombinant proteins that could diffuse into apoptotically challenged cells (Dietz, 2011; Lavu et al., 2011). Such approaches could only serve to give rise to a short term increase in the levels of anti-apoptotic proteins, but this may be sufficient to prevent cell death in the short term after an ischemic/reperfusion event in tissues like the heart.

YEAST AS A MODEL TO STUDY PCD

In addition to its long history in the food and biotechnology industries, the yeast *S. cerevisiae* may be the most widely used model organism (Botstein and Fink, 2011). As a eukaryotic cell system it has numerous advantages over other model cell types including the ability to use genetic approaches. In addition, its basic cellular processes, such as cell cycle control, DNA replication and repair, mitochondrial based respiration, and autophagy are carried out by simplified cellular process that are virtually interchangeable with the same but more complex processes of metazoan eukaryotes including humans (Perocchi et al., 2008). Thus it comes as no surprise that the description of a PCD in *S. cerevisiae* in 1997 has led to an explosion of research into the study of these processes in yeast (Madeo et al., 1997; Carmona-Gutierrez et al., 2010). Although the utility of a PCD process in unicellular eukaryotes was at first difficult to imagine, it is now widely accepted and has in fact been extensively documented to occur in a wide variety of different unicellular eukaryotes (Shemarova, 2010). Over the last few years, a large variety of studies have been instrumental in documenting many of the processes and in identifying many of the proteins involved in mediating yeast PCD (Frohlich et al., 2007; Carmona-Gutierrez et al., 2010). Although there are some differences, it would appear that yeast PCD is very similar to the mitochondrial or intrinsic apoptosis seen in metazoans (Eisenberg et al., 2007; Pereira et al., 2008). The complexity is decreased compared to mammalian apoptosis which is exemplified by the fact that most apoptotic regulators are encoded by multiple copies in humans but by single copy genes in yeast. So, yeast has single functional counterparts to many mammalian apoptotic regulators including a caspase (*YCA1*), a BH3 containing Bcl-2 family like protein (*yBH3*), an AIF (*AIF*), an OMI serine protease (*NMA111*), and an Endonuclease G (*NUC1*; Madeo et al., 2002; Fahrenkrog et al., 2004; Wissing et al., 2004; Buttner et al., 2007, 2011). As in mammalian cells, yeast will undergo apoptosis in response to a variety of different stresses including many chemicals, physical process such as altered osmolarity and pH, as well as the overexpression of genes encoding pro-apoptotic proteins including mammalian Bax and caspases (Manon et al., 1997; Lisa-Santamaria et al., 2009; Sharon et al., 2009; Carmona-Gutierrez et al., 2010; Orrenius et al., 2011). The intracellular events mediating yeast PCD also show a great deal of similarity with a number of other processes such as an increase in mitochondrial outer membrane permeability, increase in pro-apoptotic second messengers like ROS and ceramide, the release of apoptogenic factors including cytochrome c, DNA fragmentation as well as the externalization of phosphatidylserine (Manon et al., 1997; Ligr et al., 1998; Madeo et al., 1999; Yang et al., 2006; Ganesan et al., 2010; Carmona-Gutierrez et al., 2011b). In addition, naturally occurring physiological processes including ER stress and chronological aging will lead to mitochondrial and necrotic like cell death as it does in mammalian cells (Burhans et al., 2003; Fabrizio and Longo, 2008; Winderickx et al., 2008; Eisenberg et al., 2010; Madeo et al., 2010; Pan, 2011). On the other hand, ER mediated cell death in yeast is reminiscent of what occurs in a number of diseases that involves the accumulation of misfolded proteins such as Parkinson's and Huntington's (Winderickx et al., 2008; Khurana and Lindquist, 2010).

Of importance to our interests is the fact that regulated cell death, including PCD and necrosis, in yeast is also under negative regulation (Owsianowski et al., 2008; Carmona-Gutierrez et al., 2011a; Zdralevic et al., 2012). In fact many of the proteins that are known to prevent cell death in mammalian cells, such as free radical scavengers, chaperonin type proteins like HSPs, the yeast IAP (Inhibitor of Apoptosis Protein) protein (Bir1p) as well as numerous heterologous mammalian proteins such as Bcl-2 also prevent cell death (Manon et al., 1997; Moon et al., 2002; Flower et al., 2005; Walter et al., 2006). Yeast also has a well-defined stress activated autophagic process that has long been known to promote cellular longevity in response to stresses such as amino acid or glucose starvation and aging (Eisenberg et al., 2009; He and Klionsky, 2009). In spite of this, there have been very relatively few studies directed toward examining the possible role of autophagic cell death in yeast (Camougrand et al., 2003; Kissova et al., 2006; Thevissen et al., 2010; Sampaio-Marques et al., 2011).

Observed differences between yeast and mammalian cells are also of interest. For example, in a screen for yeast mutants showing increased resistance to the heterologous expression of the pro-apoptotic Bax, the *UTH1* gene was identified (Camougrand et al., 2004). Although the exact function of Uth1p has yet to be defined, deletion of this gene leads to a number of interesting pleiotropic effects including increasing replicative lifespan, increasing resistance to the autophagic inducing drug rapamycin and ER inducing stresses (Kissova and Camougrand, 2010; Ritch et al., 2010). Surprisingly, a direct mammalian ortholog of *UTH1* has yet to be found but it would be of interest to set up a suicide screen of *UTH1*Δ cells in order to identify potential functional homologs. Such screens have proven useful in the past for the identification and characterization of a number different human orthologs that can functionally complement phenotypes of a variety of different yeast mutants (Perier et al., 1994; Sato et al., 2006; Osborn and Miller, 2007). The study of yeast specific apoptotic regulators open up possibilities of increasing our understanding of the basic ancestral processes involved in regulating PCD. More recent developments including systems biology and other global approaches are being applied to understanding the processes of PCD in yeast (Munoz et al., 2012). For example a global search for yeast genes that are potential regulators of PCD was carried out by evaluating all yeast gene knockout strains for increased susceptibility to stress mediated cell death (Teng et al., 2011). Over 800 of the 6000 or so yeast genes were thus identified as

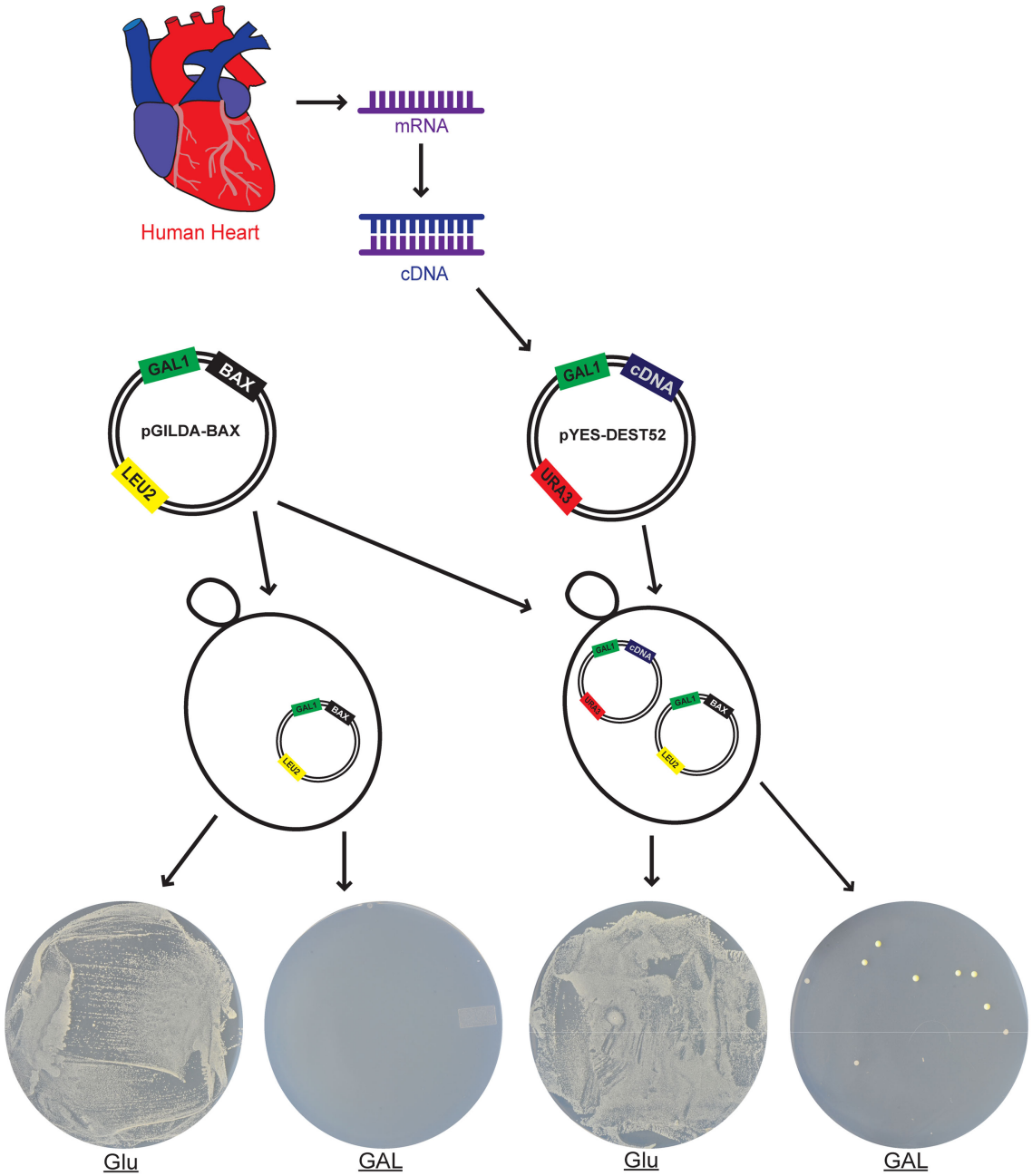

FIGURE 3 | Schematic depiction of a yeast based functional strategy to screen for and identify cardiac specific sequences capable of preventing Bax mediated cell death. Poly A^+ RNA isolated from human cardiac tissue was used to generate a cDNA library in the *URA3* selectable marker yeast expression plasmid pYES-DEST52 (Sato et al., 2006). This places the expression of the human cardiac cDNAs under the control of the galactose inducible *GAL1* promoter. Plasmid pGILDA-Bax was used to express an activated mouse Bax in yeast under the control of galactose inducible *GAL1* promoter. Yeast cells harboring pGILDA-Bax alone show normal growth when grown on glucose media but undergo cell death due to the expression of Bax when grown on galactose media. Close to 10^6 yeast transformants harboring both pGILDA-Bax as well as different cDNAs from the pYES-DEST52 cardiac cDNA library were grown on galactose media. A total of 72 different colonies containing over 60 different putative Bax suppressors were identified when the pYES-DEST52 cardiac cDNAs were isolated and their nucleotide sequence determined (Yang et al., 2006). Similar apoptotic resistant phenotypes can be achieved in the heart by subjecting the tissue to a period of mild stress in order to induce pre-conditioning. Glu, glucose; GAL, galactose.

potential pro-apoptotic genes. This suggests that many more genes are involved in the process of PCD than we know of now and these types of approaches in yeast may actually serve to identify all players in the process of PCD. Other such global strategies are now being combined with the power of yeast genetics to characterize other processes involved in PCD such as identifying the

metabolic products (metabolome) that accumulate in different process associated with different forms of PCD (Ring et al., 2012).

HUMANIZED YEAST

Humanized yeast has been extensively used to study the structure and function of human genes in an uncluttered cellular environment (Osborn and Miller, 2007; Greenwood and Ludovico, 2010; Silva et al., 2011a). For example, all mammalian cells express a multitude of G-Protein Coupled Receptors (GPCRs) and studies focused on individual members of this large gene family are further encumbered by the fact that different members of the same family are capable of functionally interacting with each other (Panetta and Greenwood, 2008). Thus the ability to functionally replace the yeast sex pheromone GPCRs and their regulatory proteins with human orthologs allows the study of individual human genes uncluttered by the presence of other members (Kong et al., 2002; Ladds et al., 2005). Given the similarity between the processes of metazoan mitochondrially induced apoptosis and yeast apoptosis (Cheng et al., 2008) it is not surprising that humanized yeast have been successfully used in the pursuit of knowledge regarding the genes involved and the processes regulating PCD (Greenwood and Ludovico, 2010; Khurana and Lindquist, 2010; Silva et al., 2011a; Zdralevic et al., 2012).

Early studies by a number of yeast researchers lead to the discovery that mammalian regulators of apoptosis including various Bcl-2 family members including pro-apoptotic Bax and anti-apoptotic Bcl-2 could be functionally expressed in yeast (Greenhalf et al., 1996; Manon et al., 1997; Tao et al., 1997). Thus Bax mediated PCD in yeast was extensively studied and all indications suggested it induced apoptosis, at least in some cells, since it lead to apoptotic hallmark events similar to what was observed in mammalian cells (Priault et al., 2003; Khoury et al., 2008). Bcl-2 was also able to prevent Bax as well as other forms of stress mediated PCD in yeast. Some of these early studies had noticed that Bax expression not only lead to typical apoptotic phenotypes but it also lead to the activation of autophagy (Camougrand et al., 2003; Kissova et al., 2006). This is indicative of some form of cross-talk and is suggestive of some of the useful avenues of knowledge that are uncovered using humanized yeast in the study of PCD. Not surprisingly other pro-apoptotic human proteins such as caspases can trigger the same types of effects in yeast (Lisa-Santamaria et al., 2009). The functional expression of Bax and of other mammalian apoptotic regulators continues to serve to uncover insights into PCD (Greenwood and Ludovico, 2010). In the case of Bax, recent work has been useful in characterizing the roles of a variety of different proteins including protein kinase C and the mitochondrial receptor Tom22 in the functional activation of Bax (Renault et al., 2011; Silva et al., 2011b). Up until recently it was thought that the yeast genome did not code for any BH3 containing proteins, so the effects of heterologously expressed Bcl-2 proteins was thought to reflect intrinsic activities of these proteins or it was thought that yeast must contain a functional that is a not structural homolog of human Bax (Priault et al., 2003; Khoury et al., 2008). More recently, an endogenously encoded BH3 domain containing protein, called yBH3p, was identified as a pro-apoptotic protein in yeast (Buttner et al., 2011). Whether it has all the functions of mammalian Bax remains to be determined.

Bax mediated lethality in yeast was exploited in other ways. Notably, yeast cells conditionally expressing Bax, most often under a galactose inducible promoter, were used in numerous suicide screens (**Figure 3**; Liu et al., 1992; Sato et al., 2006; Osborn and Miller, 2007; Greenwood and Ludovico, 2010). Copy DNA (cDNA) libraries obtained from a range of different species including bacteria, plants, and different tissues were screened in order to identify sequences that permitted yeast to grow in the presence of Bax (Greenwood and Ludovico, 2010; Laloux et al., 2010). Many of the Bax suppressors identified corresponded to obvious anti-apoptotic sequences including numerous free radical scavenging proteins (Kampranis et al., 2000; Moon et al., 2002; Camougrand et al., 2004). This is consistent with the notion that Bax is indeed inducing a PCD that has similarities to apoptosis. Other proteins identified represent a challenge since they are clearly able to prevent PCD from a number of different stresses in yeast, but the mechanism by which they carry out this function remains unknown. This class of protein includes some with well-known functions such as Vacuolar Protein Sorting 24 (VPS24) and the small GTP binding protein Ran as well as proteins that are essentially functional orphans including TMEM85 and TMEM14 (Khoury et al., 2007; Ring et al., 2008; Woo et al., 2008, 2011). Other Bax suppressors identified include the dUTP hydrolyzing enzyme dUTPase and the ceramide utilizing enzyme sphingomyelin synthase (Yang et al., 2006; Williams et al., 2011). This suggests that stress mediated cell death in yeast, like observed in mammalian cells, involves an up-regulation of the levels of apoptosis inducing second messengers including ROS, ceramide, and dUTP (Portt et al., 2011; Wilson et al., 2012). Many of the screens have reported the identification of multiple Bax suppressors but the identity of many of these have yet to be reported. For example screening of a human T cell library lead to the identification or 24 Bax suppressors but the group has only reported a detailed analysis of four of these (Eun et al., 2008; Woo et al., 2008, 2009, 2011). Similarly, we have isolated over 60 different Bax suppressors from a screen of a human cardiac library and we have since published a detailed characterization of five of these (Yang et al., 2006; Khoury et al., 2007, 2008; Ring et al., 2008; Williams et al., 2011). Parallel or subsequent analysis of a number of these Bax suppressors reveal that they are bone-fide cell survival genes since they also function as anti-apoptotic genes in mammalian cells (Fiol et al., 2007; Separovic et al., 2007; Eun et al., 2008; Woo et al., 2008, 2009, 2011; Greenwood and Ludovico, 2010; Williams et al., 2011; Wilson et al., 2012). Taken together these screens suggests that there is a lot more that remains to be discovered on the anti-apoptotic and cell survival mechanisms used by a cell and that yeast is an ideal system for this task.

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