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Mitochondrial death pathways in yeast and mammalian cells

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Abstract

In mammals, mitochondria are important mediators of programmed cell death, and this process is often regulated by Bcl-2 family proteins. However, a role for mitochondria-mediated cell death in non-mammalian species is more controversial. New evidence from a variety of sources suggests that mammalian mitochondrial fission/division proteins also have the capacity to promote programmed cell death, which may involve interactions with Bcl-2 family proteins. Homologues of these fission factors and several additional mammalian cell death regulators are conserved in flies, worms and yeast, and have been suggested to regulate programmed cell death in these species as well. However, the molecular mechanisms by which these phylogenetically conserved proteins contribute to cell death are not known for any species. Some have taken the conserved pro-death activity of mitochondrial fission factors to mean that mitochondrial fission per se, or failed attempts to undergo fission, are directly involved in cell death. Other evidence suggests that the fission function and the cell death function of these factors are separable. Here we consider the evidence for these arguments and their implications regarding the origins of programmed cell death.

Keywords

Yeast Apoptosis; mitochondria; fission; fusion; Drp1; Dnm1; caspase; metacaspase; fragmentation; cytochrome *c*

INTRODUCTION

The discovery of genes in yeast that regulate fission/division and fusion/union of mitochondria was a major advancement towards understanding the malleable nature and the dynamic morphology of these organelles. In yeast, distinct molecular complexes mediate mitochondrial fission versus fusion, and many of these factors have obvious mammalian counterparts with analogous functions in mammals (1,2). It is widely assumed that a balance between the rates of fission versus fusion is the primary determinant of the tubular shape of mitochondria in healthy cells from yeast to humans. This assumption is based primarily on genetic manipulations that alter mitochondrial morphology. For example, mutation of mitochondrial fission proteins leads to abnormally fused (netted or elongated) mitochondria apparently due to continued fusion. In contrast, inhibition or mutation of mitochondrial fusion proteins leads to abnormally short mitochondria presumably due to undeterred fission. However, there are other determinants of mitochondrial morphology, and the methodologies for quantifying these

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morphological changes are complex (3). The processes of mitochondrial fission and fusion are essential for normal cellular function in humans, as genetic mutations in these genes lead to severe developmental defects and neurological disease (4–6). In addition to their normal cellular functions in mitochondrial maintenance, mitochondrial fission and fusion proteins also regulate programmed cell death in yeast, worms, flies and mammals, consistent with the idea that mitochondria contribute importantly to evolutionarily conserved cell suicide. The conserved mitochondrial fission factor Drp1/Dnm1 mediates mitochondrial fission in a healthy state and in a dying state (1,7). Currently, it is not clear if the fission protein complex that carries out mitochondrial fission in healthy versus dying cells are identical or if their compositions are only partially overlapping. The discovery that mitochondrial fission factors also mediate the excessive mitochondrial fission during cell death, generally termed mitochondrial fragmentation, has led to speculation that mitochondrial fission itself is directly involved in permeabilization of the outer mitochondrial membrane to release of pro-death factors into the cytoplasm during cell death. Alternatively, mitochondrial fission may be dissociable or even distinct from the pro-death function of the factors that mediate mitochondrial fission.

1. ANCIENT ORIGINS OF PROGRAMMED CELL DEATH

Though not universally accepted, new evidence suggests that the origin of programmed cell death preceded multi-cellular organisms. Thus, programmed cell death may have originated, ironically, as a survival strategy for single-cell species to respond to environmental hazards, such as temperature extremes, cycles of nutrient deprivation and the inevitable infectious pathogen (8). Convincing examples of purposeful cell death outside the animal kingdom are available, but less renown. For example, it has been reported that plant embryogenesis in the Norway spruce and fungal infection-induced cell death in the lily requires metacaspase-dependent programmed cell death (9–11). Metacaspases are distantly related to animal caspases, the proteases that facilitate apoptotic programmed cell death in mammals (12). If these observations in plants are accepted as evidence of plant programmed cell death, then the existence for programmed cell death in both the animal and plant kingdoms, carried out by factors conserved across kingdoms, is consistent with a more ancient origin of cell suicide.

Evidence that budding yeast *Saccharomyces cerevisiae* have evolved self-destruct mechanisms was originally based on morphological characteristics of dying yeast cells that were suggested to resemble some of the characteristics of apoptotic mammalian cells. These apoptosis-like features reported for dying yeast include phosphatidylserine externalization on the outer leaflet of the plasma membrane, DNA degradation, nuclear fragmentation and chromatin condensation (13–18). Yeast encode orthologues of many mammalian cell death regulators, including the DNA endonuclease EndoG (19), AIF (apoptosis-inducing factor) (20), the AIF-like factor Ndi1/AMID (21) superoxide dismutase (SOD) (22), and the serine protease Omi/HtrA2 that promotes caspase activation in mammals (23,24). However, it is unclear if the molecular mechanisms by which these conserved factors promote cell death in yeast are in any way analogous to mammals. Furthermore, Bcl-2 family proteins and aspartate-cleaving caspases that constitute the apoptotic cell death machinery in mammals have not been convincingly identified in yeast. Thus, many investigators doubt that yeast cell death represents the predecessor of apoptosis in mammals, where the morphological changes characteristic of apoptosis are the direct result of caspase activation. However, a growing list of alternative caspase-independent death pathways are beginning to be elucidated in mammals, but even less is known about analogous pathways in other species.

1.1. Protease-dependent cell death

Mammalian caspases can be subdivided into two functional groups, those involved in inflammatory responses and innate immunity (e.g. caspases-1, 4 and 5) and those that regulate

programmed cell death (e.g. caspase-2, 3, 6, 7, 8, 9), though the line between these two groups has become blurred. For example, mammalian caspases involved in immune recognition may function as pro-death proteases while carrying out their role in immunity (25,26). Furthermore, caspases involved in apoptosis may also have roles in healthy cells (27,28). Thus, it is not a great leap to suggest that first-responder mechanisms for single-cell species conceivably reflect the origins of innate immune recognition responses. While sequence analysis predicts that mammalian caspases arose from more ancient proteases related to metacaspases, the unanswered question is whether or not metacaspases arose in single-cell species at least in part for the purpose of cell suicide, or if cell death is a more recently acquired function of caspases for the purpose of sculpting complex multi-organ structures and preventing cancer.

Like plants and bacteria, yeast do not encode the equivalent of mammalian caspases, but do encode a metacaspase, *MCA1*/*YCA1* (12), which is more closely related to peptidases of bacteria and plants than to mammalian caspases. Nevertheless, deletion of the *Saccharomyces cerevisiae* metacaspase results in protection from a variety, but not all death stimuli (29–35). Thus, the existence of a conserved pro-death protease in yeast fuels the idea that single-cell eukaryotes have an evolutionarily conserved programmed cell death pathway similar to mammals (Figure 1). We favor the possibility that programmed cell death is an important function of metacaspases, consistent with the observation that yeast M1 and M2 killer viruses induce yeast cell death that is mediated in part by the yeast metacaspase *YCA1*/*MCA1* (30, 35).

Several hundred caspase substrates have been identified in mammals, and the mechanisms by which a few of these promote cell death are partially delineated (36–39). In contrast, there are no known target substrates of yeast Mca1/Yca1 that function equivalently to caspase-cleaved mediators of mammalian apoptosis to support the model that yeast undergo a caspase-like death. However, there is also a paucity of caspase substrates identified in flies and worms, where caspases are well accepted mediators of programmed cell death. Despite these gaps in knowledge, genetic evidence provides support for the pro-death function of yeast metacaspase *MCA1*/*YCA1* in several cell death paradigms, including ROS-mediated damage (10,31,40), chronological aging (33), virus infection (30,35), DNA damage (34), and dysregulated mRNA stability (32).

Esp1 is another caspase-like yeast protease implicated in programmed cell death, and is conserved in mammals (41). This cysteine protease of yeast was recently reported to cleave yeast Mcd1, orthologue of human cohesin Rad21/Mcd1 involved in the attachment of sister kinetachores for chromatid cohesion. In mammalian cells or *in vitro*, this 635 amino acid protein is a substrate of caspase-3 and -7, which cleaves Rad21 after the sequence $DSPD^{279}$ in anaphase cells and preceding chromatin condensation during cell death (42). Furthermore, the C-terminal cleavage product of human Rad21 can trigger mammalian cell death. A somewhat analogous situation was reported for yeast (41). Although the critical cleavage site in yeast Mcd1 was not identified, a C-terminal cleavage fragment of Mcd1 was reported to translocate to mitochondria and trigger cytochrome *c*-dependent cell death in yeast.

Perhaps we should not expect yeast cell death facilitated by metacaspases or other proteases to directly reflect mammalian programmed cell death mechanisms. Rather, we speculate that the upstream steps critical for sensing nutrient deprivation and responding to invading pathogens are better conserved aspects of cell death pathways in yeast and mammals. Certainly, many of the signaling cascades (e.g. PI3K VPS34), growth control mechanisms (e.g. TOR kinase) and metabolic pathways are among the most conserved cellular processes across species. We may find that the proteolytic steps that occur downstream in a given cell death pathway are more important for the purpose of packaging and disposing of cell corpses, rather than for initiating cell death (or for healthy cell functions), and are less analogous between

species out of necessity as dictated by the specific environments inhabited by yeast and mammalian cells.

1.2. Are Bcl-2 family proteins universally conserved?

In yeast, the protective effect of human Bcl-2 was first demonstrated in strains lacking superoxide dismutase (43). However, most studies have used yeast as a type of incubator for cellular apoptosis regulators, under the assumption that yeast lack endogenous apoptosis regulators such as Bcl-2 family proteins that could otherwise interfere with the assay. For example, the mammalian pro-death Bcl-2 family member Bax also promotes yeast cell death (and growth arrest) that is inhibited by anti-apoptotic Bcl-2 and Bcl-x_L when these mammalian proteins are co-expressed in yeast (44). However, human Bcl-2 and Bcl-x_L can protect yeast against mitochondrial cell death in the absence of pro-apoptotic mammalian factors (18).

How might mammalian Bcl-2 family proteins inhibit cell death in yeast? Unless Bcl-2 proteins act independently of all other host proteins in yeast, such as directly altering membrane structures, then they presumably interface with evolutionarily conserved components of yeast. Based on studies in mammalian cells, candidates include yeast proteins involved in nutrient signaling, autophagy or mitochondrial energetics, which are highly conserved processes carried out by highly conserved proteins. Consistent with this idea, the pro-apoptotic Bcl-2 family protein Bax was suggested to induce autophagy instead of apoptotic death in yeast (45).

Bcl-2 proteins are widely thought to carry out their anti- and pro-apoptotic activities by interacting with other Bcl-2 family members. If true, the ability of human Bcl-2 proteins to inhibit cell death in yeast would imply conservation of Bcl-2-like functions in yeast. Thus, it is possible that yeast in fact do encode functional/structural equivalents of Bcl-2 family proteins, despite the absence of significant amino acid sequence similarity (18,46,47). Consistent with this idea, 3-dimensional structure determination of several virus-encoded anti-apoptotic factors with no significant amino acid sequence similarity to each other or to Bcl-2 proteins revealed a Bcl-2-like protein fold (48–50). These structures, together with the prokaryotic Bcl-2-like structure of *Diphtheria* toxin, lead one to consider the possible existence of many more yet unrecognized Bcl-2-like structures in diverse species. Even more important will be the discovery of any biochemical functions that reveal how the novel 3-dimensional structure of Bcl-2 proteins acts to directly or indirectly alter membrane structure and cell death/survival.

2. BENEFITS OF PROGRAMMED CELL DEATH FOR UNI-CELLULAR SPECIES

If programmed cell death evolved for the purpose of cell suicide, then what is the evidence that yeast cells have a purpose in dying? The most obvious answer is that the death of selected individuals serves as a long-term survival advantage for the species. It has been suggested that programmed cell death serves to eliminate yeast cells that are incompetent for mating (51), susceptible to viruses (30,35) and generally less fit due to mutations or aging (33,52,53). An intriguing model system was developed to investigate the advantages of programmed cell death in populations of yeast cells (54). This study linked the ability to die with the ability to genetically adapt to an adverse environment. Fabrizio et al. (52) reported that if the majority of a population of starving yeast has the ability to die early (before they would otherwise die from nutrient depletion), then the entire yeast culture is more likely to eventually establish a new population equilibrium and survive long-term. For this to occur, the dying cells are suggested to employ a programmed death pathway that is activated by mitochondrial superoxide, and the ‘debris’ from dying cells provides nutrients for the rare surviving (better adapted) genetic variants (54,55). If this is true, then the ability of yeast to undergo programmed

cell death would provide an evolutionary advantage for the species. Herker et al. (33) suggest that this cell death process requires the yeast metacaspase *MCA1/YCA1*.

One could argue that the rare variant that survives in a culture of dying yeast is itself a "cheater", a self-preservationist that has simply gained the ability to resist death but is not otherwise better fit in the new environment (55,56). However, this is apparently not the case because a culture of death-resistant yeast has a minimal chance of establishing a long-term culture (33,52). For example, cultures of yeast knockout strains lacking the metacaspase gene *MCA1/YCA1* (*yca1Δ*) have increased short-term survival, but have reduced long-term survival. Similarly, yeast strains that overexpress *SOD1* (superoxide dismutase 1) or mammalian Bcl-2, have improved short-term survival, but fail to maintain long-term survival, presumably because nutrients are exhausted by overpopulation, and the entire population fails (33,52,53). The delayed cell death in these cultures presumably does not allow sufficient time or appropriate conditions for the adaptation, selection or nurturing of potential survivors, resulting in death of the entire population within 40–60 days. These findings offer possible explanations for why it is disadvantageous to the species when the majority of individuals have an extended lifespan, and why programmed cell death may have been required prior to the origin of multi-cellular organisms.

3. MITOCHONDRIA AND PROGRAMMED CELL DEATH

Mitochondria play a central role in mammalian cell death, not only because their disruption results in an energy crisis, but also because they harbor factors that actively promote some types of programmed cell death. Pro- and anti-apoptotic Bcl-2 family members are thought to be the core regulators of mitochondrial outer membrane permeability, a process assumed to release pro-death factors during mammalian cell death (57). It is widely accepted that during mammalian apoptosis, Bax translocates from the cytosol to mitochondria and directly or indirectly permeabilizes the mitochondrial outer membrane resulting in release of cytochrome *c* and other factors that trigger caspase activation, though the mechanisms are not fully delineated (58–60). Preceding or simultaneous with mitochondrial outer membrane permeabilization, normal tubular mitochondria are commonly observed to undergo fragmentation into short punctate structures during mammalian cells apoptosis (7,61–65) (Figure 2A). The connection between cell death and mitochondrial morphology changes is currently debated.

3.1. Mitochondrial Fragmentation During Cell Death From Yeast To Mammals

Given the close link between mitochondrial fission/division and programmed cell death, and the conservation of mitochondrial fission genes across species, we investigated the possibility that dying yeast cells may exhibit mitochondrial morphology changes similar to mammalian cells. Indeed, yeast mitochondria undergo rapid fragmentation when treated with acetic acid (18), a death stimulus previously reported to mimic overgrowth conditions and trigger apoptosis-like death in yeast (15). Following treatment, yeast mitochondria change from tubular structures to beads-on-a-string within 5–10 minutes and finally to isolated fragments within 1–3 hours (Figure 2B). Treatment with the anti-fungal agent amiodarone, H_2O_2, or ethanol also induces yeast mitochondrial fragmentation in yeast (32,66,67). These morphology changes in yeast may parallel the thread-to-grain transition observed with mammalian mitochondria during apoptosis (68). In addition to mammals and yeast, mitochondrial fragmentation also occurs during programmed cell death in other model organisms, including the *Drosophila melanogaster* (69,70) and *Caenorhabditis elegans* (71,72). Thus, it appears that mitochondrial fragmentation is an evolutionarily conserved response when cells face threatening insults, implying an evolutionarily conserved death pathway involving these ancestral organelles.

If mitochondrial fragmentation is conserved during cell death, then what purpose does it serve? Some have suggested, but not all agree, that fragmentation facilitates the release of cytochrome *c* from mammalian mitochondria to facilitate caspase activation and cell death. Alternatively, mitochondrial fission during cell death could serve as a survival attempt rather than a mediator of cell death. One study in mammalian cells found that mitochondrial damage caused by high levels of Ca^{2+} can be limited if that mitochondrion is first divided into segments, not all of which have the capacity to overload with Ca^{2+}, though there was no protection from cell death in these assays (73). The implications are that mitochondrial fission and fusion factors regulate mitochondrial homeostasis, but perhaps independently of their direct roles in fission and fusion. Thus, mitochondrial fragmentation per se may not promote cell death, but instead it is the factors that mediate mitochondrial fission in healthy cells that are responsible for cytochrome *c* release in dying cells, presumably because they adopt alternative functions. In any case, the evidence that cytochrome *c* plays a role in caspase activation or in cell death in worms or flies is lacking, except for specific cell types during fly spermatogenesis (74). Whether or not cytochrome *c* or other factors released from mitochondria contribute to cell death or to activation of the *MCA1*/*YCA1* metacaspase in yeast also remains controversial, although several studies found that cytochrome *c* is released during yeast cell death, and in some cases is suggested to play a causal role (41,75–81).

Mitochondrial fragmentation during cell death is presumed to occur by a mechanism that is at least related to mitochondrial fission/division in healthy cells except carried to greater extremes. This conversion of mitochondrial morphology from reticular tubules to punctate spots closely correlates with translocation of pro-apoptotic Bax from the cytosol to mitochondria and with cytochrome *c* release from mitochondria (82). Furthermore, Bax and the Bax-like protein, Bak, localize at constriction points (predicted fission sites) on mitochondria in apoptotic mammalian cells (61,83). While Bax appears to promote fission/division during mammalian cell death, it has the opposite effect in healthy mammalian cells where Bax promotes mitochondrial fusion (84). However, the biochemical mechanisms that distinguish the fusion versus fission functions of Bax are not known.

Consistent with the possibility that enhanced fission/division of mitochondria can contribute to apoptosis, several reports have shown that decreased fusion of mitochondria sensitizes mammalian cells to apoptosis (85–87). Conversely, enhanced fusion of mitochondria can protect or delay apoptosis (87,88). This evidence is consistent with a positive relationship between mitochondrial fission/division and cell death (89–92). However, mitochondrial fission also occurs in normal healthy cells without detrimental consequences (93).

3.2. Mitochondrial Fission Versus Mitochondrial Fission Factors in Cell Death

The machinery responsible for mitochondrial fission/division in healthy cells was identified in yeast as a complex of proteins, Dnm1, Mdv1/Net2, Caf4 and Fis1 (2,94–103)). Deletion of any one of these fission proteins, except Caf4, causes yeast mitochondria to adopt a large net-like structure (100). Human Drp1, and its yeast homologue Dnm1, are dynamin-like, large GTPases that appear to constrict mitochondrial organelles. In mammalian cells, Drp1 co-localizes with Bax and Bak at these constriction sites during cell death (7,104–106). Depletion of Drp1 by RNAi or overexpression of the dominant negative mutant of human Drp1 (K38A/E) inhibits the excessive mitochondrial fragmentation typical of apoptotic cells and delays mammalian cell death (7,62,85,91,104,107). Dominant negative Drp1 or RNAi knockdown of Drp1 also inhibits or delays other events associated with mammalian cell death including cytochrome *c* release from mitochondria, mitochondrial membrane depolarization, and caspase activation (7,91,107). Yeast Dnm1/Drp1 also promotes yeast cell death, based on the observation that *DNM1*-knockout strains are resistant to several different death stimuli and have significantly delayed mitochondrial fragmentation during yeast cell death (18,30). In

addition, overexpression of a dominant-negative Dnm1 mutant (equivalent to dominant negative human Drp1) also protects yeast from cell death (18). Furthermore, inhibition of Drp1 in worms can inhibit mitochondrial fragmentation during cell death (71). These studies suggest that division of mitochondria or other function of Drp1/Dnm1 can play a causal role in programmed cell death and that this process is conserved from yeast to mammals.

While it is clear that Drp1/Dnm1 have the capacity at least under some circumstances to promote cell death, it is not clear if Drp1/Dnm1 promote cell death via their fission functions or other, yet unknown activity. A small molecule inhibitor (Mdivi) of Drp1/Dnm1, but not of dynamin, was shown recently to inhibit mitochondrial fragmentation in yeast and mammals, and to inhibit Bax-dependent cytochrome *c* release and cell death in mammalian cells (108). The authors concluded that the fission function of Drp1 is dissociable from its ability to permeablize the outer mitochondrial membrane, in part because Mdivi inhibits cytochrome *c* release from isolated mitochondria, where cytochrome *c* release occurs in the absence of mitochondrial fission.

In addition to its pro-death function, mitochondrial division mediated by Drp1/Dnm1 is required to produce new mitochondria that subsequently localize adjacent to synapses along neuronal processes (109,110). Based on knockdown studies, the application of ABT-737, a small molecule inhibitor of anti-apoptotic Bcl-x_L, and by overexpression of dominant negative Drp1, a picture emerges in mammalian neurons where endogenous Bcl-x_L activates Drp1, leading to increased numbers of mitochondria at synapses and dramatically enhanced synaptic activity (110). Given the pro-death activity of Drp1, this was initially unexpected, but fitting with the beneficial effects of may other pro-death molecules, including Bax and Bak (111, 112). Conversely, Bcl-2 and Bcl-x_L can also have pro- as well as anti-death activity (36,113). Although confirmation of this pro-death function in physiological or developmental settings has not yet been reported, the ability of ABT-737 to protect against rundown of synaptic activity during tetanus in acute preparations supports this model (114). Taken together, these studies imply that regulation of mitochondrial fission/division is of central importance in normal cellular functions as well as in cell death (Figure 1).

Yeast Mdv1 and Caf4 can facilitate the interaction between Dnm1 and Fis1 on mitochondria (97,98,100,115–118). Dnm1 interacts with the WD40 repeat domain of Mdv1 or Caf4, while the N-terminal domain of Mdv1 or Caf4 interacts with the Fis1 protein (2,101–103). Although Fis1 is conserved from plants to humans, there are no obvious homologues of Mdv1 or Caf4 in mammals, consistent with new protein structures that offer an explanation for this species specificity (95,99,117,119). Although mammals lack sequence homologs of yeast Mdv1 and Caf4, mammalian Drp1 also interacts with mammalian Fis1 to control mitochondria fission (105,120). Fis1 of *S. cerevisiae* is an 18 kD protein that is anchored to the outer mitochondrial membrane by a C-terminal hydrophobic-basic domain characteristic of transmembrane domains found in Bcl-2, Tom5 and other proteins targeted to the outer mitochondrial membrane (95,121). Consistent with its fission function in yeast, depletion of endogenous human Fis1 decreases mitochondrial fission (85,105,122,123). Decreased mitochondrial fission resulting from Fis1 knockdown can protect mammalian cells from certain apoptotic stimuli (85,124), and overexpression of human Fis1 in mammalian cell lines can promote mitochondrial fission and apoptosis in response to certain death stimuli (120,125–127). Similar to overexpression of Drp1, mitochondrial fragmentation induced by Fis1 overexpression was found to protect from Ca^{2+}-induced cell death due to blockage in efficient transmission of Ca^{2+} signals (128). However, like Drp1, Fis1 also has a pro-survival function, as knockdown of mammalian Fis1, at least in some cell types, results in cell senescence (129,130). Taken together, these studies suggest that mitochondrial fission and fusion modulate cell death differently depending on the specific death stimulus.

Despite the evidence described above, mitochondrial fission factors are not universally accepted as important mediators of cell death. In mammals, inhibition of mammalian Drp1 or Fis1-mediated mitochondrial fragmentation was reported to delay but not prevent cytochrome *c* release and cell death, and had no effect on the release of other pro-apoptotic molecules from mitochondria, including Smac/Diablo, Omi/Htr2 and others (91,107). Inhibition of mitochondrial fragmentation in these studies only delayed but did not ultimately protect cells from death, as these cells did not survive in a clonagenic assay. However, the harsh and continuous death stimuli being applied in these studies likely preclude the possibility of extended or clonagenic survival. Similar early arguments were made about Bcl-2, as overexpressed Bcl-2 often delays death without increasing clonagenicity, though now it is clear that anti-apoptotic Bcl-2 proteins contribute importantly to ultimate cell survival in many physiological settings. Nevertheless, it is still unclear how fragmentation of mitochondria mediated by Drp1 and Fis1 selectively affects cytochrome *c* release but not the release of other factors from the mitochondrial intermembrane space. Another important factor that controls cytochrome *c* release from mammalian mitochondria is Opa1 (131,132). This effect of Opa1 may be a consequence of its role as a regulator of cristae junctions and cristae remodeling in mitochondria. While Opa1 is conserved from mammals to yeast (Mgm1), its role in yeast cell death is unresolved.

3.3. Mitochondrial Fission Is Not Sufficient For Cell Death In Yeast Or Mammals

Since the link between apoptosis and the mitochondrial fission factor Drp1 was initially uncovered (7), investigators have been searching for the relationship between mitochondrial fission/fragmentation and apoptosis. It is now clear that mitochondrial fission is an early event during mammalian apoptosis induced by multiple different physiological and pharmacological insults (7,61–65). Nevertheless, there are multiple examples where mitochondrial fission/fragmentation alone is either not sufficient to induce cell death, or the normal fission machinery is distinct form the apoptotic fission machinery. For example, yeast Fis1 is required for normal mitochondrial fission in healthy cells, but not for mitochondrial fission during yeast cell death (18). In contrast, one study reported that Fis1 is required for ethanol-induced mitochondrial fragmentation (67). Barring differences between assay protocols, this discrepancy is presumably due to yet unknown genetic differences. Embryonic fibroblasts from knockout mice lacking the dynamin-like GTPases mitofusin 1 (Mfn1) or Mfn2, which promote mitochondrial fusion, have profoundly fragmented mitochondria (133). While these cells survive in this state, they can be more sensitive to cell death if treated with an insult. A portion of Mfn-deficient mitochondria has reduced membrane potential and defective retention of mitochondrial DNA, indicating that mitochondrial fusion is required for organelle maintenance (134). We found a disconnection between fission and death, as yeast lacking Fzo1 (*fzo1Δ*), homologue of human Mfn-1 and -2, were not prone to cell death even though their mitochondria are profoundly fragmented (Y Fannjiang, B Qi and JMH, unpublished). While ectopic expression of CED-9 (worm Bcl-2) in mammalian cells triggers prominent and sustained fusion of mitochondria, it fails to block apoptosis in mammalian cells (125). Some interpret this and related findings to indicate that regulation of mitochondrial morphology may be a more conserved function of Bcl-2 family proteins, consistent with the lack cell death phenotypes in *Drosophila* with defective Bcl-2 family genes. Strikingly, the potent anti-apoptotic factor vMIA encoded by cytomegalovirus induces mitochondrial fragmentation but inhibits cytochrome *c* release and apoptosis (64,135,136). This unexpected effect of vMIA is apparently due to its ability to also inhibit the “day-job” fusion activity of Bax (137). Similarly, overexpression of mouse GDAP1 (ganglioside-induced differentiation associated protein 1), a mitochondria-localized protein that is commonly mutated in Charcot-Marie-Tooth disease, promotes mitochondrial fission without inducing apoptosis (138). Furthermore, mitochondrial fission proteins are required to efficiently divide mitochondria into four spores derived form a single diploid yeast cell during sporulation, yet this process occurs without inducing cell

death (139). Thus, mitochondrial fission per se is not sufficient to induce cell death and presumably has other important functions such as mitochondrial biogenesis. However, the evolutionarily conserved role of mitochondrial fission proteins in cell death raise the interesting possibility that the processes of mitochondrial fission in healthy cells and of mitochondrial fragmentation during programmed cell death are molecularly related, though distinct.

3.4. Mitochondrial Fragmentation and Mitochondrial Destruction

Although mitochondrial fission/fragmentation per se is not necessarily predictive of cell death (see section 3.3), fission still could potentially be required for cell death. Even if cytochrome *c* release is not mediated by a fission-related process, organelle division could still contribute importantly to cell demise. We found that yeast mitochondrial fragmentation occurs very shortly (seconds to minutes) following addition of a death stimulus (18) (Figure 2B). However, yeast cell death did not occur until hours to days later, suggesting that some critical process transpires between these two time points. Instead, the cell death endpoint correlates temporally with the loss of mitochondrial markers assessed by immunoblot analyses, even though loss of membrane potential often occurred much earlier (18). This coincidence between cell death and the disappearance of mitochondrial markers is consistent with the hypothesis that mitochondrial degradation is the critical event in yeast cell death. Accordingly, mitochondrial loss would constitute the commitment point to cell death, beyond which the cell cannot return to live another day because mitochondria are essential organelles. While we often think of glycolytic yeast as having no need for mitochondria, this scenario provides a good example of the other essential functions performed by mitochondria even in glycolytic yeast, as well as mammalian cells, such as synthesis of iron-sulfur clusters. As implied by this definition of commitment point, yeast cells with fragmented mitochondria can opt to restore their normal tubular mitochondrial morphology and ultimately live. For those yeast that stay the course towards death, mitochondrial loss appears to be mediated by vacuoles, based on electron microscopy and by genetic studies (18,45,140). A similar model was proposed earlier for mammalian neurons (141). If true, then the important question is, what are the signaling events that ultimately lead to mitochondrial loss? Mitochondrial fission was recently suggested to contribute to mitochondrial degradation by triggering authophagy, possibly signaled by occasional depolarization of only one of the two daughter mitochondria following a fission event (142). However, if mitochondrial degradation is a natural consequence of mitochondrial dysfunction, then the question becomes, what is the cause of irreparable mitochondrial damage in dying cells? A gene expression analysis in synchronized yeast cultures yielded the striking finding that most genes in the genome are expressed in a cyclical pattern, and that DNA synthesis did not occur during periods of oxidative phosphorylation, possibly to protect against DNA damage (143,144). While destructive reactive oxygen species (ROS) produced by the electron transport chain on the inner membrane is a popular model, other possibilities remain. Perhaps mitochondria are victims of assault during cell death, or even the victims of self-inflicted damage (e.g. ROS production, excessive fission), but these mechanisms may have first evolved in unicellular species in part to orchestrate destruction of the entire cell.

4. PERSPECTIVES

While we now appreciate that mitochondrial fragmentation is an evolutionarily conserved step during cell death, the molecular details are not delineated. The conservation of mitochondrial fission/fragmentation during cell death across diverse species (yeast, worms, flies and mammals) is consistent with an ancient and conserved role for mitochondria in controlling programmed cell death. What are the critical events downstream of mitochondrial fission/fragmentation that trigger mitochondrial dysfunction/destruction and cell death? What is the difference between mitochondrial fission in healthy and dying cells? Why do healthy mitochondria undergo fission and fusion constantly while dying cells fail to rectify the

imbalance between fission and fusion? What is the fundamental difference between a fragmented mitochondrial segment versus a tubular mitochondrion? What is the relationship between mitochondrial morphology and mitochondrial energetics in healthy cells? How do mitochondrial fission factors contribute to cell death when cytochrome *c* release is dispensable? What is the biochemical function of Bcl-2 family proteins (e.g. CED-9, Bcl-x_L) when they interact with mitochondrial fission and fusion factors (e.g. Drp1, Mfn2) (110,125)? Is the normal cellular/biochemical function of Bcl-2 family members to regulate mitochondrial dynamics? If true, what are the yeast counterparts of Bcl-2? Interestingly, expression of mammalian Bcl-2 and Bcl-x_L proteins in yeast cause a fused or netted mitochondrial morphology (18). Therefore, yeast may serve as an excellent model for exploring the link between these two fundamental processes, programmed cell death and mitochondrial morphology.

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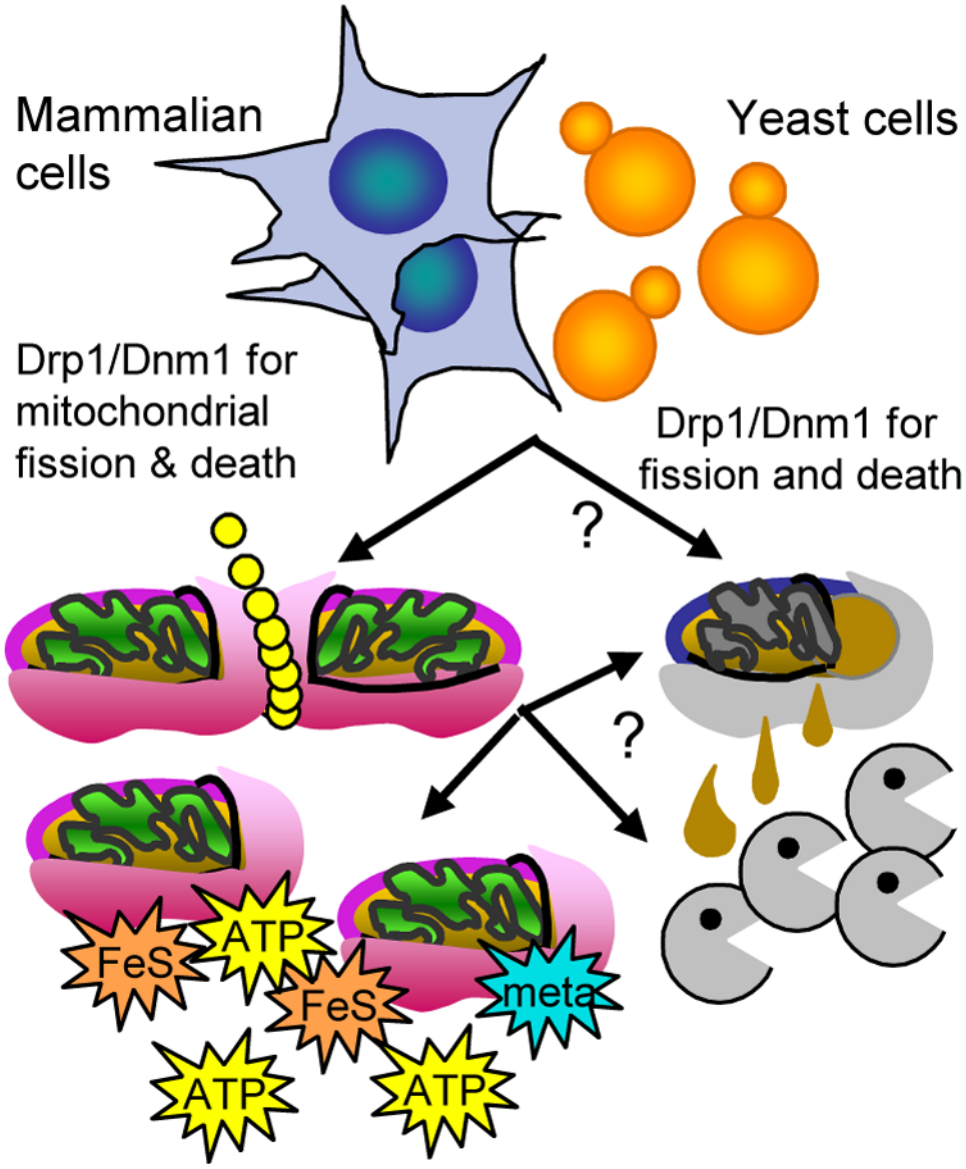

Figure 1. Conserved overlapping cell survival and death pathways

Mitochondrial fission factor Drp1/Dnm1 may have distinguishable biochemical interactions that facilitate mitochondrial fission in healthy and dying cells.

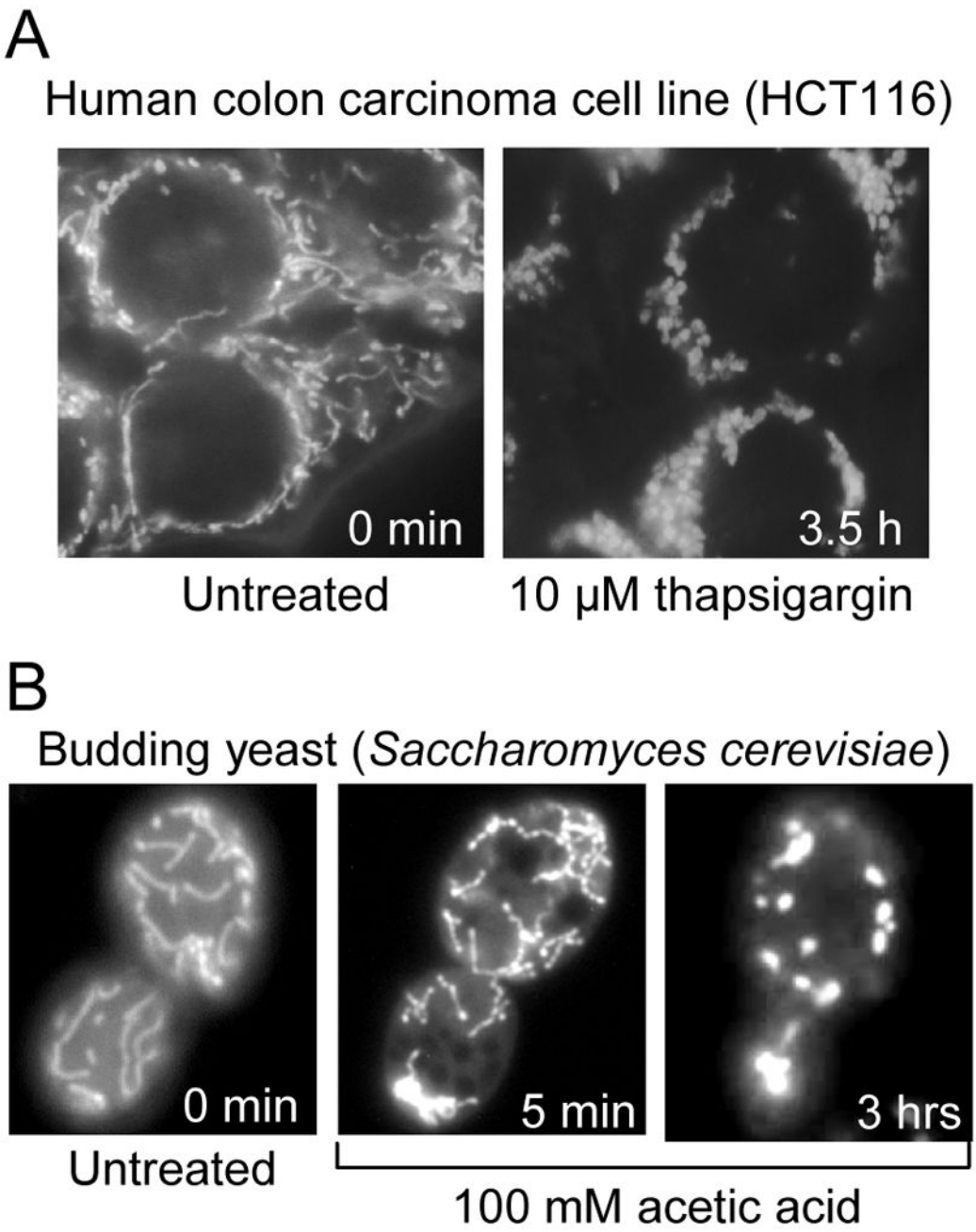

Figure 2. Mitochondrial fragmentation during death of both humans to yeast cells

(A) Fluorescence microscopy of HCT116 colon carcinoma cells stained with 100 nM Mitotracker CMXROS (Molecular Probes) for 15 minutes to visualize mitochondria before and after thapsigargin treatment. Cells were fixed and imaged with an inverted microscope (Nikon TE200) microscope at 100×. (B) Log phase wild type yeast (BY4741 strain background) expressing mitochondria-targeted GFP (106) were imaged live before and after treatment with 100 mM acetic acid using an upright fluorescence microscope (Nikon Eclipse 800) at 100×. Yeast with fragmented mitochondria can be observed starting at 1 h post-treatment, and the vast majority of cells exhibited fragmented mitochondria by 3 hours after treatment with acetic acid.