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News and Commentary

The role of mitochondria in apoptosis induction in *Caenorhabditis elegans*: more than just innocent bystanders?

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Bcl-2 proteins are key regulators of apoptosis in animals as diverse as *Caenorhabditis elegans* and mammals. This commentary is written in honor of Stan Korsmeyer, whose name we automatically associate with Bcl-2 proteins. Stan Korsmeyer's work was instrumental in the identification and functional characterization of mammalian Bcl-2, the founding member of the family of Bcl-2 proteins, as well as of Bax-like and BH3-only proteins.[1–4] In addition, important concepts of apoptosis regulation, for example the realization that pro- and antiapoptotic Bcl-2 proteins antagonize each others' functions, emerged from his studies.[5] This pioneering work forms the foundation of our current understanding of Bcl-2 proteins and, as discussed below, continues to provide inspiration for those seeking to expand our knowledge regarding this important protein family.

The Apoptotic Function of Mitochondria

An active role for mitochondria in apoptosis induction is well established in mammals. [6,7] In many types of mammalian cells, the release of cytochrome *c* from the mitochondrial intermembrane space (IMS) in response to apoptotic stimuli is required for the assembly and activity of the apoptosome, which is composed of the adaptor protein Apaf-1 and the initiator caspase Caspase-9.[8] Specifically, cytochrome *c* released from the IMS binds to WD-40 repeats in the C-terminal half of the Apaf-1 protein, thereby allowing Apaf-1 oligomerization and binding to Caspase-9. The subsequent apoptosome-dependent processing and activation of effector caspases such as Caspase-3 is a necessary event in a cell's commitment to the cell-death fate. Therefore, mitochondria play a crucial, active role in apoptosis induction in mammals.

In invertebrate models of apoptosis, such as the fly *Drosophila melanogaster* and the worm *C. elegans*, it is less clear what role mitochondria play during apoptosis and, in particular, during apoptosis induction.[9,10] While key regulators of apoptosis in *Drosophila* as well as *C. elegans* have been found in association with mitochondria, the significance of these associations has not been rigorously tested. A number of investigators have explored whether cytochrome *c* is released from the IMS in response to apoptotic stimuli in *Drosophila* but no evidence in support of cytochrome *c* release has so far been obtained. In addition, findings that cytochrome *c* can stimulate the activity of the *Drosophila* apoptosome, which is composed of the Apaf-1-like adaptor protein Dark and the initiator caspase Dronc, *in vitro* are controversial. Furthermore, it has recently been demonstrated that in contrast to the assembly of the mammalian apoptosome, the assembly of the *Drosophila* apoptosome does not require binding of cytochrome *c* to the WD-40 repeats of the Dark protein.[11] However, the analysis of *Drosophila* mutants lacking one of two *Drosophila cytochrome c* genes, the *cyt-c-d* gene, indicates that cytochrome *c* is required for the activation of effector caspases during spermatid differentiation, a process that involves an apoptosis-like process and is dependent on Dark and Dronc.[12] These results suggest that, at least under certain circumstances (i.e. during spermatid differentiation), the *Drosophila* apoptosome does require cytochrome *c* for its assembly and/or activity.

At least to our knowledge, it has yet to be investigated whether cytochrome *c* is released from the IMS in response to apoptotic stimuli in *C. elegans*. Recent biochemical studies suggest that the *C. elegans* apoptosome, which is composed of the Apaf-1-like adaptor protein CED-4, facilitates the autoprocessing and activation of the caspase CED-3 in the absence of cytochrome *c* (CED-3 is currently the only *C. elegans* caspase with an apparent function in apoptosis). Specifically, purified recombinant CED-4 was found to be sufficient for proCED-3 processing and hence, CED-3 activation *in vitro*.[13] Furthermore, unlike Apaf-1 and Dark, the CED-4 protein lacks WD-40 repeats (which have been shown to be required for cytochrome *c* binding to Apaf-1) and, for this reason, most likely is unable to bind cytochrome *c*. That the outer membrane of *C. elegans* mitochondria can become permeable to certain factors at a late stage of apoptosis is suggested by the finding that CPS-6 and WAH-1, *C. elegans* homologues of the mammalian proteins EndoG and AIF, respectively, can be released from the IMS in a CED-3-dependent manner in apoptotic cells.[14,15]

Apoptosome Activation: Direct and Indirect Pathways

If not through cytochrome *c* binding, how is the assembly and activity of the apoptosome regulated in *Drosophila* and *C. elegans*? While there is currently no satisfying answer to this question in *Drosophila*, an alternative mechanism for regulating apoptosome activity has been described in *C. elegans*.

Rather than being induced by apoptotic stimuli, the assembly and activity of the *C. elegans* apoptosome appears to be blocked in healthy cells.[10] Specifically, in healthy *C. elegans* cells, CED-4 is sequestered to the outer mitochondrial membrane (OMM) by binding to the antiapoptotic Bcl-2-like protein CED-9, which localizes to the OMM. The interaction between CED-9 and CED-4 blocks apoptosome assembly and consequently proCED-3 processing and activation. In response to apoptotic stimuli, the BH3-only protein EGL-1 binds to the CED-9-CED-4 complex, which induces a conformational change in CED-9 that results in the release of CED-4. CED-4 released from the complex subsequently assembles into the active apoptosome. Therefore, apoptosome assembly and activity in *C. elegans* is directly regulated by members of the family of Bcl-2 proteins, the BH3-only protein EGL-1 and the Bcl-2-like protein CED-9. We will refer to this mechanism of apoptosome regulation as the 'direct pathway' (Figure 1a).

Apoptosome assembly and activity in mammals is also regulated by members of the family of Bcl-2 proteins but in an indirect way.[6,7] The release of cytochrome *c* from the IMS in response to apoptotic stimuli is mediated by the proapoptotic Bcl-2 family member Bax, the activity of which in healthy cells is blocked by antiapoptotic Bcl-2 proteins. In response to apoptotic stimuli, BH3-only proteins are activated and inhibit antiapoptotic Bcl-2 proteins by physically interacting with them. Consequently, Bax is relieved from the block imposed by antiapoptotic Bcl-2 proteins. Active Bax subsequently mediates cytochrome *c* release from the IMS, thereby triggering apoptosome assembly. We will refer to the cytochrome *c*-dependent activation of the apoptosome as the 'mitochondrial' or 'indirect pathway' (Figure 1b). How Bax mediates cytochrome *c* release remains to be resolved. One of the mechanisms proposed is through a process called mitochondrial fragmentation (see below).

So, while mammals and worms mainly use the same players to regulate the assembly and activity of the apoptosome (i.e. members of the family of Bcl-2 proteins, Apaf-1-like adaptor proteins, caspases), they use them in different ways. As discussed below, recent studies in worms challenge this view and instead indicate that a mitochondrial or indirect pathway also plays a role in apoptosis induction in worms. Furthermore, recent studies in mammals hint at the possibility that a direct pathway may function in mammals as well.

Mitochondrial Fragmentation During Apoptosis

Mitochondria are double membrane-bound organelles that are multifunctional and that form tubular networks in many cell types. For example, mitochondria are essential for ATP generation, the biogenesis of iron-sulfur proteins, and various metabolic processes. Mitochondria are also highly dynamic organelles that constantly divide and fuse.[16] The relative rates of mitochondrial membrane fission and fusion determine the morphology of mitochondria and changes in these rates can have dramatic effects on mitochondrial morphology. Indeed, mitochondria rapidly alter their morphology in response to various stimuli. It is still unclear what the consequences of changes in mitochondrial morphology are. One plausible model is that they affect one or more of the various functions of mitochondria. This model is supported by studies in the yeast *Saccharomyces cerevisiae* and in mammalian cells and the finding that respiration and hence, ATP generation, is compromised in mitochondria that are defective in fusion.[16]

One type of stimulus that can induce changes in mitochondrial morphology is apoptotic stimuli. Specifically, tubular networks of mitochondria in cultured mammalian cells and in cells in *C. elegans* embryos have been shown to 'fragment' into punctiform organelles during apoptosis, a process referred to as 'mitochondrial fragmentation'[17] (Figure 2). In both systems, mitochondrial fragmentation in response to apoptotic stimuli can occur in the absence of caspase activity, which indicates that this change in mitochondrial morphology is not merely a consequence of caspase activation and the general destruction of the apoptotic cell. Indeed, it has been proposed that mitochondrial fragmentation is causally involved in apoptosis induction. This model is mainly based on studies in which investigators artificially changed mitochondrial morphology by perturbing mitochondrial fission. To that end they used Drp1, a dynamin-related, large GTPase required for mitochondrial fission, as a tool. Specifically, reducing the activity of Drp1 in cultured mammalian cells by expressing a dominantly-interfering *Drp1* construct blocked mitochondrial fragmentation in response to apoptotic stimuli and delayed cytochrome *c* release and cell death.[18] Similarly, reducing the activity of the *C. elegans* Drp1 homologue DRP-1 in *C. elegans* embryos by expressing a dominantly-interfering *drp-1* construct blocked mitochondrial fragmentation in response to apoptotic stimuli and prevented cell death in about 20% of the cells. Furthermore, the overexpression of the wild-type *drp-1* gene in *C. elegans* embryos induced excessive mitochondrial fragmentation and resulted in the apoptotic death of cells in the absence of apoptotic stimuli.[19] These results indicate that Drp1/DRP-1-mediated mitochondrial fragmentation in cultured mammalian cells and in *C. elegans* is not strictly required for apoptosis induction but is necessary for its efficient execution. Furthermore, these results suggest that DRP-1-mediated mitochondrial fragmentation can be sufficient for apoptosis induction in *C. elegans*. Hence, mitochondria play a more active role during apoptosis induction in *C. elegans* than previously recognized.

The Roles of the Mitochondrial Fission and Fusion Machineries in Apoptosis Induction

Is it the process of mitochondrial fragmentation *per se* or an apoptotic function of Drp1/DRP-1 unrelated to its role in mitochondrial fission that is required for efficient apoptosis induction? This question has been addressed in a number of studies using cultured mammalian cells. Mitochondrial fragmentation observed in apoptotic cells could be the result of increased mitochondrial fission, decreased mitochondrial fusion or a combination thereof.[16] Based on this knowledge, investigators tested whether altering the function of components of the mitochondrial fusion machinery or components of the mitochondrial fission machinery other than Drp1 can affect

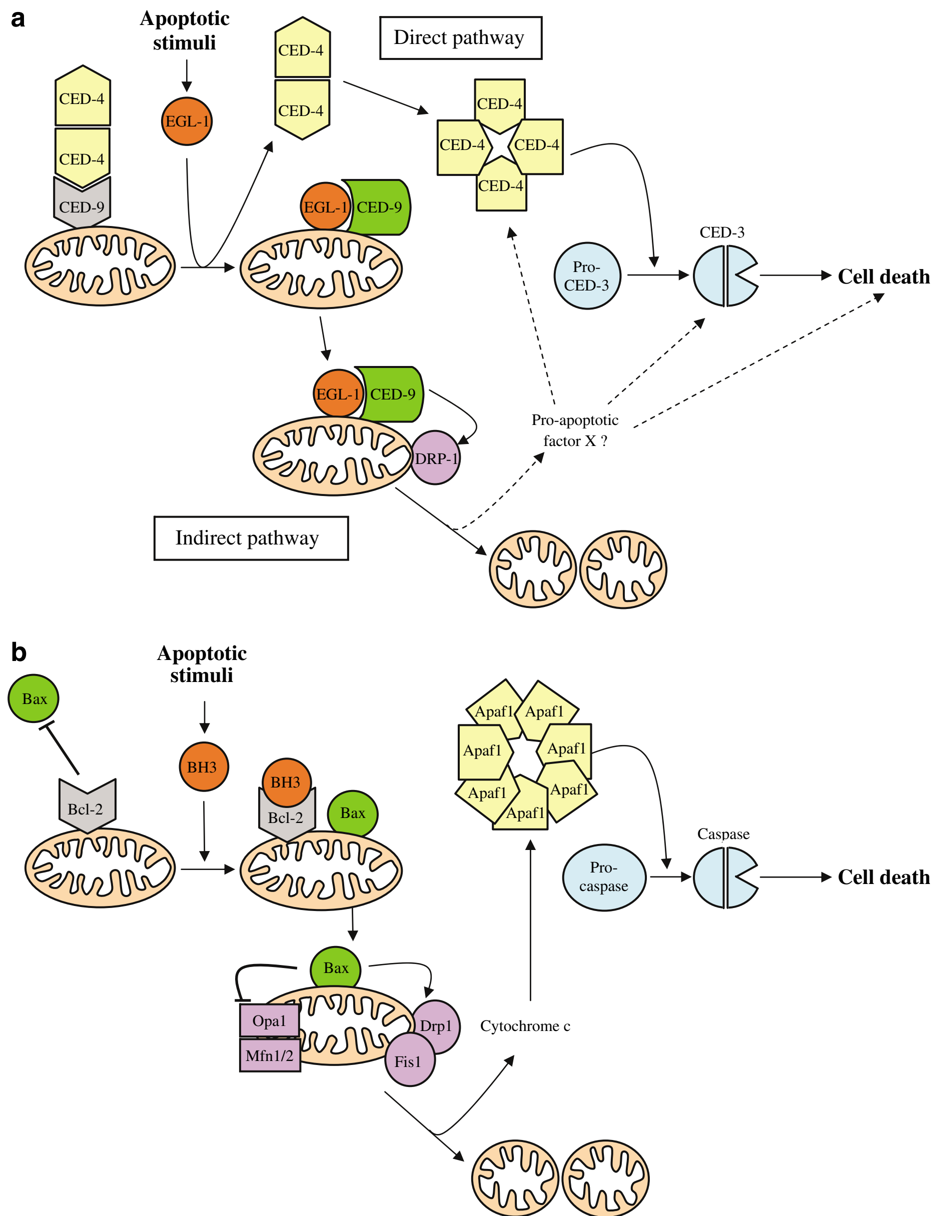

Figure 1 Molecular models of apoptosis induction. Simplified models for the molecular interactions occurring during the induction of apoptosis are shown. (**a**) Molecular model of the induction of apoptosis in *C. elegans*. In healthy cells, CED-9 blocks the ability of CED-4 to assemble into the active apoptosome and to facilitate proCED-3 processing and activation. In response to apoptotic stimuli, EGL-1 binds to the CED-9-CED-4 complex, which results in the release of CED-4, apoptosome assembly, and some CED-3 activation (direct pathway). EGL-1 binding to CED-9 also triggers a conformational and functional change in CED-9, presumably resulting in DRP-1 activation and DRP-1-mediated mitochondrial fragmentation. Mitochondrial fragmentation promotes apoptosis induction by enhancing the activity of the apoptosome or CED-3, or by acting in parallel to the apoptosome and CED-3 (indirect pathway). It remains to be determined whether the proapoptotic activity of DRP-1 is related to its role in mitochondrial fission. (**b**) Molecular model of the induction of apoptosis in mammals. In healthy cells, Bcl-2 blocks the activity of Bax. In response to apoptotic stimuli, BH3-only proteins bind to Bcl-2, which results in Bax activation, Bax translocation to the OMM, cytochrome *c* release, apoptosome assembly and caspase activation. Bax activation also results in mitochondrial fragmentation, which presumably is mediated through the activation of the fission proteins Drp1 and Fis1 and/or the inhibition of the fusion proteins Opa1, Mfn1 and Mfn2. Mitochondrial fragmentation promotes apoptosis induction by facilitating cytochrome *c* release. It remains to be determined whether the proapoptotic activity of Drp1 and Fis1 and the antiapoptotic activity of Opa1, Mfn1 and Mfn2 are related to their role in mitochondrial fission and fusion

apoptosis induction. Results from these studies clearly indicate that components of the fission and fusion machineries other than Drp1 play a role in apoptosis induction. However, results from these studies have so far failed to provide a satisfying answer to the question whether it is mitochondrial fragmentation *per se* or a function of the fission and fusion machineries unrelated to their roles in regulating mitochondrial morphology that is involved in apoptosis induction. For example, as in the case of reducing Drp1 activity, reducing the activity of Fis1 (a protein required for mitochondrial fission), or overexpressing either *Mfn1* or *Mfn2* (two genes that encode dynamin-related proteins required for mitochondrial fusion) in cultured mammalian cells blocks mitochondrial fragmentation and delays cytochrome *c* release

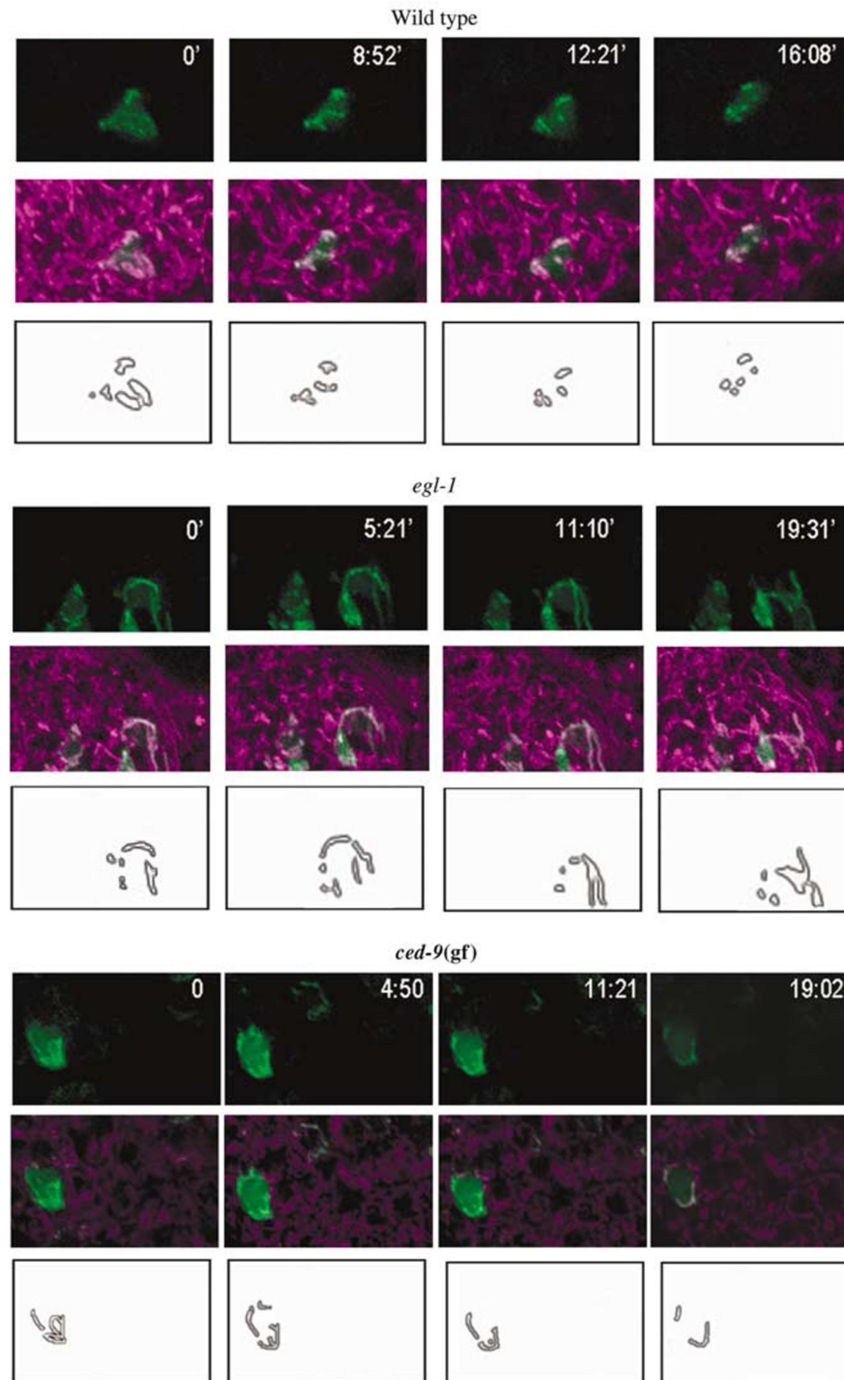

Figure 2 Mitochondrial morphology changes in *C. elegans* cells destined to die. Representative time-lapse series of confocal mitoGFP, mitoGFP/rhodamine overlay and schematic of mitochondrial morphology (from top to bottom) of wild-type, *egl-1(n1084 n3082*lf*)*, and *ced-9*(*n1950*gf) animals carrying a stable P_{egl-1}*mitogfp* transgene. The P_{egl-1}*mitogfp* transgene labels mitochondria specifically in cells destined to die. Images represent maximum intensity projections from four consecutive confocal image planes (0.5 μm). Time in minutes. Note that in wild-type animals, the morphology of mitochondria in cells destined to die changes from a reticulum to punctiform organelles. This is not observed in the *egl-1*(lf) or *ced-9*(gf) mutant background (Figure adapted from Jagasia *et al.*[19])

and cell death in response to apoptotic stimuli.[20,21] In contrast, as occurs with overexpression of wild-type *drp-1* in *C. elegans*, overexpressing *Fis1* or reducing the activity of Opa1 (another dynamin-related protein required for mitochondrial fusion) induces mitochondrial fragmentation and cell death in the absence of apoptotic stimuli.[21–23] Furthermore, reducing the activity of Mfn1 and Mfn2 in cultured mammalian cells increases the cells' sensitivity to apoptotic stimuli.[20] These results demonstrate that Drp1, Fis1, Mfn1, Mfn2, and Opa1 can affect apoptosis induction in cultured mammalian cells. However, cell death, but not mitochondrial fragmentation induced by reducing Opa1 activity or overexpressing *Fis1*,

was blocked by the overexpression of Bcl-2 and Bcl-x_L, respectively.[22,23] There are at least two possible explanations for this observation: (i) mitochondrial fragmentation might not be sufficient for cell death in the presence of high levels of Bcl-2 or Bcl-x_L (and, hence, presumably low levels of Bax activity); (ii) the apoptotic function of mitochondrial fission and fusion proteins might be distinct from their role in regulating mitochondrial morphology. Clearly, additional studies are necessary to distinguish between these possibilities.

Members of the Family of Bcl-2 Proteins Regulate Mitochondrial Fragmentation in Apoptotic Cells

As mentioned above, reducing the activity of Drp1 or Fis1, or increasing the activity of Mfn1 or Mfn2 in cultured mammalian cells delays cytochrome *c* release and hence, most likely apoptosome activation. Therefore, it has been proposed that mitochondrial fission proteins promote Bax-mediated cytochrome *c* release from the IMS and that mitochondrial fusion proteins act to antagonize this process.[17] This hypothesis is supported by the finding that in response to apoptotic stimuli, Bax colocalizes with components of the fission and fusion machineries, such as Drp1 and Mfn2, to discrete foci on mitochondria that possibly represent future sites of mitochondrial fission. Furthermore, the overexpression of *Bax* induces mitochondrial fragmentation in cultured mammalian cells, implying that Bax can functionally interact with the fission and fusion machineries in response to apoptotic stimuli (Figure 1b). It remains to be determined how and in what way Bax affects the activity of components of the fission and fusion machineries and how these functional interactions might facilitate cytochrome *c* release.

Unlike in cultured mammalian cells, it has not yet been explored whether fission and fusion proteins other than DRP-1 play a role in apoptosis induction in *C. elegans*. Therefore, we will limit our discussion to DRP-1 and DRP-1-mediated mitochondrial fragmentation. As in cultured mammalian cells, members of the family of Bcl-2 proteins appear to play a pivotal role in the induction of mitochondrial fragmentation in response to apoptotic stimuli in *C. elegans*.[19] Specifically, a complex of the BH3-only protein EGL-1 and the Bcl-2-like protein CED-9 appears to adopt a proapoptotic, Bax-like function and induce DRP-1-mediated mitochondrial fragmentation in response to apoptotic stimuli (Figures 1a and 2). How EGL-1 and CED-9 induce DRP-1-mediated mitochondrial fragmentation remains to be determined. That EGL-1 and CED-9 can regulate mitochondrial morphology is furthermore supported by recent work by Martin *et al.*[24] Martin *et al* observed that the overexpression of CED-9, but not CED-9 and EGL-1, in cultured mammalian cells can promote mitochondrial reorganization most likely by enhancing mitochondrial fusion. In addition, they found that the overexpression of CED-9 can partially antagonize mitochondrial fragmentation in cultured mammalian cells exposed to apoptotic stimuli. Furthermore, it was demonstrated that overexpressed CED-9 can interact with overexpressed mammalian Mfn2 in cultured mammalian cells. While the significance of these findings remains to be determined they confirm that EGL-1 and CED-9 can regulate mitochondrial morphology. These findings also hint at the possibility that EGL-1- and CED-9-induced mitochondrial fragmentation in apoptotic cells in *C. elegans* is not only the result of the activation of DRP-1-mediated mitochondrial fission but also the inhibition of FZO-1- (FZO, FZO mitochondrial fusion protein-related; http://www.wormbase.org) mediated mitochondrial fusion (FZO-1 is the *C. elegans* homologue of *S. cerevisiae* Fzo1 and mammalian Mfn1 and Mfn2).

A Direct as Well as Indirect Pathway is at Work in *C. elegans*

Like in mammals, the apoptosome is also a likely target of mitochondrial fragmentation in apoptotic cells in *C. elegans*. Mitochondrial fragmentation in response to apoptotic stimuli occurs in the absence of a functional *ced-4* gene and hence, independently of the activity of the apoptosome. However, apoptosis induced by excessive DRP-1-mediated mitochondrial fragmentation is dependent on a functional *ced-4* gene. These observations suggest that mitochondrial fragmentation is induced prior to the activation of the apoptosome and promotes apoptosome-dependent cell death. Mitochondrial fragmentation might promote apoptosome-dependent cell death by directly enhancing the activity of the apoptosome or, alternatively, by acting in parallel to the apoptosome (Figure 1a).

Through what mechanism or mechanisms might mitochondrial fragmentation enhance the activity of the apoptosome or act in parallel to the apoptosome to promote cell death in *C. elegans*? As discussed above, while it has not been tested experimentally whether cytochrome *c* is released from the IMS in response to apoptotic stimuli in *C. elegans*, there are reasons to believe that *C. elegans* cytochrome *c* might not play a significant role in apoptosis induction. However, it is possible that mitochondrial fragmentation in response to apoptotic stimuli facilitates the release from the IMS of a proapoptotic factor or factors other than cytochrome *c*. WAH-1 and CPS-6 are potential candidates for such proapoptotic factors; but as their release from the IMS depends on the activity of the caspase CED-3, it is unlikely that they are the culprits.[14,15] One reason why the real culprits might still be at large is that many mitochondrial proteins have functions that are essential in healthy cells and that most essential *C. elegans* genes have so far not been tested in a rigorous way for a possible proapoptotic function. Alternatively, it is feasible that it is changes in specific mitochondrial functions (i.e. respiration, biogenesis of iron-sulfur proteins etc.) rather than changes in the permeability of the OMM that are induced by mitochondrial fragmentation in response to apoptotic stimuli and that promote cell death in *C. elegans*. As the fragmentation of mitochondria during apoptosis is a process conserved in mammals and *C. elegans*, the identification and analysis of the mechanisms through which mitochondrial fragmentation promotes cell death in *C. elegans* might also contribute to our understanding of the apoptotic function of this process in mammals.

In summary, the recent work on the role of mitochondrial fragmentation in *C. elegans* apoptosis indicates that a mitochondrial or indirect pathway for apoptosis induction

exists in *C. elegans*. The normal function of this indirect pathway appears to be to enhance the activity of the direct pathway. However, the activation of the indirect pathway can be sufficient to induce apoptosis in a manner that is dependent on the direct pathway (Figure 1a).

Why Two Pathways in *C. elegans*?

Why does *C. elegans* need two pathways when mammals need only the indirect pathway to efficiently induce apoptosis? One possibility is that two pathways are put to work in *C. elegans* in order to 'speed things up'. In contrast to cultured mammalian cells, in which the apoptotic process can take hours if not days, apoptosis is a very rapid process in *C. elegans*: it takes only about 20 min from the first sign of apoptosis until the doomed cell is engulfed by a neighboring cell. We favor the hypothesis that worms and mammals might not be that different and that both pathways, the direct and indirect pathway, might be at work in mammals as well. The idea that a direct pathway also functions in mammals fell out of favor mainly because of the lack of evidence for a physical interaction between antiapoptotic Bcl-2 proteins and Apaf-1.[25] However, the recent analysis of mice expressing a mutant cytochrome *c* protein unable to bind to Apaf-1 (and hence, most likely unable to activate the apoptosome), suggests that one or more cytochrome *c*-independent, Apaf-1-dependent pathways do exist for the activation of caspases in mammals.[26]

Mitochondria have long been thought to not play a role in apoptosis induction in *C. elegans;* however, the recent studies on mitochondrial fragmentation have revealed that they are more than just innocent bystanders. The time may also have come to revisit the role of mitochondria in apoptosis induction in mammals and to consider that mitochondria might 'only' be innocent bystanders in a pathway functionally equivalent to the direct pathway in *C. elegans*.

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