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Caenorhabditis elegans drp-1 and *fis-2* Regulate Distinct Cell-Death Execution Pathways Downstream of *ced-3* and Independent of *ced-9*

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SUMMARY

The dynamin family of GTPases regulate mitochondrial fission and fusion processes and have been implicated in controlling the release of caspase activators from mitochondria during apoptosis. Here we report that profusion genes *fzo-1* and *eat-3* or the profission gene *drp-1* are not required for apoptosis activation in *C. elegans*. However, minor proapoptotic roles for *drp-1* and *fis-2*, a homolog of human Fis1, are revealed in sensitized genetic backgrounds. *drp-1* and *fis-2* function independent of one another and the Bcl-2 homolog CED-9 and downstream of the CED-3 caspase to promote elimination of mitochondria in dying cells, an event that could facilitate cell-death execution. Interestingly, CED-3 can cleave DRP-1, which appears to be important for DRP-1's proapoptotic function, but not its mitochondria fission function. Our findings demonstrate that mitochondria dynamics do not regulate apoptosis activation in *C. elegans* and reveal distinct roles for *drp-1* and *fis-2* as mediators of cell-death execution downstream of caspase activation.

INTRODUCTION

Genetic studies in *C. elegans* have led to the identification of a central killing pathway that is conserved between nematodes and humans (Horvitz, 1999). In cells destined to undergo apoptosis, the BH3-only proapoptotic protein EGL-1 is upregulated and binds to CED-9, an antiapoptotic Bcl-2 homolog, resulting in the disassociation of CED-4, a mammalian Apaf-1 homolog, from the CED-4/CED-9 complex tethered on the surface of mitochondria (Horvitz, 1999). CED-4 subsequently oligomerizes and activates the CED-3 caspase zymogen (Yan et al., 2006), which then orchestrates numerous cell disassembly and cleanup processes, including fragmentation of chromosomal DNA (Parrish et al., 2001; Wang et al., 2002) and removal of cell corpses (Reddien and Horvitz, 2004).

The cell-death activation process in mammals appears to be far more complex and involves release of proapoptotic factors from the intermembrane space of mitochondria. For example, Bcl-2 family proteins impinge on the structure of mitochondria, causing dramatic changes in mitochondria size and cristae structure and ultimately increased permeability of the outer mitochondrial membrane to caspase activators such as cytochrome c and Smac/Diablo (Antignani and Youle, 2006; Cereghetti and Scorrano, 2006). In addition, dynamin family GTPases, such as DRP1, which is required for mitochondrial fission, and OPA1 and MFN1/FZO1, which control inner and outer mitochondrial membrane fusion events, respectively, have been reported to mediate some of the structural rearrangements of mitochondria observed during apoptosis (Cereghetti and Scorrano, 2006; Chan, 2006). Downregulation of MFN1/FZO1 and concurrent activation of DRP1 was shown to cause dramatic fragmentation of the mitochondrial network during apoptosis (Frank et al., 2001; Karbowski et al., 2002, 2004), whereas disruption of OPA1 oligomers leads to remodeling of inner membrane cristae (Cipolat et al., 2006; Frezza et al., 2006). These changes in mitochondrial network and structure, in some cellular contexts (Frank et al., 2001; Frezza et al., 2006; Lee et al., 2004), but not in others (Delivani et al., 2006; Estaquier and Arnoult, 2007; Parone et al., 2006), appear to affect the release of cytochrome c from the mitochondrial intermembrane space and subsequent caspase activation. Fis1, which may recruit DRP1 to the outer mitochondrial membrane (Okamoto and Shaw, 2005), has also been suggested to mediate mitochondrial fission and apoptosis signaling in yeast (Fannjiang et al., 2004; Mozdy et al., 2000) and in mammals (Alirol et al., 2006; James et al., 2003; Lee et al., 2004; Parone et al., 2006). Recently, *C. elegans drp-1* was reported to promote *ced-9*-dependent mitochondrial fission and apoptosis, possibly by releasing caspase activating factors from mitochondria (Jagasia et al., 2005), leading to the hypothesis that apoptotic mitochondrial fission is an evolutionarily conserved aspect of caspase activation. However, this hypothesis has yet to be substantiated by genetic analysis. As a result, the exact roles of mitochondrial fission and fusion processes in apoptosis and their positions in the cell-death pathway with respect to caspase activation and Bcl-2 family proteins remain unclear (Parone and Martinou, 2006).

Here, we report systematic genetic and cell biological characterization of the contribution of mitochondrial fission and fusion processes to programmed cell death in *C. elegans.* Surprisingly, we found that loss of the mitochondrial dynamin genes, *drp-1*,

fzo-1, and *eat-3* (an OPA1 homolog), does not affect the activation or the kinetics of programmed cell death in *C. elegans*, even though loss of *drp-1* blocks mitochondrial fission and loss of *fzo-1* or *eat-3* prevents mitochondrial fusion and causes excessive mitochondrial fission. However, minor and independent roles for *drp-1* and *fis-2* (a Fis1 homolog) downstream of CED-3 activation in promoting mitochondrial elimination and cell-death execution were uncovered in sensitized genetic backgrounds. Furthermore, we find that DRP-1 can be cleaved by CED-3 in vitro, and such cleavage appears to be important for DRP-1's pro-apoptotic function in vivo, but not its mitochondrial fission function, suggesting that the proapoptotic function of DRP-1 can be separated from its mitochondrial fission function. Our results argue against a conserved, caspase-activating role for mitochondrial dynamins and highlight distinct roles for *drp-1* and *fis-2* downstream of caspase activation.

RESULTS

drp-1, *fzo-1*, and *eat-3* Regulate Mitochondrial Fission and Fusion in *C. elegans*

The *C. elegans* genome contains three genes, *fzo-1*, *eat-3*, and *drp-1*, which encode orthologs of MFN1/FZO1, OPA1, and DRP1, respectively. Two homologs of Fis1, *fis-1* and *fis-2*, exist in the worm, and they share a similar degree of sequence homology to the human and yeast Fis1 proteins (Figure S1 available online). For each of these genes, we obtained deletion allele(s) that disrupt the respective coding sequences and are expected to be strong loss-of-function (*lf*) or null mutations (Figure S2).

fzo-1(tm1133), *eat-3(ad426)*, and *eat-3(tm1107)* animals exhibit slow growth, reduced brood sizes, and high percentages of embryonic lethality (Avery, 1993), probably due to compromised mitochondrial functions important for the vitality of the cells (Chan, 2006). The *eat-3(ad426)* mutation causes a Val 328 to Ile substitution within the GTPase domain (Avery, 1993; D.G.B. and D.X., unpublished data). *drp-1(tm1108)* animals also exhibit reduced brood sizes and a high percentage of embryonic lethality, whereas *fis-1(tm1867)*, *fis-1(tm2227)*, *fis-2(gk363)*, and *fis-2(tm1832)* animals, as well as *fis-1(tm1867); fis-2(gk363)* double mutant animals, appear superficially wild-type (data not shown). Staining of live embryos with tetramethyl rhodamine ester (TMRE), a mitochondria-specific dye, and thin section electron microcopy analysis revealed striking differences in the overall connectivity of the mitochondrial network in embryos of the various mutant backgrounds (Figures 1A–1N). Compared to wild-type *N2* animals, TMRE-stained mitochondria in *drp-1(tm1108)* embryos appeared clumpy and highly fused (Figures 1A and 1B), and the number of mitochondria observed in EM sections of *drp-1(tm1108)* embryos was low. But individual mitochondria were often very long, with an increased longitudinal mean length of 2.28 μm, compared with 0.95 μm mean length in *N2* animals (Figures 1I, 1J, and 1N). These results suggest a reduction in mitochondrial fission in *drp-1(tm1108)* animals (Labrousse et al., 1999). Conversely, mitochondria appeared highly fragmented in *fzo-1(tm1133)*, *eat-3(ad426)*, and *eat-3(tm1107)* embryos (Figures 1F and 1G and data not shown). Electron micrographs of *fzo-1(tm1133)* and *eat-3(ad426)* embryos displayed an increased number of spherical mitochondria with rather uniform length (mean lengths of 0.38 μm and 0.44 μm, respectively) (Figures 1L and 1M). Therefore, *fzo-1* and *eat-3* appear to be required for mitochondrial fusion in *C. elegans*. In addition, mitochondria in *eat-3(ad426)* embryos had disrupted cristae structures (Figure S3), suggesting that, like OPA1 in mammals, EAT-3 is required for maintenance of mitochondrial cristae (Frezza et al., 2006). Mitochondria in the *fzo-1(tm1133); drp-1(tm1108)* double mutant were highly connected and indistinguishable from those in *drp-1(tm1108)* single mutant embryos (compare Figures 1B and 1H), indicating that *drp-1* is required for the mitochondrial fragmentation observed in *fzo-1* mutants (Bleazard et al., 1999). Similar mitochondrial morphologies were observed in body wall muscle cells, the germline, and the intestinal cells of each mutant strain (data not shown). The defects in mitochondrial morphology observed in *drp-1(tm1108)*, *fzo-1(tm1133)*, and *eat-3(ad426)* animals are similar to those described for loss-of-function mutants of their respective homologs in yeast, mouse, and humans (Chan, 2006). Mitochondrial connectivity appears normal in *fis-1(tm1867)*, *fis-1(tm2227)*, *fis-2(gk363)*, and *fis-2(tm1832)* single mutants and in the *fis-1(tm1867); fis-2(gk363)* double mutant (Figures 1C, 1D, 1E, and 1K and data not shown), suggesting that, unlike in yeast and mammals (Okamoto and Shaw, 2005; Chan, 2006), *C. elegans fis* genes are not required for mitochondrial fission. However, it is possible that *fis-1* and *fis-2* function redundantly with other unknown genes to regulate mitochondrial fission.

Cell Death Occurs Normally in the Absence of Mitochondrial Fission or Fusion in *C. elegans*

We next examined the kinetics of programmed cell death in various mitochondrial fission and fusion mutants by conducting a time course analysis of cell-corpse appearance during embryo development. In this assay, mutants strongly defective in cell death have few cell corpses at all stages of embryonic development (Ellis and Horvitz, 1986; Stanfield and Horvitz, 2000), mutants that are weakly defective in cell death often display a delay in cell-corpse appearance or reduced cell-corpse numbers (Parrish et al., 2001; Stanfield and Horvitz, 2000; Wang et al., 2002), and mutants defective in antiapoptotic genes or genes involved in cell-corpse engulfment have increased cell-corpse numbers at all embryonic stages (Hedgecock et al., 1983; Ellis et al., 1991; Hengartner et al., 1992; Bloss et al., 2003). Although *fzo-1(tm1133)*, *eat-3(ad426)*, and *eat-3(tm1107)* animals had highly fragmented mitochondria (Figure 1), the numbers of cell corpses in these mutants were normal at all stages of embryonic development (Figure S4A), indicating that excessive mitochondrial fragmentation per se does not cause ectopic cell deaths in *C. elegans*. We confirmed this finding in a sensitized genetic background, *ced-1(e1735)*, where engulfment of apoptotic cells is blocked and a small increase in cell deaths will result in a greater increase in the number of persistent cell corpses (Hedgecock et al., 1983; Ellis et al., 1991; Bloss et al., 2003) (Figure S4B). The cell-corpse numbers or the kinetics of cell-corpse appearance were also unaffected in *drp-1(tm1108)* animals (Figure S4C), even though mitochondria were constitutively fused (Figure 1). *fis-1(tm1867)*, *fis-2(gk363)*, and *fis-2(tm1832)* single mutants, and *fis-1(tm1867); fis-2(gk363)* double mutant animals also had cell-corpse profiles that did not significantly

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