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Report

Bcl-2 Proteins EGL-1 and CED-9 Do Not Regulate Mitochondrial Fission or Fusion in *Caenorhabditis elegans*

David G. Breckenridge,[1,3,*] Byung-Ho Kang,[2,3] and Ding Xue[1,*]

[1]Department of Molecular, Cellular, and Developmental Biology
University of Colorado
Boulder, CO 80309
USA

[2]Department of Microbiology and Cell Science
Interdisciplinary Center for Biotechnology Research
University of Florida
Gainesville, FL 32611
USA

Summary

The Bcl-2 family proteins are critical apoptosis regulators that associate with mitochondria and control the activation of caspases. Recently, both mammalian and *C. elegans* Bcl-2 proteins have been implicated in controlling mitochondrial fusion and fission processes in both living and apoptotic cells. To better understand the potential roles of Bcl-2 family proteins in regulating mitochondrial dynamics, we carried out a detailed analysis of mitochondria in animals that either lose or have increased activity of *egl-1* and *ced-9*, two Bcl-2 family genes that induce and inhibit apoptosis in *C. elegans*, respectively. Unexpectedly, we found that loss of *egl-1* or *ced-9*, or overexpression of their gene products, had no apparent effect on mitochondrial connectivity or mitochondrial size. Moreover, loss of *ced-9* did not affect the mitochondrial morphology observed in a *drp-1* mutant, in which mitochondrial fusion occurs but mitochondrial fission is defective, or in a *fzo-1* mutant, in which mitochondrial fission occurs but mitochondrial fusion is restricted, suggesting that *ced-9* is not required for either the mitochondrial fission or fusion process in *C. elegans*. Taken together, our results argue against an evolutionarily conserved role for Bcl-2 proteins in regulating mitochondrial fission and fusion.

Results

Mitochondrial Morphogenesis Is Not Affected in *egl-1(lf)* or *ced-9(lf)* Mutants

Recently, the *C. elegans* proapoptotic BH3-only Bcl-2 protein EGL-1 has been implicated in promoting mitochondria fission during apoptosis [1]. In addition, the *C. elegans* antiapoptotic Bcl-2 protein CED-9 was shown to mediate mitochondria fission during apoptosis in one study [1] but was found to promote mitochondria fusion in healthy cells in another [2], calling into question of the exact physiological roles of *C. elegans* Bcl-2 family proteins in regulating mitochondria dynamics. To address the critical issue of whether Bcl-2 proteins regulate normal mitochondrial fission or fusion process in *C. elegans*, we carried out a comprehensive analysis of mitochondria morphology and structure in animals that either lose or have increased activity of *egl-1* or *ced-9*. First, we visualized mitochondria in early *C. elegans* embryos that were stained with the mitochondria-specific dye tetramethylrhodamine ethyl ester (TMRE); the large blastomere size in early embryos permits clear visualization of the mitochondrial network. In N2 (wild-type) animals, in which mitochondrial fission and fusion processes are balanced, mitochondria appeared as a large network, evenly distributed through out each cell (Figure 1A) [1, 3–7]. In *drp-1(tm1108)* mutant animals, which are null for the DRP-1 protein expression and defective in mitochondrial fission [7], mitochondria appeared as highly connected clusters and asymmetrically distributed in individual blastomeres (Figure 1B), which results from ongoing mitochondrial fusion in the absence of mitochondrial fission [6]. In contrast, in *fzo-1(tm1133)* animals, which harbor a deletion in the *fzo-1* gene and in which mitochondrial fusion is compromised but mitochondrial fission continues [7], the mitochondrial network was disrupted into highly fragmented, punctiform organelles (Figure 1C). Thus, a defect in either the mitochondrial fission or fusion process is clearly identifiable in this assay.

Mitochondria in *egl-1(n3082)* animals, which carry a strong loss-of-function (*lf*) mutation in *egl-1*, appeared undistinguishable from those in wild-type animals (Figure 1D), although somatic programmed cell death is abolished in these animals [8]. Similarly, the mitochondrial network appeared unaffected in *ced-9(n1950 gf)* animals (Figure 1E), which carry a gain-of-function (*gf*) mutation (a G169E substitution) in the *ced-9* gene that prevents EGL-1 from binding to CED-9 [9, 10] and thus blocks *C. elegans* programmed cell death [11]. We also analyzed mitochondria morphology in two *ced-9(lf)* mutants: *ced-9(n1653ts)* and *ced-9(n2812)*. The *n1653* mutation causes a Y149N substitution in CED-9 that reduces its association with CED-4 at the restrictive temperature (25°C) and compromises its apoptosis inhibitory activity [12], leading to ectopic apoptosis. *n2812* is an early nonsense mutation in the *ced-9* gene [13] and a putative null allele that abolishes expression of *ced-9* in *C. elegans* [14]. *ced-9(n2812)* animals are embryonic lethal as a result of excessive apoptosis but can be maintained and analyzed in the *ced-3(lf)* or *ced-4(lf)* mutant background, which blocks apoptosis [11]. As shown in Figures 1F–1H, we observed no significant difference in mitochondrial morphology in *ced-9(n1653ts)*, *ced-4(n1162) ced-9(n2812)*, or *ced-9(n2812); ced-3(n717)* embryos compared to that in N2 embryos or that in *ced-3(n717)* or *ced-4(n1162)* embryos or *ced-9(n1653ts)* embryos at the permissive temperature (Figure S1 available online). We quantified the connectivity of mitochondria in N2, *drp-1(tm1108), fzo-1(tm1133), egl-1(n3082)*, and *ced-9(n2812); ced-3(n717)* blastomeres by generating line intensity plots and calculating the frequency of major TMRE fluorescent spikes (Figure S2; method described in Supplemental Experimental Procedures). In N2 blastomeres, TMRE fluorescent signals varied in frequency, with an average of 0.49 fluorescent spikes/μm (Figure S2). TMRE fluorescent signals were very broad and of low spike frequency in *drp-1(tm1108)* blastomeres (average frequency of 0.16 fluorescent spikes/μm; Figure S2), consistent with large

*Correspondence: breckenridge.david@gmail.com (D.G.B.), ding.xue@colorado.edu (D.X.)
[3]These authors contributed equally to this work

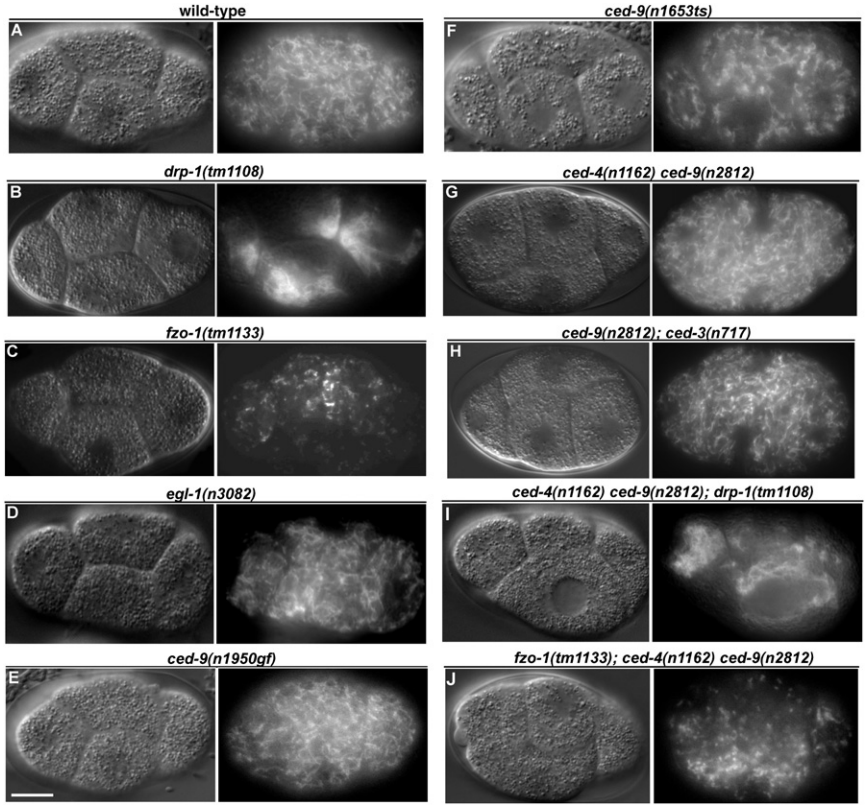

Figure 1. The Mitochondrial Network Is Altered in *fzo-1* and *drp-1* Mutants but Unaffected by Mutations in *egl-1* and *ced-9*

Animals were stained with tetramethylrhodamine ethyl ester (TMRE), a mitochondrial-specific dye, and blastomeres at the four-cell embryonic stage were imaged. Embryos were visualized by differential interference contrast (DIC, left) and rhodamine fluorescence (right) microscopy. Representative images are shown. Compared to wild-type embryos (A), *drp-1(tm1108)* embryos (B) have a highly connected mitochondrial network, whereas mitochondria appeared highly fragmented in *fzo-1(tm1133)* embryos (C). Mitochondria in *egl-1(n3082)* (D), *ced-9(n1950 gf)* (E), *ced-9(n1653ts)* at the restrictive temperature (F), *ced-4(n1162) ced-9(n2812)* (G), and *ced-9(n2812); ced-3(n717)* (H) embryos were indistinguishable from those observed in wild-type embryos. Loss of *ced-9* has no effect on the mitochondria morphology in *drp-1(tm1108)* or *fzo-1(tm1133)* animals. The mitochondrial network in the *ced-4(n1162) ced-9(n2812); drp-1(tm1108)* embryo (I) and in the *fzo-1(tm1133); ced-4(n1162) ced-9(n2812)* embryo (J) is similar to that seen in *drp-1(tm1108)* embryos (B) and *fzo-1(tm1133)* embryos (C), respectively. The scale bar represents 10 μm.

clumps of mitochondria asymmetrically distributed within cells. In contrast, *fzo-1(tm1133)* embryos displayed high frequency of TMRE signal spikes, averaging 2.29 spikes/μm, delineating punctiform mitochondria evenly distributed throughout the cells (Figure S2). The frequency of TMRE signal spikes in *egl-1(n3082), ced-9(n1950 gf)* or *ced-9(n2812); ced-3(n717)* blastomeres was similar to that of N2 animals (an average frequency of 0.44 spikes/μm and 0.48 spikes/μm in *egl-1(n3082)* and *ced-9(n2812); ced-3(n717)* blastomeres; Figure S2). Taken together, these results suggest that loss of *egl-1* or *ced-9* function does not affect mitochondria dynamics and morphology in *C. elegans*.

Of note, a recent report showed that mitochondria appeared highly fragmented in *ced-9(n1653ts)* embryos at the restrictive temperature [2]. However, in that study, embryos were examined at a later stage of development and the mitochondrial fragmentation observed could have been the result of widespread ectopic apoptosis [1, 7], rather than a requirement for *ced-9* to maintain the integrity of the mitochondrial network. Importantly, CED-9 protein is ubiquitously expressed in embryos as early as the two-cell stage [14]. If CED-9 is required to maintain normal mitochondrial networks, its role should be uncovered in early embryos. The expression pattern of EGL-1 is not well understood, but *egl-1* transcription has been shown to be upregulated in several cells destined to die [15]. Nonetheless, our results suggest that the activity of *egl-1* is not required for normal mitochondrial morphogenesis.

We carried out electron microscopy (EM) analysis to confirm the TMRE staining results in Figure 1 and to investigate whether *egl-1* or *ced-9* might play subtle roles in regulating mitochondrial dynamics. In 2D images of EM sections from N2 embryos, mitochondria appeared in a variety of shapes and sizes, ranging from small spherical organelles to longer dumbbell-shaped organelles (Figure 2A), and with a mean longitudinal length of 0.94 μm (Figure 2F). As expected, mitochondria in *drp-1(tm1108)* embryos were very long, with fewer individual mitochondria observed in each cell (Figure S3A) and a mean mitochondrial length of 2.28 μm (Figure 2F) [7]. *fzo-1(tm1133)* embryos displayed only small and spherical mitochondria, with a mean mitochondrial length of 0.38 μm (Figure S3B and Figure 2F). However, mitochondria in *egl-1 (n3082)*, *ced-9(n1950 gf)*, *ced-9(n1653ts)*, and *ced-9(n2812); ced-3(n717)* embryos appeared similar to those observed in N2 embryos and in all cases had mean longitudinal mitochondrial lengths that were not significantly different from those of N2 animals (Figures 2B–2E). Mitochondria in the germline, gut, and muscle cells of adult *egl-1(lf)*, *ced-9(lf); ced-3(lf)*, or *ced-9(gf)* mutants also appeared to be normal (data not shown). The mitochondrial morphology in N2, *drp-1(tm1108)*, *fzo-1(tm1133)*, *egl-1(n3082)*, and *ced-9(n2812); ced-3(n717)* animals was confirmed by serial EM sectioning and 3D reconstruction from the serial images (Figure 3 and Figure S4). Again, mitochondria in N2, *egl-1(n2812)*, and *ced-9(n2812); ced-3(n717)* animals varied in shape and size and were evenly distributed throughout the cell. In contrast, mitochondria in *drp-1(tm1108)* embryos were long, highly interconnected, and clustered around the nucleus, whereas mitochondria in *fzo-1(tm1133)* embryos were small, puntiform, and evenly distributed. Altogether, these results confirm that *egl-1* and *ced-9* do not have a detectable role in regulating mitochondrial fission or fusion in *C. elegans.*

ced-9 Does Not Promote *drp-1*-Dependent Mitochondrial Fission or *fzo-1*-Dependent Mitochondrial Fusion

If CED-9 somehow has both profission and profusion activities as previously reported [1, 2], it is conceivable that loss of *ced-9*