Mitochondrial pathway of apoptosis is ancestral in metazoans

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The mitochondrial pathway of apoptosis is the major mechanism of physiological cell death in vertebrates. In this pathway, proapoptotic members of the Bcl-2 family cause mitochondrial outer membrane permeabilization (MOMP), allowing the release of cytochrome c, which interacts with Apaf-1 to trigger caspase activation and apoptosis. Despite conservation of Bcl-2, Apaf-1, and caspases in invertebrate phyla, the existence of the mitochondrial pathway in any invertebrate is, at best, controversial. Here we show that apoptosis in a lophotrochozoan, planaria (phylum Platyhelminthes), is associated with MOMP and that cytochrome c triggers caspase activation in cytosolic extracts from these animals. Further, planarian Bcl-2 family proteins can induce and/or regulate cell death in yeast and can replace Bcl-2 from these animals. Further, planarian Bcl-2 family proteins can induce and/or regulate cell death in yeast and can replace Bcl-2 proteins in mammalian cells to regulate MOMP. These results suggest that the mitochondrial pathway of apoptosis in animals predates the emergence of the vertebrates but was lost in some lineages (e.g., nematodes). In further support of this hypothesis, we surveyed the ability of cytochrome c to trigger caspase activation in cytosolic extracts from a variety of organisms and found this effect in cytosolic extracts from invertebrate deuterostomes (phylum Echinodermata).

In Drosophila, the role of MOMP and cytochrome c in apoptosis is more controversial. It has been observed that cells undergoing apoptosis in Drosophila do not undergo MOMP as detected by release of cytochrome c (12), although this finding has been challenged (13). However, no release of cytochrome c was observed in Drosophila S2 cells during stress-induced apoptosis (14). ARK, the Drosophila APAF-1 homolog, is required for the activation of DRONC (15), the presumptive initiator caspase and caspase 9 ortholog, which, in turn, activates the effector caspase DRICE. The mechanism of ARK activation is unknown. ARK possesses a WD region (16); however, attempts to show cytochrome c-mediated activation of caspases in cytosolic extract from Drosophila cells (14, 17) have consistently failed. Indeed, knockdown of cytochrome c in Drosophila S2 cells had no effect on ARK-dependent caspase activation or apoptosis (14, 17, 18). In contrast, genetic studies have suggested that caspase activation involved in Drosophila sperm differentiation (19) and in apoptosis in pupal eye development (20) may depend on cytochrome c. However, biochemical evidence that cytochrome c activates ARK in any setting is lacking. Although it has been suggested that ARK forms an oligomeric apoptosome in the absence of cytochrome c (21), there is no evidence for caspase activation by this structure. Reaper and Hid induced changes in mitochondrial morphology (13, 22) and cytochrome c distribution (13); however, cytochrome c redistribution was shown to be caspase-dependent and is thus unlikely to be involved in the initiation of the apoptotic program. Nevertheless, a role for the mitochondrial fission machinery in apoptosis was identified, suggesting that at least some aspects of the mitochondrial pathway of apoptosis exist in insects. Therefore, a role for cytochrome c in activating the APAF1 homolog in insect apoptosis remains, at best, controversial.

In this study, we examined the mitochondrial pathway of apoptosis in a lophotrochozoan, the freshwater planarian (phylum Platyhelminthes), and in an invertebrate deuterostome, the purple sea urchin, Strongylocentrotus purpuratus (phylum Echinodermata). Our results support a revised phylogeny of the mitochondrial pathway of apoptosis, in which this pathway is an-
central in the animals and may have been lost in some phyla, including those of the Ecdysozoa.

**Results**

Planarian cells die in response to several agents, including γ-radiation, and this death proceeds via apoptosis, as determined by ultrastructural changes and DNA fragmentation (by terminal deoxynucleotidyl transferase dUTP nick end labeling) (23). To determine whether planarian cells, including the cycling planarian stem cells known as neoblasts (24, 25), undergo death via the mitochondrial pathway of apoptosis, a single cell suspension enriched in planarian (*Schmidtea mediterranea*) neoblasts was prepared and cultured with or without the broad-spectrum caspase inhibitor qVD-OPh, followed by γ-radiation. Apoptosis was assessed 24 h later by the binding of Annexin V to externalized phosphatidylserine before loss of plasma membrane integrity [assessed by uptake of propidium iodide (PI)] and the loss of mitochondrial membrane potential, ΔΨm. We observed an increase in Annexin V+ PI+ cells following irradiation (Fig. 1A), which was effectively blocked by preincubation with qVD-OPh (Fig. 1B). We also observed a reduction of ΔΨm in irradiated cells (Fig. S1).

Cytosolic extracts prepared from another planarian (*Dugesia dorotocephala*), 24 h after irradiation of the whole organism, cleaved the synthetic caspase substrate Ac-DEVD-afc (Fig. 1C). This cleavage was blocked by qVD-OPh, but not by a mixture of inhibitors that block members of other protease families (Fig. 1C), suggesting that irradiation had induced a caspase responsible for orchestrating apoptotic death. To test this idea, cytosolic extracts from untreated or irradiated planaria were incubated with mammalian iCAD, a known caspase substrate, or iCAD mutated at its caspase cleavage sites (26). The cytosolic extracts from irradiated animals cleaved wild-type iCAD to the same fragments observed with recombinant human caspase 3. Although some substrate cleavage was observed in the extract from untreated animals, cleavage was increased in the extract from irradiated animals (Fig. 1D). The cleavage was blocked by qVD-OPh and was not observed with the mutant substrate (Fig. 1D). To assess release of cytochrome c, as an indication of MOMP during apoptosis, we examined the cytosolic extracts by immunoblot and found that irradiation effectively induced release of cytochrome c into the cytosolic fraction (Fig. 1E).

Similar results were obtained by using another stressor, heat, to which these organisms are likely exposed under natural conditions. As with γ-radiation, mild heat stress induced caspase activation and cytochrome c release (Fig. S2).

Because cytochrome c mediates activation of caspases in the mitochondrial pathway of apoptosis in vertebrates, we hypothesized that it might also induce caspase activation in planaria. Cytosolic extracts were prepared from untreated *D. dorotocephala* and caspase activity was measured. Addition of mammalian cytochrome c rapidly triggered DEVDase activity, which was inhibited by broad-spectrum caspase inhibitors zVAD-fmk and qVD-OPh (Fig. 2A and B). Cytochrome c-activated extracts cleaved wild-type iCAD, but not the caspase-un cleavable mutant, in the same manner as recombinant human caspase 3 (Fig. 2C). Untreated extract induced a variable background of substrate cleavage, perhaps due to contamination with low levels of endogenous cytochrome c; however, addition of cytochrome c induced markedly more cleavage, and this cleavage was blocked by qVD-OPh. Similar results were observed when cytochrome c-activated extracts were incubated with wild-type human PARP or its caspase-un cleavable mutant (27) (Fig. 2D). Although general degradation of the substrate was observed, only cytochrome c-activated extract induced cleavage of the substrate to an 85-kDa band, dependent on the caspase cleavage site. In vertebrate extracts, cytochrome c from mammals and insects triggers caspase activation, whereas that from yeast does not (28), and this pattern was also observed in planaria extracts (Fig. 2E). Cytochrome c did not induce other protease activities, as assessed by cleavage of other synthetic caspase, calpain/proteasome, or cathepsin substrates (Fig. S3 A and B).

To determine whether planarian mitochondrial proteins such as cytochrome c can similarly trigger caspase activation, planaria (*D. dorotocephala*) mitochondria were enriched and then disrupted with water. The supernatants induced caspase activation in both planaria cytosolic extract (Fig. 2F) and in *Xenopus* egg extract (Fig. S5C). This result is consistent with an ability of planaria cytochrome c to induce DEVDase activity. We suggest that the release of cytochrome c, upon induction of apoptosis in planaria, can induce caspase activity, thus implicating the mitochondrial pathway in cell death induced by γ-radiation and heat shock in planaria.

In vertebrates, cytochrome c triggers caspase activation via oligomerization of the adapter protein APAF1 (29). A planarian protein with homology to APAF1 was identified in the *S. mediterranea* genome (Fig. S4), and the region containing the caspase-recruitment...
domain (CARD) was cloned and expressed in *Escherichia coli* as a GST-fusion protein. The corresponding human APAF1 region was similarly expressed. In mammalian cytosolic extracts, the human, but not the planarian, APAF1 CARD fragment inhibited caspase activation in response to mammalian cytochrome *c* (Fig. 2G and Fig. S5). Therefore, it is likely that the human APAF1 CARD-containing fragment acts as a competitive inhibitor of the human APAF1 apoptosome, presumably by competing for the caspase-9 CARD domain. This hypothesis is further supported by the ability of recombinant human caspase-9 CARD region to inhibit in this assay (Fig. 2G and Fig. S5). In contrast, the planarian, but not the human, APAF1 CARD fragment inhibited the ability...
of mammalian cytochrome c to trigger caspase activation in planarian extracts (Fig. 2H and Fig. S5). Thus, it is likely that cytochrome c triggers caspase activation in planarian extracts via the planarian APAF1 homolog.

Recently, antiapoptotic and proapoptotic Bcl-2 family proteins were identified in another lophotrochazoan, Schistosoma mansoni (30). We interrogated the genome of *S. mediterranea* and identified several candidates for proapoptotic Bcl-2 effector proteins (Table S1). The mammalian Bcl-2 effector proteins, Bax and Bak (31), trigger cell death in the yeast *Saccharomyces cerevisiae*, and we therefore tested the planarian candidates for such killing. Of these, one candidate (Smed-Bak-2) effectively triggered cell death (Fig. S6A), which we designate herein as “Smed Bak” based on its homology to human Bak. A mutation in Smed Bak that corresponds to an inactivating mutation in human Bak was introduced (32), and we found that this mutation inactivated its ability to kill yeast (Fig. 3A). We then tested whether this killing was antagonized by either human BCL-xL or a described *S. mediterranea* antiapoptotic Bcl-2 protein (23). Both human and planarian antiapoptotic Bcl-2 proteins blocked killing by Smed Bak and mouse Bak (Fig. 3B and Fig. S6B).

Bak and Bak are essential for MOMP in mammalian cells (33). We therefore tested whether Smed Bak could induce MOMP in Bak, Bak double-deficient mouse embryo fibroblasts (MEFs), which do not undergo MOMP in response to apoptotic signals (33). We used cells expressing the mitochondrial intermembrane-space marker Omi-mCherry, which upon MOMP is released to the cytosol and degraded by the proteasome (34). We transiently expressed fluorescent Venus-tagged Smed Bak and

![Graph](image1)

**Fig. 3.** Planaria Bak localizes to mitochondria and induces MOMP and cell death. (A and B) *S. cerevisiae* were transformed with the indicated constructs, plated in serial dilution, and induced to express the transformed gene(s). (C and D) Bax/Bak-deficient MEFs stably expressing Omi-mCherry were transfected with the indicated constructs in the presence of qVD-OPh (32 μM). At 24 h, cells were analyzed by confocal microscopy (C) or flow cytometry (D) for MOMP. Displayed is the percentage of cells that have undergone MOMP relative to the transfected population, averaged over three independent experiments ± SD. (E) HeLa cells were transfected with Venus-Smed Bak and mitochondrial targeted Cerulean (fused to the C-terminal 20 amino acids of human Bcl-xL). Cells were permeabilized with digitonin and imaged by confocal microscopy. In the merged image, the cerulean is false colored red.
observed the induction of MOMP in the cells expressing the green Venus (Fig. S7). Quantitative confocal analysis revealed that Smed Bak was at least as effective as human Bax in inducing MOMP (Fig. 3C). Importantly, both mutant Smed Bak and Smed Bak coexpressed with human Bcl-xL failed to induce MOMP. We obtained similar results using flow cytometry to detect cells that had undergone MOMP [detected by lower Omi-mCherry expression (34)] (Fig. 3D). To determine whether Smed Bak localized to mitochondria, Venus Smed Bak was coexpressed with mitochondrial-targeted Cerulean in HeLa cells (35).

Before imaging, cells were treated with digitonin to selectively permeabilize the plasma membrane and thereby release soluble cytosolic proteins. By using this approach, Smed Bak was found to colocalize with mitochondria (Fig. 3E). These results demonstrate that Smed Bak can localize to the mitochondria and induce MOMP in a Bcl-xL-regulated manner.

Our findings suggest that the mitochondrial pathway of apoptosis, as defined by cytochrome c-induced caspase activation, exists not only in vertebrates but also in lophotrochozoans. Therefore, other invertebrate phyla may display this pathway. In keeping with this idea, we found that egg cytosolic extracts from two echinoderms, purple sea urchin (S. purpuratus) and sand dollar (Dendraster excentricus), both showed robust cytochrome c-induced DEVDase activity, which was blocked by caspase inhibitors (Fig. 4A and B and Fig. S8), but not by a mixture of other protease inhibitors (Fig. S8A). Cytochrome c-activated sea urchin egg extracts cleaved iCAD, but not its caspase-uncleavable mutant (Fig. 4C). As with vertebrate (28) and planaria cytosolic extracts, mammalian and insect cytochrome c triggered DEVDase activity in sea urchin egg extracts, whereas yeast cytochrome c did not (Fig. 4D). Cytochrome c-induced caspase activity was enhanced by the addition of exogenous dATP (Fig. S8 B and C). Cytochrome c did not induce cleavage of other synthetic caspase, calpain/proteasome, or cathepsin substrates (Fig. S8D).

**Discussion**

Apoptosis and the molecules of the mitochondrial pathway for this form of cell death are conserved among the animals, but evidence has not supported the existence of cytochrome c-induced caspase activation beyond the vertebrates (5). Our studies herein suggest that if apoptosis via APAF-1-like molecules proceeds independently of MOMP in some organisms, this mechanism is likely to have derived from an ancestral pathway in which MOMP-mediated release of cytochrome c activates caspases—that is, the mitochondrial pathway as seen in vertebrates. Indeed, the most parsimonious scenario is that the mitochondrial pathway of apoptosis arose once, before the emergence of the deuterostomes, and that all or portions of the pathway have been lost in some lineages. This hypothesis is supported by the observation that proapoptotic and antiapoptotic Bcl-2 proteins in the lophotrochozoans, S. mansonii, promote or inhibit apoptosis in mammalian cells (30), whereas the nematode Bcl-2 protein, CED-9, does not (30).

Apoptosis, by morphological criteria, has unambiguously been described throughout the animals, including Sponges, Cnidaria, Platyhelminthes, Mollusks, Nematodes, Arthropods, Echinoderms, and Chordates, both invertebrate (Ciona) and vertebrate (5). In addition to the C. elegans and Drosophila homologs, homologs of caspases are described in many of these other phyla, as are Bcl-2 homologs (Table S1). It is clear, however, that sequences of genes and proteins relating to the mitochondrial pathway of apoptosis do not predict the function of this pathway in organisms of a given phylum. Our results suggest that, in addition to sequence-based phyletic analysis, an understanding of the underlying mechanism of cellular processes requires cell and biochemical approaches to determine whether complex processes such as the mitochondrial pathway are, indeed, conserved.

**Materials and Methods**

**Animals.** S. maderiterranea were maintained in Montiuc salts (3G) (pMedia). D. dorotocephala were purchased from Carolina Biological Supply and maintained in pMedia. S. purpuratus and D. excentricus were collected locally in San Diego.

**DEVDase Assay.** For each data point, 100 μg of cytosol extract was used. Samples were activated by 1 μL of 100 μM horse heart cytochrome c (Sigma) for 30 min at room temp [or insect (M. sexta), yeast (S. cerevisiae), or mitochondria lysate as indicated]. The human cytosol included 1 μL of 10 mM dATP (Invitrogen). DEVDase activity was determined by adding 100 μL of Caspase Buffer [20 mM Pipes at pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% Chaps, 10% (wt/vol) sucrose, and 10 mM DTT] with 0.1 mM Ac-DEVD-afc (SM Biochemicals). Ac-DEVD-afc cleavage was measured in a SpectraMax Gemini XPS plate reader (Molecular Devices) with excitation at 400 nm and emission at 505 nm. Readings were measured each minute for 30 min. Results are expressed as RFU per minute (37).

**Caspase Activation Assay.** To analyze cleavage of protein substrates, cytosolic extract was added to in vitro transcribed/translated wild-type or mutant (D117E, D224E) iCAD or wild-type or mutant (D214A) PARP. Samples were then subjected to SDS/PAGE and analyzed by autoradiography (iCAD) or immunoblot (PARP).

**Yeast Death Assays.** S. cerevisiae were transformed with pYES2.1 with or without pADH plasmids with the indicated inserts and selected on the appropriate dropout noninducing medium. pYES2.1 has an inducible promoter (GAL1), and pADH has a constitutive promoter (alcohol dehydrogenase). Colonies were picked, suspended in water, serially diluted 10-fold, and
plated on both inducing and noninducing medium. Plates were photographed at the indicated times.

**MOMP Assay.** Bax/Bak double-knockout MEFs stably expressing Omi-mCherry were transfected with the indicated plasmids by using Lipofectamine 2000 (Invitrogen). Transfected cells were cultured with qV-D-Oph at a concentration of 32 μM for 20–23 h. MOMP was evaluated by flow cytometry (LSR II; BD Biosciences) and analyzed by using FloJo software or a spinning disk confocal microscope (Zeiss) and Slidebook software (Intelligent Imaging Innovations).


**Supporting Information.** Detailed methods and methods not described here can be found in SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

Apoptosis Assays. To measure apoptosis in planaria cells, a cellular fraction enriched in planaria (S. mediterranea) neoblasts was preincubated with or without qVD-OPh and subjected to γ-irradiation. Twenty-four hours, later, cells were stained with Annexin V-FITC and PI and analyzed by flow cytometry.

Planaria Cytosol Preparation. D. dorotocephala (Carolina Biologicals) were starved at least 1 wk before use. Approximately 300 planaria (10 jars) were collected and washed once in pMedia (1.6 mM NaCl, 1 mM CaCl2, 1 mM MgSO4, 100 μM MgCl2, 100 μM KCl, and 1.2 mM NaHCO3), pMedia was removed, and the planaria were incubated in Calcium and Magnesium Free medium (CMF) (2.8 mM Na2HPO4, 13.6 mM NaCl, 16 mM KCl, 9.5 mM NaHCO3, 15 mM Hepes, pH 7.3) on ice for 5 min. Planaria were washed once in pMedia and transferred to a 15-mL glass homogenizer. pMedia was removed, and 1/2 pellet volume of pMedia supplemented with Complete Protease Inhibitor (Roche) was added. Planaria were homogenized with 8–10 strokes of a tight-fitting Teflon dounce. The homogenate was centrifuged for 30 min at 15,000 × g at 4 °C. The resulting supernatant was recovered as cytosolic extract, aliquoted in 100-μL aliquots, and stored at −80 °C.

Sea Urchin Cytosol Preparation. S. purpuratus were collected at the tide pools at Mission Bay, San Diego or from the ocean floor at ~60-foot depth, 1/4 mile off shore at Point Loma, CA. Urchins were either spawned immediately or transferred to a sea table with running sea water at Scripps Institute for Oceanography where they were maintained for later use. Urchins were spawned by injecting 0.5 M KCl into the soft tissue surrounding the mouth. Males were discarded, and females were placed mouth-side down on a 100-mL glass beaker filled with filtered sea water (FSW). Eggs were washed three times in FSW, then de-jellied by stirring rapidly by hand while adding 0.1 M HCl dropwise until the pH dropped to 5.0. After 1 min of continuous stirring, the pH was raised to 8.0 by using 1 M NaOH dropwise while stirring (1). Eggs were centrifuged at 500 × g, and excess FSW was removed. Sea urchin egg cytosal extract was prepared in a similar manner to Planaria, except egg lysis buffer (ELB; 250 mM sucrose, 50 mM KCl, 2.5 mM MgCl2, 20 mM Hepes-KOH, pH 7.5, 1 mM DTT, Complete Protease Inhibitor) was used instead of pMedia.

Sand Dollar Cytosol Preparation. D. excentricus were collected from the sandy bottom in ~10 feet of water off the Quivera Basin in San Diego and were transferred to the laboratory in sea water, where they were processed immediately, similarly to the sea urchin.

Human Cytosol Preparation. HEK293T cells were grown to ~90% confluence, collected by scraping, washed in PBS, and incubated in CEB lysis buffer (50 mM Pipes at pH 7.4, 50 mM KCl, 2 mM MgCl2, 5 mM EGTA, 1 mM DTT) for 20 min. Cells were dounced until >80% trypan blue positive, then centrifuged for 30 min at 15,000 × g at 4 °C. The resulting supernatant was recovered as cytosolic extract, aliquoted in 100-μL aliquots, and stored at −80 °C.

Mitochondria Lysate Preparation. Planaria (D. dorotocephala) were incubated in calcium-magnesium free medium and homogenized, and mitochondria were enriched by differential centrifugation (2). To lyse mitochondria by osmotic shock, mitochondria were pelleted, resuspended, and incubated in deionized water. Mitochondria were pelleted; supernatant was recovered and added to planaria cytosolic extract; and Ac-DEVD-afc cleavage was measured.

Recombinant Protein Preparation. Coding sequences of human Apaf-1 (aa 1–95), human caspase-9 (aa 1–167), Smed Apaf-1 (aa 1-104), (aa 1–252), (aa 1–309) were cloned into pGEX-4T (Pharmacia) and transformed into BL21 bacteria. Fresh 100-mL cultures were grown into log phase at 37 °C, moved to room temperature, and induced with 0.5 mM IPTG (Sigma) for 3 h. Bacterial pellets were lysed in 9 mL of B-per (Pierce) with Complete Protease Inhibitor (Roche) and DNase I (Invitrogen). Lysates were rocked at room temp for 30 min, centrifuged at 15,000 rpm for 30 min, aliquoted, and frozen at −80 °C. Lysates were analyzed by standard Western blot methods and probed with anti-GST Ab (Gene Tex) at a concentration of 1:2,000. The indicated human and planaria recombinant proteins were added to cytosol and diluted twofold serially before activation with cytochrome c.

MOMP Assay. Bax/Bak double-knockout MEFs stably expressing Omi-mCherry were transfected with the indicated plasmids by using Lipofectamine 2000 (Invitrogen). Transfected cells were cultured with qVD-OPh at a concentration of 32 μM for 20–23 h. MOMP was evaluated by flow cytometry (LSR II; BD Biosciences) and analyzed by using FloJo software or by using a spinning disk confocal microscope (Zeiss) and Slidebook software (Intelligent Imaging Innovations).


Fig. S1. γ-radiation induces loss of ΔΨm in planaria cells (S. mediterranea). Cell fraction enriched in planaria neoblasts was treated with or without γ-radiation. Twenty-four hours later cells were stained with Draq5, PI, and TMRE. 50 μM CCCP (a mitochondrial uncoupling agent) was used as a positive control to dissipate ΔΨm. Cells were analyzed by flow cytometry, and TMRE fluorescence (as a measurement of ΔΨm) was measured in the Draq5+, PI− cells. (A) Representative histogram. (B) Quantification of the percent cells with high ΔΨm following treatment. Each data point represents two or three independent experiments. Error bars represent SD.

Fig. S2. Heat stress induces caspase activation and cytochrome c release in planaria. Planaria (D. dorotocephala) were subjected, or not, to heat stress at 37 °C to up to 4 h, and cytosolic extract was prepared immediately. (A) Rate of cleavage of Ac-DEVD-afc by cytosolic extract from planaria subjected to heat stress for the time period indicated. (B) Rate of cleavage of Ac-DEVD-afc by cytosolic extract from untreated or heat-stressed (4 h) planaria preincubated in the presence or absence of a broad protease inhibitor mixture (see text) and then incubated with or without DMSO (vehicle) or 10 μM qVD-OPh. Error bars in A and B represent SD. (C) 35S-labeled wild-type iCAD or mutant iCAD (D117E/D224E) (m-iCAD) was incubated with extract from untreated or heat-stressed (4 h) planaria and preincubated with a protease inhibitor mixture, in the presence or absence of 10 μM qVD-OPh (Left) or with activated recombinant caspase 3 (rCasp3) (Right). Samples were resolved by SDS/PAGE and analyzed by autoradiography. Arrowhead indicates cleaved product. (D) Cytosolic extracts from untreated and heat-stressed planaria were examined for cytochrome c (cyt c) and actin content by Western immunoblot.
**Fig. S3.** Characterization of cytochrome c-induced caspase activation in planaria (*D. dorotocephala*) and frog egg (*Xenopus laevis*) cytosolic extract. (A) Rate of cleavage of Ac-DEVD-afc, Ac-VEID-afc, Ac-IETD-afc, Ac-LEHD-afc and Ac-YVAD-afc (synthetic caspase substrates) by planaria cytosolic extract preincubated in the presence or absence of 10 μM cytochrome c. (B) Rate of cleavage of suc-LY-amc (synthetic calpain/proteasome substrate) (Upper) and z-RR-amc (synthetic cathepsin substrate) (Lower) by planaria extract in the presence or absence of 10 μM cytochrome c. Note the reduced scale in Upper. Data are representative of at least three independent experiments. Error bars represent SD. (C) Rate of cleavage of Ac-DEVD-afc by frog egg cytosolic extract (*Xenopus laevis*) preincubated or not with supernatant from enriched mitochondria disrupted with water. Error bars represent SD.
Fig. S4. Alignment and domain assignment of Apaf-1 sequences. Apaf-1 of Smed (JN621807), Homo sapien (NP_863651), M. mus (NP_001036023), D. rerio (NP_571683), S. purpuratus (XP_796156), H. vulgaris (ACY71873), Drosophila melanogaster (NP_725637), and C. elegans (NP_001021202) were aligned by using MacVector's ClustalW Alignment. The domain assignments are based on human Apaf-1 (1).

Fig. S5. Cytochrome c-induced caspase activity can be inhibited by Apaf-1 CARD. Rate of cleavage of Ac-DEVD-afc by planaria cytosolic extract or human cytosolic extract preincubated with recombinant Apaf-1 fragments or the human Caspase-9 CARD. Proteins were serially diluted twofold.

Fig. S6. Planaria Bcl-2 proteins regulate yeast viability. (A) Plasmids expressing Planaria Bcl-2 family molecules were transfected into yeast to determine their ability to affect growth. Yeast were serially diluted 10-fold before plating. (B) Plasmids expressing Planaria Bcl-2 family molecules were transfected into yeast along with mouse Bax to determine their ability to protect growth.
**Fig. S7.** Planaria Bak induces MOMP. Bax, Bak double-deficient MEFs, stably expressing Omi-mCherry were transfected with the indicated constructs in the presence of qVD-OPh (32 μM). 24 h later, cells were examined by confocal microscopy.
Fig. S8. Characterization of cytochrome c-mediated caspase activation in sea urchin (*S. purpuratus*) and sand dollar (*D. excentricus*) egg cytosolic extract. (A) Rate of cleavage of Ac-DEVD-afc by sea urchin egg cytosolic extract preincubated in the absence or presence of protease inhibitors as in Fig. 1 and then incubated in the absence or presence of 10 μM mammalian (horse heart) cytochrome c. (B) Rate of cleavage of Ac-DEVD-afc by sea urchin egg cytosolic extract preincubated in the presence or absence of 1 mM dATP and/or 10 μM cytochrome c. (C) Rate of cleavage of Ac-DEVD-afc, Ac-VEID-afc, Ac-IETD-afc, Ac-LEHD-afc, and Ac-YVAD-afc (synthetic caspase substrates) by sea urchin egg cytosolic extract preincubated in the presence or absence of 10 μM cytochrome c/1 mM dATP. (D) Rate of cleavage of suc-LY-amc (synthetic calpain/proteasome substrate) (Upper) and z-RR-amc (synthetic cathepsin substrate) (Lower) by sea urchin egg extract in the presence or absence of 10 μM cytochrome c/1 mM dATP. Data are representative of at least three independent experiments. Error bars represent SD. (E) Cleavage of Ac-DEVD-afc by sand dollar egg cytosolic extract preincubated for 30 min in the presence or absence of 10 μM mammalian (horse heart) cytochrome c and 100 mM dATP with or without 10 μM zVAD-fmk. Data are representative of two independent experiments.

Table S1. Planaria NCBI accession numbers

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