Activation of mitochondrial protease OMA1 by Bax and Bak promotes cytochrome c release during apoptosis

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Intrinsic apoptotic stimuli initiate mammalian cells’ apoptotic program by first activating the proteins that have only Bcl-2 homology domain 3 (BH3), such as Bcl-2 interacting mediator of cell death (Bim) and truncated BH3 interacting death domain agonist (tBid), which in turn trigger conformational changes in BCL2-associated X (Bax) and BCL2-antagonist/killer (Bak) proteins that enable oligomer formation on the mitochondria, causing cytochrome c and other apoptogenic proteins in the intermembrane space to leak out. Leaked cytochrome c then initiates apoptotic caspase activation through a well-defined biochemical pathway. However, how oligomerized Bax and Bak cause cytochrome c release from mitochondria remains unknown. We report here the establishment of cell lines in which Bim or tBid can be inducibly expressed to initiate apoptosis in a controlled, quantitative manner. We used these cell lines to examine apoptotic events after Bax and Bak oligomerization but before cytochrome c release. The mitochondrial metalloprotease OMA1 was activated in this system in a Bax- and Bak-dependent fashion. Activated OMA1 cleaved the dynamin-like GTPase, optical nerve atrophy 1, an event that is critical for remodeling of mitochondrial cristae. Knockdown or knockout of OMA1 in these cells attenuated cytochrome c release. Thus it is clear that oligomerized Bax and Bak trigger apoptosis by causing both the permeabilization of the mitochondrial outer membrane and activation OMA1.

Smac | permeability | membrane potential | caspase

Mitochondria in mammalian cells fulfill multiple functions. They are cells’ bio-energetic center, where reducing agents generated through the Krebs cycle transfer their electrons to oxygen in a manner mediated by the electron transfer chain, a process that builds a proton gradient across the inner membrane of mitochondria. The energy of this gradient is transferred into the high-energy bond of ATP by oxidative phosphorylation of ADP through the F1/F0 ATP synthase. During apoptosis, the sole water-soluble component of the electron transfer chain, cytochrome c, is released from the intermembrane space of mitochondria to the cytosol (1). Cytosolic cytochrome c binds to the Apaf-1 protein to promote the assembly of a heptamer complex named an “apoptosome”; this complex subsequently recruits procaspase-9, which autoactivates once on the apoptosome. The activated caspase-9 then cleaves and activates downstream caspase-3 and caspase-7, which subsequently cleave many intracellular substrates for apoptosis execution (2).

In addition to cytochrome c, other proteins that normally are located in the mitochondrial intermembrane space also function in apoptosis. One such protein is second mitochondria-derived activator of caspase (Smac). When Smac is released, it binds to the BIR domain of inhibitors of apoptosis proteins to relieve their inhibition of the caspases directly or to cause their degradation (3, 4). Thus controlling the permeability of mitochondria for these apoptogenic proteins constitutes a key regulatory step for apoptosis.

The B-cell leukemia/lymphoma 2 (Bcl-2) family of proteins constitutes a protein network that regulates the release of proteins such as cytochrome c and Smac (5, 6). BCL2-associated X (Bax) and BCL2-antagonist/killer (Bak), the proapoptotic members of the family with multiple Bcl-2 homology (BH) domains, form the core of the mitochondrial membrane permeability machinery that is activated by the proapoptotic proteins that have only the BH3 domain, a process that is inhibited by the proteins whose function is similar to that of Bcl-2 itself (7, 8). In response to apoptotic stimuli, BH3-only proteins, such as Bcl-2 interacting mediator of cell death (Bim), Puma, and truncated BH3 interacting death domain agonist (tBid) directly activate Bax/Bak and lift the inhibition of Bcl-2/Bcl-xL by forming stable heterodimers to sequester them from binding Bax/Bak (7, 9–13). Activated Bax and Bak initially form homodimers and then oligomers on the mitochondrial membrane (14–18). Bax/Bak oligomers are believed to form proteinaceous or lipidic pores on the mitochondrial outer membrane that allow the passage of proteins such as cytochrome c and Smac. Although the results of in vitro liposome leakage experiments support this model, there is no direct in vivo evidence to validate such a straightforward model (19–23).

Moreover, increasing evidence indicates that the majority of cytochrome c in the mitochondrial intermembrane space is locked inside cristae by the protein complex containing optical nerve atrophy 1 (OPA1). The cristae must undergo reconfiguration to open up the neck of cristae for the bulk of cytochrome c to be released from the mitochondria after the outer membrane becomes permeable (24–26). The mitochondrial inner membrane fusion factor OPA1, a dynamin-like GTPase, plays a critical role in the remodeling of cristae. OPA1 presents in several spliced and proteolytic forms in mitochondria, and the maintenance of the relative amounts of each of these forms is known to be critical for stabilizing the cristae (27, 28). The longest form, L-OPA1, is cleaved in the periphery.
response to a variety of mitochondrial stresses, leading to the disassembly of OPA1-containing complexes and remodeling of the cristae (24, 26). It has been proposed that several different proteins cleave OPA1; these include the mitochondrial AAA proteases, presenilin-associated rhomboid-like protein (PARL), and the zinc metalloprotease OMA1 (overlapping activity with m-AAA protease) (26, 27, 29–31). However, the relationship between the pore formation on the mitochondrial outer membrane and OPA1 cleavage-mediated cristae remodeling during apoptosis, as well as the precise roles of those mitochondrial proteases in apoptosis, remain to be clarified.

To dissect the molecular details of cytochrome c release induced by BH3-only proteins, we generated cell lines in which Bim or tBid can be inductively expressed by adding doxycycline (Dox) into the culture medium. The expression of these proteins triggers apoptosis in a controlled and synchronized fashion. We used this cell-based system to characterize the mitochondrial response to the induction of Bim and tBid and found that OMA1 activation is an important step for apoptosis induction.

**Results**

**Inducible Expression of Bim Triggers Mitochondria-Mediated Apoptosis.** In an effort to dissect the detailed processes of the apoptosis pathway inside mitochondria, we engineered a U2OS human osteosarcoma cancer cell line in which the BH3-only protein Bim is inducibly expressed when Dox is added to the culture medium. As shown in Fig. 1A, there was no detectable Bim expression before the addition of Dox. Bim started to appear 2 h after the addition of Dox and reached an expression plateau at 4 h (Fig. 1A, Lower). Consistently, the cells’ vitality as measured by their intracellular ATP level started to drop at the 4-h time point. The ATP level was preserved at 90% when the pan-caspase inhibitor z-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) was included in the cultures (Fig. 1A, Upper). The activation of caspase-9 and caspase-3 seemed to follow the kinetics of Bim induction, with a peak of activation at 4 h (Fig. 1B, Top and Middle). Further, most of the poly(ADP-ribose) polymerase (PARP), a caspase-3 substrate, was cleaved at 4 h (Fig. 1B).

The induction of Bim caused a dramatic release of cytochrome c and Smac from mitochondria as measured by immunofluorescent staining (Fig. 1C) and cell fractionation followed by Western blotting analysis (Fig. S1). Both proteins were located exclusively within mitochondria before Dox addition, and the mitochondria were in the healthy tubular form. After Bim induction, the majority of the cytochrome c and Smac was released into the cytosol, and the residual protein remaining in the mitochondria showed a fragmented and aggregated form around the nuclei.

Consistent with previous reports, the expression of Bim also caused concurrent cleavage of the long form of OPA1 (L-OPA1) (Fig. 1D, lanes 3–5) and disassembly of the OPA1 complex, which was measured by adding a protein cross-linker before analysis with Western blotting (Fig. 1D, lanes 8–10).

**OPA1 Cleavage and Disassembly of the OPA1-Containing Complex Require OMA1.** Several proteases have been shown to cleave OPA1 under different stress conditions; these include the i-AAA and m-AAA proteases YME1L, ATPase family gene 3-like 2 (AFG3L2), and paraplegin, the mitochondrial inner membrane zinc protease OMA1, and PARL. Therefore we tested the role of these proteases in Bim-induced apoptosis and OPA1 cleavage (Fig. S2). We found that OMA1 was critically important for apoptosis induction by Bim or tBid expression, whereas knockdown of other mitochondrial proteases had little effect. As shown in Fig. 2, knockdown of OMA1 by stable expression of an shRNA against OMA1 blocked cell death induced by Bim expression (Fig. 2A) and prevented disassembly of the OPA1-containing complex and cleavage of L-OPA1 (Fig. 2B, Top and Bottom). Additionally, knockdown of OMA1 prevented the release of cytochrome c and Smac from the mitochondria (Fig. 2C) and the fragmentation of mitochondria (Fig. 2D).

To confirm that OMA1 is required in apoptosis, we generated another U2OS cell line in which tBid, another BH3-only protein, could be induced with Dox. These cells underwent apoptosis when Dox was added to the medium (Fig. 3A and Fig. S3). We used clustered regularly interspaced short palindromic repeats (CRISPR) technology to delete the OMA1 gene from the cell line (Fig. 3A, Lower and Fig. S4); as a consequence, cell death was inhibited upon the addition of Dox (Fig. 3A, Upper). Disassembly of the OPA1-containing complex and cleavage of L-OPA1 were prevented also (Fig. 3B). As in the Bim-expressing cells in...
level when apoptosis was blocked by disabling OMA1 (compare line in which OMA1 was knocked down and induced apoptosis by mutant (H331A) version of OMA1 back into the U2OS-Bim cell line). The ectopic expression of the shRNA-resistant wild-type OMA1, but not the H331A mutant, also restored cytochrome c release upon Bim induction was completely reversed by expression of the wild-type OMA1, but not the inactive H331A mutant (Fig. 4C). Of note, Bim accumulated to a higher level when apoptosis was blocked by disabling OMA1 (compare lanes in Fig. 4B), even though the Bak level remained constant.

The OMA1 Effect on Apoptosis Can Be Bypassed by OPA1 Knockdown. Although OMA1 seems to be the protease that cleaves L-OPA1 upon tBid expression and seems to promote disassembly of the OPA1-containing complex, we sought to confirm whether OPA1 is the downstream effector of OMA1 during apoptosis. To do so, we knocked down OPA1 with siRNA in cells in which apoptosis was blocked by the lack of OMA1. As shown in Fig. 4E, knockdown of OPA1 in cells expressing OMA1 shRNA restored the apoptosis response when Bim was induced by Dox. Both cytochrome c/Smac release and mitochondrial fragmentation were normal when OPA1 was knocked down (Fig. 4F and G and Fig. S7 A and B). Similar results were obtained with the U2OS-tBid OMA1 knockout cells (Fig. S7 C and D). These results indicate that OPA1 is indeed the downstream effector of OMA1.

Bax and Bak Function Upstream of OMA1 Activation. To test if Bax/ Bak activation is upstream or independent of OMA1 function in apoptosis, we knocked down both Bax and Bak in the cell line in which Bim was inducibly expressed. The knockdown effect of Bak and Bax was nearly complete (Fig. S4, Lower). The apoptosis response in those cells was largely eliminated following induction of Bim (Fig. 5B, Upper). Interestingly, although knockdown of OMA1 which OMA1 was knocked down, we did not detect cytochrome c or Smac release or mitochondrial fragmentation, even when tBid was induced in these cells (Fig. 3 C and D). Knockdown of OMA1 also blocked UV-induced OPA1 cleavage and cytochrome c/Smac release in HeLa cells, indicating that OMA1 plays a general role in promoting apoptosis (Fig. S5).
Percentages of cells with cytochrome c-VAD was included during the treatment for Bim and OMA1_WT cells. The P15 fractions of the cells were analyzed by Western blotting. (Fig. 5B) Whole-cell extracts of the indicated cell lines were analyzed by Western blotting. (Fig. 4A, B) Bim cells (ctrl), Bim/shOMA1 cells, Bim/shOMA1 cells rescued with wild-type OMA1 (OMA1_WT), and Bim/shOMA1 cells rescued with the protease active site mutant of OMA1 (OMA1_H331A) were treated with or without Dox for 16 h. Cell viability was determined using the Cell-Titer Glo kit. Whole-cell extracts of the indicated cell lines were analyzed by Western blotting. (C and D) The indicated cell lines were treated with or without Dox for 16 h. z-VAD was included during the treatment for Bim and OMA1_WT cells. Percentages of cells with cytochrome c and Smac release into cytosol (C) or percentages of cells with fragmented mitochondria (D) as examined by immunostaining were calculated. Mitochondria were visualized with immunostaining of TOM20. (E) Bim and Bim/shOMA1 cells were transfected with siRNA against luciferase (luci) or OPA1. Forty-eight hours later, cells were treated with or without Dox for 12 h. Cell viability was determined using the Cell-Titer Glo kit. Whole-cell extracts of Bim cells before and after 48 h of OPA1 siRNA transfection were analyzed by Western blotting. (F and G) Bim and Bim/shOMA1 cells were transfected with the indicated siRNAs and were treated with or without Dox for 8 h. z-VAD was included during the treatment for Bim cells and Bim/shOMA1 cells with OPA1 siRNA transfection. Percentages of cells with cytochrome c and Smac release into cytosol (F) and percentages of cells with fragmented mitochondria (G) as determined by immunostaining were calculated.

Discussion

Bak and Bax Oligomerization Can Cause Both Outer Membrane Permeabilization and Activation of OMA1 to Accommodate Cytochrome c Release. The current model for cytochrome c release during apoptosis centers on the formation of pores by oligomerized Bak and Bax, which are induced by the BH3-only proteins such as Bim and tBid (16, 19–23, 32). Such pores allow apoptogenic proteins in the intermembrane space of mitochondria to leak out to the cytosol passively. Such a model does not take into account that most of the cytochrome c in mitochondria actually is locked inside cristae by the OPA1-containing complexes at the neck of mitochondrial cristae. The disassembly of such a complex thus is critical for the majority of cytochrome c to gain access to the inner boundary of the mitochondrial outer membrane (24–26, 33).

The results presented here demonstrate that OMA1 is another downstream target for Bax/Bak activation, in addition to its functional role in Bak and Bax oligomerization during outer membrane pore formation. Elimination of OMA1 by either shRNA knockdown or CRISPR-mediated gene knockout significantly attenuated cytochrome c release without affecting Bak/Bak oligomerization. However, knockdown of OPA1 circumvented knockdown of OMA1, indicating that the role of OMA1 in cytochrome c release is to cleave OPA1, leading to the disassembly of the OPA1-containing complex.

OMA1 can be activated by a variety of mitochondrial stress signals (34). Both CCCP, which dissipates mitochondrial membrane potential, and oligomycin, which inhibits F0 ATP synthase and thus increases mitochondrial membrane potential, have been shown to activate OMA1, leading to the cleavage of OPA1 and mitochondrial fragmentation (31, 34). However, simply treating cells with CCCP or oligomycin does not lead to cytochrome c release. Thus, OMA1 activation is necessary, but not sufficient, for cytochrome c release. In contrast, Bak and Bax oligomerization induced by either Bim or tBid expression can cause both outer membrane permeabilization and activation of OMA1. Thus, oligomerized Bak- and Bax-induced cytochrome c release requires two steps. One is to permeabilize the outer membrane, allowing the cytochrome c that has free access to the outer membrane to leak out; the second, concurrent step is the activation of OMA1, which cleaves L-OPA1 and causes disassembly of the OPA1-containing protein complexes that hold most of the cytochrome c within cristae. The second step may not be critical for apoptosis initiation but clearly is able to accelerate caspase-9 activation and change the dynamics of apoptosis progression.

OMA1 Activation Manifested in Accelerated Autocleavage and Cleavage of Its Substrate OPA1. It is apparent that loss of mitochondrial membrane potential stunts the import process of precursor OMA1 at the outer membrane, resulting in its accumulation (Fig. S8B–D).
Interestingly, treatment with CCCP or induction of Bim or tBid also decreased the level of the active 40-kDa OMA1. Because 40-kDa OMA1 undergoes autocleavage under normal conditions (Fig. S8A), it is likely that OMA1 activation also accelerates its own degradation. The rapid elimination of OMA1 following treatment with CCCP or Bax/Bak oligomerization (Fig. 5F and Fig. S8 B–D) is caused by the blockage of OMA1 import into the mitochondrial inner membrane and accelerated autocleavage-mediated degradation of the existing OMA1. Accelerated OMA1 activity also resulted in more cleavage of its substrate, L-OPA1.

**How Does Oligomerized Bax/Bak Activate OMA1?** Because OMA1 is an inner mitochondrial membrane protease, Bax and Bak oligomers must affect the inner membrane in some way. Indeed, loss of mitochondrial inner membrane potential was observed after Bim or tBid induction (Fig. 5E and Fig. S9 A and B). However, neither the proteinaceous nor lipidic pores that have been proposed for oligomer Bax and Bak can account for such a function if such pores are located solely on the outer membrane of mitochondria. It also is hard to imagine that the permeability of the outer membrane and the activation of OMA1 on the inner membrane...
result from two separate modes of action mediated by the same protein. In addition, treatment of mitochondria with tBid completely eliminated ADP-stimulated oxygen consumption (stage III respiration) (35), suggesting that the disruption of the coupling of F1/F0 ATP synthase and mitochondrial membrane potential is similar to that seen with oligomycin treatment.

We thus propose that oligomerized Bax and Bak must interact with the contact sites of the outer and inner membranes so that they can affect outer membrane permeability, OMA1 activation, and F0/F1 ATP synthase coupling, thus accounting for all the observed effects. Such interactions cause the enhancement of OMA1 protease activity, resulting in accelerated autodegradation and more cleavage of L-OPA1. The molecular mechanism by which oligomer Bax and Bak control OMA1 activity should be an interesting topic of future studies. The resulting dissociation of cytochrome c from its normal functional sites, the dissipation of mitochondrial membrane potential, and the decoupling of electron transfer chain and oxidative phosphorylation even might be used normally to synchronize electron transfer chain activity with the required supply and the demand for cellular energy. This hypotesis is in consistent with the low energy expenditure and heat generation observed in the OMA1-knockout mice (36). However, because this disassociation also leads to oxidative damage in cells, the ensuing apoptosis might be a clean exit strategy for cells in which Bax and Bak oligomerization is too strong.

**Materials and Methods**

Reagents, plasmids, siRNA oligos, and methods for cell viability assay, transfection, lentiviral packaging and viral infection, cell culture and stable cell lines, cellular fractionation, and the protein cross-linking assay are described in SI Materials and Methods. Also see SI Materials and Methods for details of the preparation of whole-cell extract, UV irradiation, immunostaining, and TMRM staining. Data are presented as means ± SD of duplicate experiments.

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**Supporting Information**

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**SI Materials and Methods**

**Reagents.** General chemicals were obtained from Sigma unless otherwise stated. Tetramethylrhodamine (TMRM) and 1,6-bis(maleimido)hexane (BMMH) were purchased from Life Technologies. z-Val-Ala-Asp (z-VAD) was synthesized in Zhiyuan Zhang’s laboratory at the National Institute of Biological Sciences, Beijing. The following antibodies were used: anti-Bcl-2 interacting mediator of cell death (Bim) (2819; Cell Signaling), anti-caspase 9 (5502; Cell Signaling), anti-caspase 3 (9662; Cell Signaling), anti-poly(ADP ribose) polymerase (PARP) (9542; Cell Signaling), anti-β-actin–HRP (D291-7; MBL), anti-Flag-HRP (A2859; Sigma), anti-OMA1 (overlapping activity with cytochrome c) (sc-11415; Santa Cruz), anti-OMA1 (overlapping activity with m-AAA protease) (sc-168844; Santa Cruz), anti-paraplegin (sc-135026; Santa Cruz), anti-poly(A) polymerase (PAP) (9545; Cell Signaling), anti-B-cell leukemia/lymphoma 2 (BCL2)-antagonist/killer (Bak) (3814; Cell Signaling), anti–optical nerve atrophy 1 (OPA1) (612606; BD Biosciences), anti–Flag-HRP (A2859; Sigma), anti-cytocrome c (556432 for immunostaining, 556433 for Western blot; BD Pharmingen), anti–second mitochondrial-derived activator of caspase (Smac) (sc-22766; Santa Cruz), anti-translocase of the outer membrane 20 (TOM20) (sc-11415; Santa Cruz), anti–OMA1 (overlapping activity with m-AAA protease) (sc-168844; Santa Cruz), anti–paraplegin (sc-135026; Santa Cruz), and anti–ATPase family gene 3-like 2 (AFG3L2) (ab68023; Abcam). Anti-OMA1 Flag-tag at the C-terminal end. The OMA1 shRNA construct was generated by TA cloned OMA1 around the gRNA targeting site, which was TA cloned using the pGEM-T Easy Vector System (Promega) with the forward primer 5′-GAACACTGACGATGAATCCTGCAGTA-3′ and the reverse primer 5′-CAGCGATCTGTTCGCCATGCT-3′. The OMA1 guide RNA (gRNA) plasmid for the knockout experiment was constructed in the gRNA-cloning vector (Addgene) according to the manufacturer’s instructions. The target sequence was TGGAC-TCTGGCTTGCTGGAAAGGCT. The OMA1 wild-type rescue construct was generated as described in ref. 1.

**Plasmids and siRNA Oligos.** The Bim and truncated Bid (tBid) expression plasmids were constructed in the pcDNA 3.1 vector (Invitrogen). OMA1 cDNA was PCR amplified from U2OS cDNA, which was reverse transcribed from U2OS total mRNA, and was cloned into a modified pcDNA 3.1 plasmid with a 3X Flag tag at the C-terminal end. The OMA1 shRNA construct was generated in the pLKO.1 plasmid (Addgene) according to the manufacturer’s instructions. The target sequence was TGCCCAAGTACTGCAGCTGCTATTTCATGGCCCAG-3′. For paraplegin, a mixture of the three oligos was used.

**Cell Viability Assay.** Cell viability was measured using the Cell-Titer Glo kit (G7570, Promega) according to the manufacturer’s instructions.

**Transfection, Lentiviral Packaging, and Viral Infection.** Transfection of cells with plasmids or siRNAs was performed using Lipofectamine 2000 (Invitrogen) as described in the experimental procedures of ref. 3. Lentiviral packaging of the OMA1 shRNA and the OMA1 gRNA constructs and infection of the cells with the lentiviral particles were performed according to the manufacturer’s instructions.

**Cell Culture and Stable Cell Lines.** All cells were cultured in DMEM containing 10% (vol/vol) FBS. The U2OS_tetR stable cell line was generated as described in ref. 4. The Bim or tBid expression plasmid was transfected into the U2OS_tetR cells, and cells were selected with 1 mg/mL G418 (Calbiochem) or 500 μg/mL hygromycin (Roche) to establish the Bim or tBid inducible expression cell line. An RFP-tagged adenine nucleotide translocase (ANT) expression plasmid was transfected into the tBid cell line, and cells were selected with 1 mg/mL G418. The OMA1 stable knockdown cell line was generated by transfecting the OMA1 shRNA plasmid into the U2OS_Bim cells and selecting the cells with 2 μg/mL puromycin (InvivoGen). The OMA1 wild-type and H331A mutant rescue cell lines were generated by transfecting the rescue constructs into the stable knockdown cells and selecting the cells with 500 μg/mL hygromycin. U2OS_tBid cells were infected with lentiviral particles containing the OMA1 gRNA plasmid, followed by transfection of the caspase 9 plasmid (Addgene) to establish the tBid/OMA1 KO cells; cell colonies were verified by sequencing the genomic DNA sequence of OMA1 around the gRNA targeting site, which was TA cloned using the pGEM-T Easy Vector System (Promega) with the forward primer 5′-GAACACTGACGATGAATCCTGCAGTA-3′ and the reverse primer 5′-TACATTTTGTCGTGATTCTCCTC3′. Bim/shBaxXBak cells were established by infecting the Bim cells with both Bax and Bak shRNA lentiviral particles. Infected cells were selected initially by sorting with flow cytometry to pick out cells with GFP expression and then were selected with 2 μg/mL puromycin.

**Cellular Fractionation and Protein Cross-Linking Assay.** Harvested cell pellets were resuspended in buffer A [20 mM Hepes, 40 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1× mixture protease inhibitor (Roche)] containing 250 mM sucrose, the volume of which was five times that of the cell pellet. After incubation on ice for 15 min, cells were broken by passage through a 22-gauge needle 25 times. The resulting mixtures were centrifuged at 15,000 × g for 10 min at 4 °C. The supernatants were saved as the whole-cell extract. For paraplegin, a mixture of the three oligos was used.

**Preparation of Whole-Cell Extract.** Whole-cell extract was prepared by lysing the cell pellet in lysis buffer [50 mM Tris (pH 8.0), 137 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1× mixture protease inhibitor] on ice for 30 min and then centrifuging the lysate at 18,000 × g for 20 min at 4 °C. The resulting supernatant was saved as the whole-cell extract.

**Preparation of Whole-Cell Extract.** Whole-cell extract was prepared by lysing the cell pellet in lysis buffer [50 mM Tris (pH 8.0), 137 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1× mixture protease inhibitor] on ice for 30 min and then centrifuging the lysate at 18,000 × g for 20 min at 4 °C. The resulting supernatant was saved as the whole-cell extract.
**UV Irradiation.** Cells were plated in 6-cm dishes for Western blot analysis or in Lab-Tek eight-chambered slides (Thermo Scientific) for immunostaining. UV irradiation (254 nm) was performed in a Stratagene Stratalinker at 200 mJ/cm$^2$.

**Immunostaining.** Cells were seeded in Lab-Tek eight-chambered slides (Thermo Scientific) and treated as needed on the following day. Cell were washed in PBS for 10 min and fixed in 2% PFA for 30 min at room temperature followed by three washes in PBS and incubation in PBS containing 0.1% Triton X-100 for 10 min. Primary antibodies were diluted in 5% BSA in PBS and incubated with the cells at 4 °C overnight. Cells then were washed three times in PBS and were incubated with secondary antibodies at room temperature for 1 h. After another three washes in PBS, the slides were covered and sealed and were examined with a Zeiss LSM 510 confocal microscope.

**TMRM Staining.** Cells were seeded in Lab-Tek eight-chambered cover glasses (Thermo Scientific) and were treated as needed on the following day. Then cells were incubated with 50 nM TMRM for 30 min. Cells were washed twice in warmed PBS and examined with a Zeiss LSM 510 confocal microscope. For flow cytometry analysis, cells were seeded in six-well plates and were treated and stained with TMRM as described above. Cells then were trypsinized and examined using a BD FACS Aria II flow cytometer with excitation at 561 nm and emission at 575 nm. Cells harboring stronger TMRM fluorescence intensity than the minimal fluorescence of 70% of the cells without doxycycline (Dox) treatment were counted as TMRM$^+$ cells.


![Fig. S1. Inducible expression of Bim triggers cytochrome c and Smac release. U2OS_Bim cells were treated with Dox for 8 h or were left untreated. P15 fractions were analyzed by Western blotting using anti-Bim, anti-Smac, anti-cytochrome c, and anti-VDAC antibodies.](image-url)
Fig. S2. Testing of the effect of mitochondrial proteases on Bim-induced apoptosis and OPA1 cleavage. (A, C, and E) U2OS_Bim cells were transfected with the indicated siRNAs for 48 h and then were treated with Dox for 12 h or were left untreated. Cell viability was determined using the Cell-Titer Glo kit. Data are presented as means ± SD of duplicate experiments. Whole-cell extracts of Bim cells with the indicated transfection for 48 h were analyzed by Western blotting (A and C). (B and D) U2OS_Bim cells were transfected with the indicated siRNAs for 48 h. Whole-cell extracts were analyzed by Western blotting using anti-OPA1 and anti-β-actin–HRP antibodies.

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Fig. S3. Inducible expression of tBid triggers mitochondria-mediated apoptosis. (A) A U2OS cell line with a stably transfected tBid transgene (U2OS_tBid) was treated with Dox in the presence or absence of z-VAD for the times indicated. Cell viability was determined using the Cell-Titer Glo kit. Data are presented as means ± SD of duplicate experiments. tBid and VDAC levels in the P15 fractions of U2OS_tBid cells after Dox treatment for the indicated times were examined by Western blotting. (B) U2OS_tBid cells were treated with Dox for the indicated times. Whole-cell extracts were analyzed by Western blotting using anti-caspase 9, anti-caspase 3, anti-PARP, and anti-β-actin-HRP antibodies. (C) U2OS_tBid cells were treated with Dox for 4 h in the presence of z-VAD or were left untreated. Immunostaining was performed using anti-cytochrome c (fluorescent secondary antibody with excitation at 633 nm, shown in yellow) and anti-Smac (blue) antibodies. ANT_RFP was examined together with the immunostained proteins. (Scale bars: 10 μm.) (D) U2OS_tBid cells were treated with Dox for the indicated times. The P15 fractions of the cells were cross-linked with 10 mM BMH where indicated and were analyzed by Western blotting using the indicated antibodies.
**Fig. S4.** Verification of tBid/OMA1 KO cells. (A) The genomic DNA sequence of OMA1 in tBid/OMA1 KO cells (KO_S1, KO_S2) around the gRNA targeting site (shown in blue) was aligned with that of wild-type tBid cells. (B) tBid and tBid/OMA1 KO cells were treated with 10 μM carbonylcyanide m-chlorophenylhydrazone (CCCP) for 90 min or were left untreated. Whole-cell extracts were analyzed by Western blotting using anti-OPA1 and anti–β-actin–HRP antibodies.

**Fig. S5.** OMA1 is required for UV-induced OPA1 cleavage and release of cytochrome c/Smac in HeLa cells. (A) Western blot analysis of OMA1 knockdown efficiency in HeLa cells. (B) HeLa cells transfected with the indicated siRNAs were treated with UV for 90 min. Whole-cell extracts of the cells were analyzed by Western blotting using anti-OPA1 and anti–β-actin–HRP antibodies. (C) HeLa cells transfected with the indicated siRNAs were treated with UV for 90 min. Immunostaining was performed using anti-cytochrome c (green) and anti-Smac (red) antibodies.
Fig. 56. Cytochrome c/Smac release and mitochondrial fragmentation require OMA1 protease activity. (A and B) The indicated cell lines were treated with Dox for 16 h or were left untreated. z-VAD was included during the treatment for Bim and OMA1_WT cells. Immunostaining was performed using anti-cytochrome c (green) and anti-Smac (red) antibodies (A) or anti-TOM20 antibody (B). (Scale bar: 10 μm.)
Fig. S7. The effect of OMA1 on Bim- and tBid-induced apoptosis can be bypassed by OPA1 knockdown. (A and B) Bim and Bim/shOMA1 cells were transfected with the indicated siRNAs and were treated with Dox for 8 h or were left untreated. z-VAD was included during the treatment of Bim cells and Bim/shOMA1 cells with OPA1 siRNA transfection. Immunostaining was performed using anti-cytochrome c (green) and anti-Smac (red) antibodies (A) or anti-TOM20 antibody (B). (Scale bar: 10 μm.) (C) tBid and tBid/OMA1 KO cells were transfected with siRNA against luciferase (luci) or OPA1. Forty-eight hours later, cells were treated with Dox for 12 h or were left untreated. Cell viability was determined using the Cell-Titer Glo kit. Data are presented as means ± SD of duplicate experiments. (D) tBid and tBid/OMA1 KO cells were transfected with the indicated siRNAs and were treated with Dox for 8 h or were left untreated. z-VAD was included during the treatment for tBid cells and tBid/OMA1 KO cells with OPA1 siRNA transfection. Immunostaining was performed using anti-Smac (blue) and anti-cytochrome c (fluorescent secondary antibody with excitation at 633 nm, shown in yellow) antibodies. ANT_RFP was examined together with the immunostained proteins. (Scale bar: 10 μm.)
Fig. 5B. Bim and tBid induction stabilizes the OMA1 precursor and activates OMA1 activity. (A) Bim/shOMA1 cells were transfected with Flag-tagged wild-type OMA1 or the protease active site mutant (H331A) of OMA1 for 48 h. Whole-cell extracts were analyzed by Western blotting using anti-Flag-HRP and anti-β-actin-HRP antibodies. (B–D) The indicated cell lines were transfected with Flag-tagged OMA1 for 48 h. Cells were treated with Dox for 8 h or were left untreated (A–C) or were treated with 10 μM CCCP for 90 min or were left untreated (D). Whole-cell extracts were analyzed by Western blotting using anti-Flag HRP, anti-OPA1, and anti-β-actin-HRP antibodies.
Fig. 59. Bax and Bak are required for tBid- and Bim-triggered, but not CCCP-triggered, OMA1 activation. (A and B) The indicated cell lines were treated with Dox for 8 h or were left untreated or were treated with 10 μM CCCP for 90 min. (A) z-VAD was included for Bim cells with Dox treatment. TMRM staining followed by flow cytometry analysis was performed. The P5 constricted area was the cell population we considered as TMRM+ cells. (B) TMRM staining was performed followed by imaging analysis. (Scale bar: 20 μm.) (C) Bim and Bim/shBax&Bak cells were treated with 10 μM CCCP for 90 min or were left untreated. TMRM staining was performed followed by imaging analysis. (Scale bar: 50 μm.) (D) Bim and Bim/shBax&Bak cells were transfected with Flag-tagged OMA1 for 48 h and then were treated with 10 μM CCCP for 90 min or were left untreated. Whole-cell extracts were analyzed by Western blotting using anti-Flag-HRP, anti-OPA1, and anti-β-actin-HRP antibodies.