

Review

Mitochondrial targeting of tBid/Bax: a role for the TOM complex?

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The release of pro-apoptotic proteins from the mitochondria is a key event in cell death signaling that is regulated by Bcl-2 family proteins. For example, cleavage of the BH3-only protein, Bid, by multiple proteases leads to the formation of truncated Bid that, in turn, promotes the insertion/oligomerization of Bax into the mitochondrial outer membrane, resulting in pore formation and the release of proteins residing in the intermembrane space. Bax, a monomeric protein in the cytosol is targeted to the mitochondria by a yet unknown mechanism. Several proteins of the outer mitochondrial membrane have been proposed to act as receptors for Bax, among them the voltage-dependent anion channel, VDAC, and the mitochondrial protein translocase of the outer membrane, the TOM complex. Alternatively, the unique mitochondrial phospholipid, cardiolipin, has been ascribed a similar function. Here, we review recent work on the mechanisms of activation and the targeting of Bax to the mitochondria and discuss the advantages and limitations of the methods used to study this process.

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Apoptosis can be executed using two different, but partially overlapping pathways.¹ The extrinsic pathway is initiated by the assembly of death receptors on the plasma membrane triggered by their binding of specific ligands. This leads to the activation of initiator caspases-8/10, which can directly activate executioner caspases, but can also recruit the intrinsic pathway by engaging mitochondrial effectors to amplify the cell death process. The intrinsic pathway can also be triggered by a variety of stress signals, and it culminates in the permeabilization of the outer mitochondrial membrane (OMM), often considered as the 'point of no return' in apoptosis signaling.² The OMM permeabilization allows the efflux of multiple pro-apoptotic proteins from the mitochondrial intermembrane space, among them cytochrome *c* that can then interact with Apaf-1 in the cytosol to form a molecular platform on which initiator caspase-9 is activated.^{1,2}

The past 10 years have seen considerable efforts to decipher the molecular pathways leading to permeabilization of the OMM, and it was early recognized that the Bcl-2 family of proteins play a prominent role in the regulation and execution of this process.³ As a result of this work, three different types of Bcl-2 family proteins have been identified: (1) the pro-apoptotic mediators, namely Bax and Bak; (2) the anti-apoptotic effectors, notably Bcl-2, Bcl-X_L and Mcl-1; and (3) a host of Bcl-2 homology domain 3 (BH3-only) proteins,

which control either both the pro- and anti-apoptotic family members or only a specific member of one group.³ The exact mechanisms of action and interplay of all these proteins are still a matter of vibrant debate.

Much work has been dedicated to the final mediators of OMM permeabilization, the pro-apoptotic proteins Bax and Bak. They share a similar structure that consists mainly of α -helical segments as well as three BH-domains, the signature of the Bcl-2 family of proteins. Importantly, Bax and Bak differ in their subcellular localization. Hence, Bax is mainly present in the cytosol of healthy cells, whereas Bak is anchored by a C-terminal transmembrane segment to either the OMM or endoplasmic reticulum (ER) membranes. During apoptosis, both proteins change conformation and insert two central helices into the membrane. These conformers can assemble into higher oligomers to promote formation of the pores through which the pro-apoptotic proteins of the intermembrane space are released. Although these conformational rearrangements are already quite complex for Bak, the situation with Bax is even more intricate, because it must undergo both conformational changes and targeting to the OMM.⁴ Therefore, the activation and OMM targeting of Bax have to be precisely controlled and regulated to ensure efficient apoptosis execution. The mechanisms proposed to be involved in this process are the topic of this review.

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Abbreviations: ANT, adenine nucleotide translocase; Drp-1, dynamin-related protein 1; ER, endoplasmic reticulum; IMM, inner mitochondrial membrane; MEF, mouse embryonic fibroblasts; Mfn, mitofusin; MTS, mitochondrial targeting signal; OMM, outer mitochondrial membrane; SAM, sorting and assembly machinery; tBid, truncated Bid; TIM, translocase of the inner mitochondrial membrane; TOM, translocase of the outer mitochondrial membrane; VDAC, voltage-dependent anion channel

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Experimental Approaches to Study Bax Activation and OMM Targeting

The experimental analysis of the significance and mechanisms of Bax/Bak-mediated OMM permeabilization has relied mainly on three different approaches, each having its particular advantages and limitations. The model closest to human physiology is without doubt a pure genetic system of a mammalian organism. The breakthrough in mouse genetics has allowed the creation of many knockout systems that were instrumental to unravel the physiological significance of Bax, Bak and various BH3-only proteins for both embryonic development and apoptosis regulation. The disadvantage of this approach lies in the complexity and redundancy of the signaling networks involved. This is particularly true for the Bcl-2 family of proteins, which consists of about 20 members with partially overlapping functions.

A similar problem is associated with the use of mammalian cell cultures, the standard experimental model for most apoptosis researchers. In contrast to the true knockout situation, in which one or more genes are deleted, the use of mammalian cell cultures allows for downregulation of protein(s), most often by RNAi or antisense technology. With this approach, protein depletion can be achieved to varying extents. Hence, there is always a risk that the depletion achieved by this approach is insufficient to completely silence the function of the protein in question. The alternative use of chemical inhibitors is limited by the notorious lack of specificity of almost all known inhibitors.

Models with reduced complexity, like heterologous and reconstituted systems, on the other hand, allow the study of molecular mechanisms at high resolution, but many aspects of complex regulatory networks are difficult to address. Reconstituted systems used to study Bax activation and targeting involve isolated mitochondria or liposomes composed of mixtures of different phospholipids. Although the use of isolated mammalian mitochondria might be complicated because of the presence of inherent Bcl-2 family proteins, liposomes offer the advantage of being easily controllable in terms of composition. These liposomes are typically loaded with proteins or dextrans, and characteristics of the release of these molecules are usually monitored on the addition of recombinantly expressed and purified Bcl-2 family proteins. The activation of Bax and Bax-induced protein release in the reconstituted systems can be monitored by a variety of methods, including analysis of the incorporation and assembly state of Bax, kinetics and the extent of release of entrapped molecules, and other biophysical methods. However, one limitation of these reconstituted systems lies in the choice of the components used, and care has to be taken to use a system that faithfully reflects the physiological situation.

Models of reduced complexity also include heterologous systems; especially, the heterologous expression of the mammalian Bcl-2 proteins in yeast or *Drosophila* has allowed studies of the molecular requirements for their activation and mitochondrial targeting as other, potentially interfering Bcl-2 proteins are not constitutively present in such systems, making them excellent experimental models to study the process of Bax activation without the need to overcome the anti-apoptotic Bcl-2 barrier. Another major advantage of the

heterologous systems is their accessibility to genetic manipulation, which is performed much more easily than in mammalian organisms. For example, the availability of a variety of mutant yeast strains has been important for such studies. However, only a combination of multiple approaches with careful assessment of the methods used will properly reveal the significance and organization of the signaling networks that control this important process.

Mechanisms of Bax Activation

To translocate to the mitochondria, cytosolic Bax has to be activated by specific mechanisms during apoptosis signaling. Two different, but not mutually exclusive, concepts have been elaborated, which propose either direct activation of Bax by a BH3-only protein (direct activation)⁵ or the relief of inhibition of constitutively active Bax by sequestration of anti-apoptotic Bcl-2 family members (indirect activation).⁶

The prevailing concept of OMM permeabilization involves the interaction of Bax with a BH3-only activator protein, like the truncated fragment of the cytosolic protein Bid (tBid), Bim or Puma. The interaction of their BH3-only domain with Bax is believed to trigger the translocation of Bax to the mitochondria followed by its insertion into the OMM, the oligomerization and the release of intermembrane space proteins (Figures 1a–d).¹ How such a direct activation would function at the molecular level is not understood, although it is conceivable that the transient interaction of a BH3-domain with Bax could result in conformational changes of the Bax protein. Interestingly, the

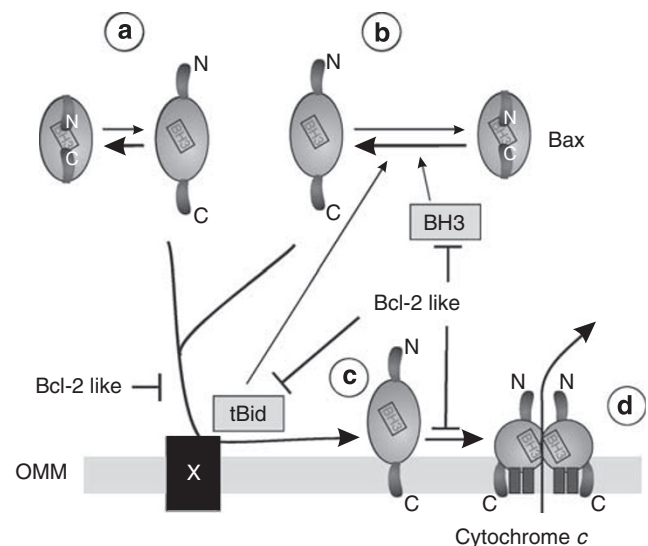


Figure 1 Hypothetical model of conformational change-mediated activation of Bax. Bax resides in the cytosol as a monomeric soluble protein. During thermal breathing the C-terminus (harboring the BH3-domain of Bax) is exposed (a). In the absence of anti-apoptotic Bcl-2 family proteins, this is sufficient for Bax integration into the OMM and cytochrome *c* release. A BH3-only protein may bind to the BH3-domain of Bax to stabilize its conformation in the cytosol. Alternatively, tBid might recruit Bax to the OMM and activate it (b). Here, Bax recognizes its receptor (X) and is inserted into the OMM (c). Inserted Bax oligomerizes to form pores through which intermembrane space proteins are released (d). Anti-apoptotic Bcl-2 proteins inhibit this activation by direct binding and neutralizing the BH3-only proteins and, therefore, Bax insertion

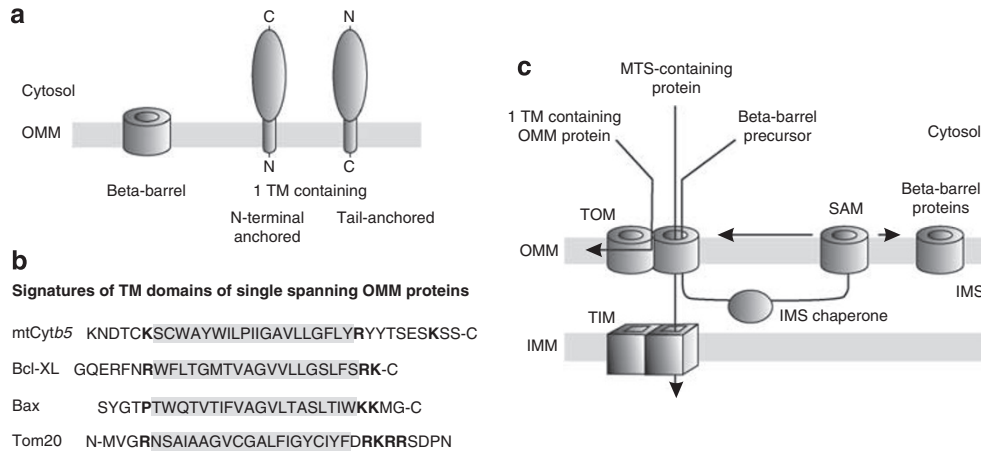


Figure 2 Composition and biogenesis of the outer mitochondrial membrane. (a) The OMM houses two distinct classes of proteins, β -barrel proteins and proteins containing an N- or a C-terminus single transmembrane segment. (b) Signatures of transmembrane domains anchoring OMM proteins. The C-termini of the human OMM proteins: mtCytb5, Bcl-X_L, and Bax and the N-terminus of human Tom20 are depicted. Gray highlighted sequences indicate the transmembrane domain. The positively charged amino acids flanking this region are shown in bold and could resemble the signature of the OMM single spanning transmembrane proteins. (c) Various precursor proteins use the pore formed by Tom40 of the TOM complex for their translocation through the OMM. These precursors are sorted to distinct subcompartments using the TIM complex. Beta-barrel precursors are translocated to the intermembrane space through the TOM complex. They are bound by IMS chaperones that deliver the proteins to the SAM machinery, which mediates the insertion of the β -barrel proteins into the OMM and their assembly, making them functional. OMM proteins containing one transmembrane segment presumably insert at the interface of the Tom40 dimer of the TOM complex. OMM, outer mitochondrial membrane; IMS, intermembrane space; SAM, sorting and assembly machinery; TIM, translocase of the inner mitochondrial membrane; MTS, mitochondrial targeting signal

BH3-domain of Bax is hidden in a hydrophobic groove that is occupied by the C-terminal α -helix.^{7,8} This C-terminal α -helix is required for targeting of Bax to mitochondria and represents a C-tail membrane anchor found in many mitochondrial outer membrane proteins (Figure 2a and b).⁹ Exposure of this domain involves isomerization of the conserved proline directly in front of the transmembrane (Figure 2b) and is most probably the key step in the activation of Bax. Once inserted into the membrane, the contact with the bilayer might induce conformational changes in the Bax protein, leading to the insertion of α -helices 5 and 6 into the bilayer.^{3,10} This restructured protein may then homo-oligomerize and create the pores through which pro-apoptotic intermembrane space proteins can be released. Experimental support for the direct activation model comes mainly from studies using reconstituted systems, consisting of recombinant proteins and isolated mitochondria from various sources or artificial liposomes. A recent study by the group of David Andrews using fluorescence resonance energy transfer (FRET) technology in combination with pure liposomes could resolve the chain of events with high resolution.¹¹ In their system, tBid interacted rapidly with the liposome membrane, and this membrane-bound tBid was required to activate Bax. These findings suggest that tBid initiates Bax activation in close proximity to the membrane, starting the slower process of membrane insertion and oligomerization of Bax. Hence, Bax is directly activated by tBid in this system.

The indirect activation model proposes that sequestration of the anti-apoptotic Bcl-2 proteins by BH3-only proteins is sufficient to trigger the activation/translocation of Bax to the mitochondria. This implies that Bax would be constitutively active, and that permanent inhibition is required to keep it in check. This concept has mainly derived from studies showing that the anti-apoptotic Bcl-2 proteins have much higher affinity

for BH3-only proteins than their pro-apoptotic relatives.^{10,12} Similarly, depletion of several activating BH3-only proteins (knockout of Bid and Bim, Puma knockdown) does not affect Bax activation and translocation during apoptosis, arguing that these activators are not essential for this process. However, although these studies showed that Bax activation/translocation was independent of the BH3-only proteins mentioned above, the significance of other BH3-only proteins for Bax activation has not yet been addressed.

How could this controversy between the two opposing concepts of Bax activation be resolved? The main difference between them is that they are based on results of experiments using different model systems. Work with the reconstituted systems consisting of liposomes and recombinantly expressed proteins convincingly shows that an activating function of certain BH3-only proteins is required for Bax action. In contrast, the indirect model implies that Bax gets activated when its inhibition by anti-apoptotic proteins is relieved. This discrepancy suggests that an important component required for Bax activation is lacking in the reconstituted systems. Studies using heterologous systems might resolve this issue. When overexpressed in *Drosophila* or various yeast strains, Bax is able to kill on its own, also in the absence of activating BH3-only protein.^{13,14} Similarly, addition of purified monomeric Bax can trigger cytochrome c release from isolated yeast mitochondria^{15,16} most probably because of the absence of anti-apoptotic Bcl-2 proteins in these mitochondria. This implies that Bax is constitutively active in the absence of anti-apoptotic Bcl-2 proteins. Importantly, the release of cytochrome c from the isolated yeast mitochondria can be accelerated by the inclusion of tBid, indicating a potentiating effect of tBid on Bax.^{15,16} Hence, the results with the heterologous systems suggest that, indeed, both models might be correct. Bax can target and

permeabilize mitochondria also in the absence of activating BH3-only protein(s) that, however, can potentially accelerate the process. Mechanistically, it is conceivable that the C-terminal membrane anchor flips out of the hydrophobic pocket of Bax with low frequency during thermal breathing of the molecule, allowing Bax to target the mitochondria (Figure 1a). An activating BH3-only protein might transiently bind to the BH3-domain of Bax and enhance its targeting efficiency by stabilizing a conformation of Bax with an exposed C-terminal membrane anchor (Figure 1b). If correct, C-tail exposure of Bax (in the absence of tBid), and hence its activation, should be a temperature sensitive process. Indeed, at 37 °C a substantial fraction of Bax is constitutively active in the absence of anti-apoptotic Bcl-2 proteins in the heterologous, but not in the reconstituted, system.^{15,16} Therefore, we speculate that the difference between the heterologous and the reconstituted systems could be explained by the presence of a factor in the yeast mitochondria offering a high-affinity binding site that is missing in the reconstituted system. This missing factor is most probably a constituent of the OMM.

Are There Bax Receptors in the OMM?

During apoptosis signaling, Bax targets the OMM for permeabilization. This preference is important, as it ensures the integrity of the other cellular membrane systems during the early phase of the apoptotic process. If this would not be the case, the apoptotic phenotype would become necrotic, for example, if the plasma membrane would also be permeabilized by Bax. Hence, how does Bax recognize the proper membrane in which to be inserted? The mitochondrial outer membrane houses, apart from its lipids, only a small set of proteins that can typically be divided into two classes: (1) membrane proteins with either one or only a few α -helical transmembrane segments and (2) β -barrel membrane proteins (Figure 2a). Bax is a member of the first group, displaying a similar pattern of positive charges at the side of the transmembrane segment that faces the intermembrane space, as it is found in other well-characterized single membrane spanning proteins.

The lipid composition of the OMM is similar to that of the ER. The only phospholipid specific to the mitochondria is cardiolipin, the signature lipid of the inner membrane of bacteria, chloroplasts and mitochondria. The role of cardiolipin in apoptosis signaling attracted much attention recently. We proposed that the binding of cytochrome *c* to cardiolipin in the inner mitochondrial membrane (IMM) limits the amounts of cytochrome *c* that can be released during apoptosis.¹⁷ In addition, a recent study suggested that cardiolipin might play a role in the activation of caspase-8 in the extrinsic pathway of apoptosis by acting as a receptor for the pro-caspase-8.¹⁸ A strict requirement of cardiolipin for targeting of Bax and tBid to the mitochondria has been suggested by experiments using reconstituted systems consisting of artificial liposomes and recombinant proteins.¹⁹ However, most studies have concluded that there is very little cardiolipin in the OMM. Typical values for cardiolipin in OMM fractions vary between 0.3 and 1.4% of the total phospholipids, when determined for mitochondria derived from rat liver and yeast, respectively.^{20,21} Importantly, the concentrations of cardiolipin,

ranging from 4–40% of the total phospholipids that were used in the liposomal studies exceeded by far those measured in the OMM fractions.^{11,19,22} Moreover, a strict requirement of cardiolipin for tBid/Bax-induced cytochrome *c* release has not been demonstrated in more physiological systems. The few well-controlled studies published suggest that a reduced level of cardiolipin in the mitochondria accelerates cell death without changing the efficacy of Bax targeting.²³ Similarly, the expression of Bax in yeast cells is lethal also in mutants completely devoid of cardiolipin because of deletion of *CRD1* encoding the terminal enzyme of cardiolipin biosynthesis.²⁴ One possible explanation for these conflicting results is that cardiolipin in the reconstituted system mimics a yet missing component that acts as the mitochondrial receptor for Bax in the intact cell. A similar conclusion was drawn in a recent report from T. Kuwana's group²⁵ reappraising the requirement for cardiolipin in a reconstituted system. Analyzing highly purified OMM vesicles, they found that cardiolipin was present only in trace amounts, and that mitochondrial outer membrane proteins could fully replace cardiolipin in reconstituted outer membrane proteoliposomes to allow tBid/Bax-induced cytochrome *c* release.

What Is the Missing Component Required for Bax Targeting?

The first of several suggested protein candidates for a mitochondrial Bax receptor (Table 1) was the voltage-dependent anion channel (VDAC1), also known as mitochondrial porin. Using immobilized Bcl-X_L, Tsujimoto and colleagues isolated VDAC1 and demonstrated that Bax accelerated the efflux of ¹⁴C-sucrose from reconstituted VDAC1 proteoliposomes, whereas Bcl-X_L inhibited this exchange.²⁶ Subsequent work by Korsmeyer's group reported that Bak can bind to VDAC2, but that this interaction leads to closure, rather than opening, of the VDAC channel.²⁷ However, more recently a possible role for the VDACs as receptors for Bax was challenged by the observation that ablation of VDAC isoforms 1, 2, and 3 did not affect apoptosis signaling in fibroblasts isolated from VDAC 1–3 knockout mice.²⁸

Another potential Bax receptor could be the mitochondrial fission and fusion machinery. Using time-lapse analysis, it was found that GFP-Bax targets mitochondria at sites in which they divide.²⁹ More specifically, Bax was found at foci on the mitochondria that contains Drp1, the dynamin mediating mitochondrial fission, and mitofusin 2 (Mfn2), another dynamin implicated in mitochondrial fusion. Furthermore, a recent report indicates that Bak interacts with Mfn2 and Mfn1 during apoptosis, although it is unclear how such interactions influence signaling.³⁰ Thus, it has been shown that cells lacking both Mfn1 and Mfn2 undergo apoptosis in response to several stimuli, including tBid, with similar, if not enhanced, kinetics as compared with control cells.^{31,32} Moreover, a recent study reported that overexpression of Mfn1 protects cells from apoptosis, though one would expect that cell death would be enhanced if Mfn1 can act as a receptor for tBid/Bax.³³

Various components of the translocase of the outer mitochondrial membrane (TOM complex) have also been considered as possible Bax receptor candidates. Tom22, a

Table 1 Possible tBid/Bax receptors: pros and cons

tBid/Bax receptors	For	Against
<i>β</i> -Barrel proteins VDAC	<ul style="list-style-type: none"> -Bax/Bak accelerated opening of VDAC reconstituted in proteo-liposomes²⁶ -Bax/Bak-induced cyt <i>c</i> release was blocked in VDAC1-deficient yeast²⁶ 	<ul style="list-style-type: none"> -tBid/Bax-induced cyt <i>c</i> release was similar from wt and VDAC 1-3 KO mice²⁸ -Oligomerization of Bax was detected in VDAC-deficient yeast mitochondria⁸ -Bax expression released cyt <i>c</i> from VDAC-deficient yeast mitochondria^{13,15}
Tom40	<ul style="list-style-type: none"> -Inactivation of ts-sensitive mutant of Tom40 inhibited tBid/Bax-induced cyt <i>c</i> release from yeast mitochondria upon mild incubation conditions (15 min, 30°C)¹⁵ -Direct interaction of Tom40 with tBid/Bax was detected using IP¹⁵ -Antibodies against Tom40 inhibited the insertion of tBid/Bax and cyt <i>c</i> release in rat liver mitochondria⁴² -Antibodies against TOM complex inhibited the insertion of tBid/Bax and cyt <i>c</i> release in Bax/Bak DKO MEFs¹⁵ 	<ul style="list-style-type: none"> -Inactivation of ts-sensitive mutant of Tom40 did not affect Bax-induced cyt <i>c</i> release from yeast mitochondria during harsh incubation conditions (1 h, 37°C)¹⁶ -Incomplete shRNA knockdown of Tom40 in HeLa cells did not affect Bax/Bak oligomerization during TNF-induced apoptosis⁴⁴
Sam50	<ul style="list-style-type: none"> -Long-term shRNA knockdown of Sam50 in HeLa cells caused a slight reduction in Bax/Bak oligomerization⁴⁴ 	<ul style="list-style-type: none"> -Inactivation of ts-sensitive mutant of Sam50 did not affect Bax-induced cyt <i>c</i> release from yeast mitochondria during harsh incubation conditions (1 h, 37°C)¹⁵
<i>Proteins with transmembrane domain</i>		
Tom70	<ul style="list-style-type: none"> -Loss-of-function mutations in <i>Drosophila</i> attenuated Bax-induced phenotype⁴³ 	<ul style="list-style-type: none"> -Proteolytic removal of Tom70 did not affect tBid/Bax-induced cyt <i>c</i> release in yeast¹⁵ -shRNA knockdown of Tom70 in HeLa cells did not affect Bax/Bak oligomerization during TNF-induced apoptosis⁴⁴
Tom20	<ul style="list-style-type: none"> -Loss-of-function mutations in <i>Drosophila</i> attenuated Bax-induced phenotype⁴³ -Antibodies against Tom20 inhibited the insertion of tBid/Bax and cyt <i>c</i> release in rat liver mitochondria⁴² 	<ul style="list-style-type: none"> -Proteolytic removal of Tom20 did not affect tBid/Bax-induced cyt <i>c</i> release in yeast¹⁵
Tom22	<ul style="list-style-type: none"> -Tom22 was found to interact with the N-terminus of Bax³⁴ -Antisense knockdown of Tom22 in the GBM cells abolished Bax association with mitochondria³⁴ -Direct interaction with Bax was detected using IP³⁴ -Antibodies against Tom22-inhibited association of tBid/Bax with rat liver mitochondria^{34,42} 	<ul style="list-style-type: none"> -Proteolytic removal of Tom22 did not affect tBid/Bax-induced cyt <i>c</i> release in yeast¹⁵
Drp1/Mfn2	<ul style="list-style-type: none"> -Time-lapse analysis revealed GFP-Bax clusters at foci of mitochondrial fragmentation in HeLa and Cos-7 cells²⁹ 	<ul style="list-style-type: none"> -Proteolytic removal of exposed domains of OMM proteins did not affect tBid/Bax-induced cyt <i>c</i> release in yeast¹⁵ -Bax-induced cyt <i>c</i> release was similar in mitochondria from yeast carrying mutations in proteins regulating mitochondrial morphology as in wt mitochondria¹⁶
<i>Lipids</i>		
Cardiolipin	<ul style="list-style-type: none"> -tBid/Bax-induced permeabilization of artificial liposomes required cardiolipin^{11,19,22} -tBid, but not Bid BH-3 peptide or oligomerized Bax, induced cardiolipin-dependent permeabilization of OMM vesicles and liposomes²⁵ -Cardiolipin directed tBid to reconstituted liposomes and to the mitochondria from CHO cells⁴⁷ 	<ul style="list-style-type: none"> -Yeast mitochondria lacking cardiolipin showed similar tBid/Bax-induced cyt <i>c</i> release as wt mitochondria^{15,24} -tBid-induced activation of Bax turned independent of cardiolipin when OMM proteins were included in the vesicles²⁵ -shRNA knockdown of cardiolipin synthase in HeLa cells accelerated cell death and cyt <i>c</i> release from mitochondria without changing tBid association with OMM²³

central subunit of the translocase was found as interaction partner of the N-terminus of Bax in a yeast two hybrid-screen. Subsequent analysis showed that injected antibodies against Tom22 can inhibit cell death efficiently in the glioblastoma multiforme cells.³⁴ We have recently used a heterologous system consisting of yeast mitochondria and purified proteins to address the targeting specificity of Bax. Importantly, either the expression of Bax in yeast cells¹³ or the exposure of isolated yeast mitochondria to Bax and tBid, resulted in incorporation of Bax into the OMM and the release of cytochrome *c*¹⁵. Hence, it appears that Bax can also insert into the OMM of yeast mitochondria. As yeast mitochondrial proteins are different from their mammalian homologs, this insertion occurs most probably through a conserved pathway for insertion of C-tail-anchored proteins rather than by direct protein–protein interaction.

Owing to the availability of unambiguous and well-characterized mutants in yeast, this heterologous system should allow the study and identification of OMM component(s) able to serve as potential Bax receptor(s) on the yeast mitochondria. Our analysis led us to rule out an involvement of cardiolipin, as cytochrome *c* was released efficiently also from mitochondria isolated from *CRD1*-deleted mutants lacking this phospholipid¹⁵. Similarly, the proteolytic removal of the OMM proteins exposing external domains toward the cytosol (such as Mfn2, Tom22 and Drp1) did not affect tBid/Bax-induced cytochrome *c* release. Hence, the only remaining receptor candidates were mitochondrial β -barrel proteins that are resistant to externally added proteases. However, the most abundant β -barrel proteins in the OMM, the VDACs, were found not to be required for Bax targeting, as cytochrome *c* release triggered by tBid/Bax proceeded efficiently also in the mitochondria from the yeast mutants depleted of VDAC 1 and 2, which are the two isoforms normally found in yeast.¹⁵

The family of β -barrel proteins is rather small, consisting of six different proteins in yeast and presumably also in mammals. Beta-barrel proteins are derived from bacterial outer membrane proteins and are found in non-plant eukaryotes exclusively in the mitochondrial outer membrane.³⁵ This makes them plausible candidates for proteins that would allow specific targeting of Bax to the mitochondria. All β -barrel proteins are synthesized in the cytosol and transported by the assistance of the TOM complex into the mitochondrial intermembrane space (Figure 2c). Here, the precursor proteins interact with small chaperones handing them over to the sorting and assembly (SAM) machinery that mediates the insertion and assembly of the β -barrel proteins into the outer membrane.^{36,37} Interestingly, both the SAM and the TOM complex contain a β -barrel protein as their main component, that is Sam50 and Tom40. Other members of this class include the VDACs as well as Mmm2 and Mdm10, two proteins that are involved in TOM complex assembly and mitochondrial morphology in yeast.³⁸ The mammalian homologs of these proteins have not yet been identified. As Tom40 and Sam50 are indispensable for cell viability, mammalian knockout models are not available. Only in yeast, there are mutants that allow functional analysis of these proteins with adequate precision. Such mutants carry temperature sensitive proteins that turn nonfunctional on incubation at non-permissive temperature.³⁹ When analyzing tBid/Bax-induced

cytochrome *c* release from mutant yeast mitochondria, the normal cytochrome *c* release was observed when the mitochondria containing a heat-labile version of Tom40 were incubated at the permissive temperature. However, exposure of these mitochondria to the nonpermissive temperature decreased tBid/Bax-induced cytochrome *c* release significantly, in contrast to findings with wild-type mitochondria that showed similar release kinetics under both conditions.¹⁵ In addition, antibodies against the TOM complex were able to block insertion/activation of Bax as well as cytochrome *c* release in permeabilized Bax/Bak double-knockout MEFs. Importantly, the requirement for functional Tom40 was bypassed, when a chemically oligomerized form of Bax was used to trigger cytochrome *c* release. On the basis of this and other results, it was concluded that the TOM complex is important for tBid/Bax-induced cytochrome *c* release in both yeast and mammalian cells.¹⁵

The TOM complex is a well-conserved 450 kDa protein assembly, which is equipped with receptors, structural components and a dimer of the Tom40 protein, resembling the general translocation pore through which precursor proteins are transported over the OMM. To date, all comparative analyses indicate that the general mechanism of protein import into mitochondria and, hence, probably, also that of the TOM complex *per se* are conserved. Moreover, the function of the TOM complex might not be restricted to the translocation of precursor proteins across the OMM. It was recently suggested that this complex plays a direct role also in the outer membrane insertion of proteins with one transmembrane segment, like Bax with an exposed C-tail (Figure 2c); this function involves the Tom40 protein⁴⁰. A lateral opening of the β -barrel to release transmembrane segments into the outer membrane appears thermodynamically unfavorable. Hence, it was suggested that transmembrane segments insert at the interface of the Tom40-dimer.⁴¹ A role for the TOM complex as a conserved receptor for Bax would also be in line with results of other studies in both mammalian systems and *Drosophila*. Hence, Vallete's group showed earlier that blocking the TOM complex with antibodies against Tom22 inhibited the interaction of Bax with rat liver mitochondria *in vitro* as well as Bax-induced apoptosis in a glioblastoma cell line.³⁴ The same group has later shown that Bax integration into the OMM requires both Tom22 and Tom40.⁴² Similarly, studies of apoptosis in *Drosophila melanogaster*, representing a heterologous system in which human Bax was expressed, revealed that certain TOM subunits were required for efficient cell death in this system.⁴³

Are Protein Transporters not Involved in Bax Targeting?

Other studies have concluded that the TOM complex is not required for mitochondrial Bax targeting in either yeast or mammalian model systems. Thus, Meisinger and colleagues¹⁶ reported that inactivation of the known protein import and sorting machineries of the outer membrane of yeast mitochondria did not impair its permeabilization by human Bax. Furthermore, in a recent publication in *Cell Death and Differentiation*, Ross *et al.*⁴⁴ reported that downregulation of various subunits of the TOM and SAM complexes by RNAi technology in stably transfected HeLa cells had no influence

on TNF-induced Bax targeting or apoptosis. This approach led to a 80–90% reduction in the protein levels of Tom22 and Tom40 seen in control cells, whereas Sam50 was more efficiently depleted. Exposure of the cells to TNF led to similar oligomerization of both Bak and Bax, as measured by blue native PAGE. Only when cells were depleted of Sam50 for longer time periods, a slight decrease in TNF-induced oligomerization of Bax was observed, suggesting that either Sam50, or a substrate of Sam50 might play a role in Bax oligomerization or targeting. Using isolated mitochondria from depleted cells, they also tested whether radiolabeled Bak and a constitutively active Bax were oligomerizing with similar kinetics as compared with control mitochondria *in vitro*. Depletion of the various subunits did not inhibit the formation of a 450 kDa complex, into which both Bax and Bak were assembling. However, it should be noted that this 450 kDa complex is not equivalent to the oligomers formed by Bax or Bak during apoptosis, which are smaller in size. It is also not clear whether the *in vitro*-translated radiolabeled form of Bax used in this study had the same folding as the monomeric Bax found in the cytosol. Proteins with a folding similar to oligomerized Bax could bypass a requirement for the TOM complex, in line with observations in yeast in which incubation of mitochondria with Bax at 37 °C allowed such a bypass.^{15,16} However, the most pertinent question is whether depletion of Tom40 to 10% of its initial abundance was sufficient to completely silence Tom40 function in the system. In fact, our own study indicated that 10% of Tom40 remaining after downregulation was sufficient to mediate almost full import capacity of precursor proteins into the mitochondrial matrix of HEK293 cells. Likewise, mitochondrial targeting of GFP-Bax during staurosporine-induced apoptosis in the Tom40-depleted HEK293 cells occurred with similar kinetics as in nondepleted cells (Katharina M. Walter and Martin Ott, unpublished data). Therefore, extreme caution must be exerted when interpreting results of experiments with incompletely depleted mitochondrial protein import components. Even if a defect is observed for one import pathway, it is possible that another pathway, converging on the same components, can still operate properly because of different kinetics. In contrast, carefully controlled antibody interference allows specific functions to be blocked more efficiently. With such an approach, a requirement of TOM subunits for Bax insertion into the OMM could be demonstrated earlier.^{15,34,42} However, for further investigations of the role of the TOM complex in mitochondrial protein import and tBid/Bax-induced cytochrome *c* release, it will be important to develop new strategies to manipulate the TOM complex in mammalian cells. A possible approach could be to inhibit the TOM complex using the expression of nanobodies from titrable promoters.⁴⁵ Similarly, a reconstitution approach starting with purified OMM vesicles and fractions thereof obtained either by depleting the fraction of certain components using immunoprecipitation or by chromatographic methods, should allow us to unravel the molecular identity of the physiological mitochondrial Bax receptor (possibly Tom40).

Apart from the controversy about a requirement of the TOM complex for Bax targeting, two findings of the study by Ross *et al.*⁴⁴ seem particularly interesting: (1) the defect of Bax oligomerization seen after long-term depletion of Sam50 and

(2) the formation of a 450 kDa complex with radiolabeled Bax and Bak in isolated mitochondria *in vitro*. The first observation points to the possibility that a substrate of Sam50, or Sam50 itself, might play a role in Bax oligomerization. Potential Sam50 substrates include the Tom40 protein, but also other not yet identified β -barrel protein. Second, it will be important to identify subunits of the 450 kDa complex that bind radioactive Bax and Bak upon mitochondrial import. Notably, the size of this complex is identical to the size of the solubilized TOM complex, although it is certainly possible that another, yet unidentified complex is responsible for the binding of these proteins.

Do BH3-Only Proteins Direct Bax to the Mitochondria?

Another remaining question concerns the role that the BH3-only proteins might play in the process of Bax targeting to the mitochondria. Although it is clear that they can potentially activate Bax, it is also possible that they can assist in Bax targeting to the OMM by providing local activation at the membrane. In line with this hypothesis is the observation that when BimS was targeted to the mitochondria, it triggered apoptosis much more potently than did a nontargeted analog.⁴⁶ Similarly, tBid was early demonstrated to target mitochondria at the contact sites between the OMM and IMM.⁴⁷ Until now, the molecular properties of the contact sites have not been well characterized. In addition to metabolic contact sites, consisting of VDAC-ANT (adenine nucleotide translocase) complexes, contact sites can also be established between the TOM and TIM complexes during protein transport.⁴⁸ Therefore, the possibility exists that tBid could target the TOM complex on mitochondria and cooperate with the translocase in the insertion of Bax. Such Bax/Bak-independent targeting of a BH3-only protein would be in line with a recent report showing that the BH3-only protein Bad can target mitochondria, possibly by using VDAC or Bcl-X_L to trigger alterations in mitochondrial calcium signaling.⁴⁹

Another mechanism of Bax insertion into the OMM is the binding of cytosolic Bax to mitochondrial, oligomeric Bax, presumably by an interaction of the BH3-domains of two Bax molecules.^{42,50} This might accelerate the permeabilization of the OMM in a self-amplification loop. Importantly, this does not require the presence of targeting signals in the newly inserting Bax molecules. This phenomenon would further complicate the analysis of Bax targeting in the presence of a mitochondrial Bax-like protein, because it would conceal the involvement of other factors required for initial Bax recruitment. How all these different pathways operate simultaneously will be difficult to address in a mammalian cell-based system. However, the use of simplified systems might allow such studies and contribute to our understanding of this fundamental process in apoptosis signaling.

Concluding Remarks

Although mitochondrial permeabilization is a key event in apoptosis signaling, important details of this process are still unclear. There is general agreement that the pro-apoptotic Bcl-2 proteins, Bax and Bak, are the terminal mediators of OMM permeabilization, and that BH3-only family members are often critical for their activation, and perhaps also for Bax

translocation to the mitochondria. It also appears that certain OMM constituents, notably members of the TOM complex, might serve as tBid/Bax receptors on the outer membrane. As a result of Bax insertion into the OMM, pores are formed that mediate the release of pro-apoptotic proteins from the intermembrane space into the cytosol. The molecular nature of such pores, that is whether they are proteinaceous or lipidic, is currently not known. Hence, further work is needed to finally resolve these important events in cell death signaling.

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