OUTLOOK

MOMP in the absence of BH3-only proteins

Ana J. García Sáez[1] and Andreas Villunger[2,3]

[1]Interfaculty Institute of Biochemistry (IFIB), University of Tübingen 72076, Tübingen, Germany; [2]Division of Developmental Immunology, Biocenter, Medical University Innsbruck, A-6020 Innsbruck, Austria; [3]Tyrolean Cancer Research Institute, A-6020 Innsbruck, Austria

The minimum requirement for mitochondrial apoptosis has been controversial ever since the discovery of BCL-2 as a cell death regulator. In this issue of *Genes & Development*, O'Neill and colleagues (pp. 973–988) end a long-standing debate by creating a cellular system free of BCL-2 family proteins, thereby identifying the outer mitochondrial membrane rather than BH3-only proteins as the only requirement for BAX/BAK activation and mitochondrial outer membrane permeabilization (MOMP).

Early in the new millennium, two schools of thought emerged as to how pro- and anti-apoptotic BCL-2 family proteins control BAX/BAK-dependent mitochondrial outer membrane permabilization (MOMP), which leads to caspase activation and apoptosis. Several high-profile studies pushed two different, seemingly incompatible models. The "direct activation" model suggested that, within the BCL-2 family, proapoptotic BH3-only proteins exist in two distinct flavors; i.e., "sensitizers," which are present in an inactive state and need to be post-translationally modified or transcriptionally induced (e.g., BAD and NOXA), and "direct activators" (BID and BIM), which are sequestered by BCL-2 in steady state until displaced by "sensitizers" upon apoptotic cell stress. Free BIM or BID then activate BAX/BAK via transient "noncanonical" protein–protein interactions to trigger MOMP (Letai et al. 2002). A variation of this theme, the "embedded together" model, incorporates an active role of the mitochondrial membrane in the complex regulation of interactions between BCL-2 family proteins that happen in parallel to regulate apoptosis (Lovell et al. 2008).

The alternative "neutralization model" posited that BCL-2 prosurvival proteins sequester and inhibit BAX/BAK by direct binding in steady state until conditions of stress activate BH3-only proteins and disrupt this interaction to allow BAX/BAK to acquire an active conformation and induce MOMP (Willis et al. 2007). In an attempt toward reconciliation, the "unified model" tried to combine both concepts in a hierarchical manner and suggested that the active conformation of BAK/BAK requires a transient interaction with BH3-only proteins. Once activated, BAX/BAK can be inhibited by BCL-2-like proteins, which can also interact with BH3-only proteins. Notably, the model also established that prosurvival BCL-2 proteins inhibited BAX/BAK more efficiently than BH3-only proteins (Llambi et al. 2011).

The "direct activation" model has found ample support in cell biological studies as well as in vitro analyses using recombinant proteins, lipid vesicles, or isolated mitochondria to study MOMP. In vitro, recombinant full-length BAX, purified without detergents, exists in a monomeric, soluble, and inactive form even in the presence of membranes. Addition of substoichiometric amounts of a "direct activator" BH3-only protein or BH3 peptide induces potent BAX activation, including membrane insertion, oligomerization, and membrane permeabilization, blocked effectively by BCL-X and the like (Kuwana et al. 2005; Lovell et al. 2008). However, over the years, the list of BH3-only proteins with "direct activator" potential became increasingly long (adding PUMA, BMF, and NOXA, leaving only BAD, BIK, and HRK as "sensitizers" behind), suggesting that experimental design can strongly impact BH3 peptide or BH3-only protein-mediated BAX/BAK activation or MOMP in in vitro assays (Hockings et al. 2015).

While the "neutralization model" clearly also had strong arguments on its side, recent structural studies actually tilted the balance in favor of the "direct activation" model by demonstrating direct binding of the BH3 peptides of the "direct activators" BID and BIM to the hydrophobic groove of BAX and BAK (Czabotar et al. 2013, 2014). In addition, interaction with the BH3 peptides triggered a conformational change in BAX that was associated with dimer formation (the minimal unit needed) before assembling into higher-order oligomers in the presence of membranes or detergents (Subburaj et al. 2015).

[*Keywords*: anti-apoptotic Bcl-2 proteins; BH3-only; Bax/Bak activation; outer mitochondrial membrane]
Corresponding author: andreas.villunger@i-med.ac.at
Article is online at http://www.genesdev.org/cgi/doi/10.1101/gad.281519.116.

Although each model had its strengths, a certain degree of ambiguity persisted as to the minimal requirements for MOMP. Mouse genetics also failed to resolve these issues due to the high degree of redundancy within the family. Results on compound mutants—e.g., BH3-only double-knockout or triple-knockout mice or cells ± RNAi—as well as BH3 domain knock-in exchange studies were simply not fully conclusive (Villunger et al. 2012), although experiments using cells from compound mutant mice suggested that direct activation might not be essential, at least for the activation of BAK (Senft et al. 2015).

Luo and coworkers (O'Neill et al. 2016) used CRISPR/Cas9 technology to tackle the issue of BAX/BAK activation in mammalian cells in a pragmatic and highly efficient way. They generated a cell system that lacks all BH3-only proteins as well as the possible culprits p53 and Rb. Not surprisingly, these cells are highly resistant to apoptotic stimuli. However, they do undergo BAX/BAK-driven MOMP and caspase activation upon genetic or pharmacologic neutralization of BCL-2 proteins with kinetics and rates comparable with those of wild-type cells. These observations make a convincing argument against the need for direct activation of BAX/BAK by any known BH3-only protein, p53, or Rb for the induction of MOMP.

Taking the system to the next level, the investigators then generated BCL-2 allKO HCT116 cells lacking all established members of the BCL-2 family. Remarkably, these cells appear to grow normally and show no major problems in cell cycle progression. Thus, we need to consider that mitochondrial dynamics, autophagy, Ca^{++} homeostasis, and respiratory capacity—all processes in which individual BCL-2 family proteins have been implicated repeatedly—can easily proceed without them.

Even further rattling the fundamental beliefs in apoptosis research, reconstitution assays in BCL-2 allKO cells show that BAX and BAK share identical localization to mitochondria in the cell, documenting that their distribution in healthy wild-type cells is regulated by interactions within the family, in agreement with the "retrotranslocation" hypothesis (Edlich et al. 2011). The one and only requirement for MOMP is indeed the presence of the outer mitochondrial membrane and the C-terminal end (helix 9) in BAX or BAK, as its deletion prevents mitochondrial binding and subsequent formation of higher oligomers and MOMP.

At a minimum, the study by Luo and colleagues (O'Neill et al. 2016) prompts a re-evaluation of the work with reconstituted systems, eliminates a role of p53 or Rb in BAX/BAK activation, and forces us to reconsider the role of "direct activator" BH3-only proteins in this process. While it is clear that there can be direct interaction between cleaved BID and BAX as a potent trigger of its activity, one should keep in mind that BAX easily becomes active in the test tube. Changes in pH, mild heat, exposure to detergents, oxidized lipids, impurities, or too high protein concentration during purification can drive BAX activation and pore formation in the membranes of liposomes and giant unilamellar vesicles in the absence of any other BCL-2 protein. In addition, we also know that membrane-bound active BAX can help to recruit more soluble BAX to the membrane and activate it in a feed-forward loop, facilitating MOMP.

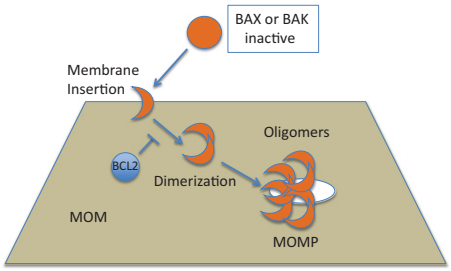

Figure 1. In the absence of any other BCL-2 family protein, proapoptotic BAX or BAK spontaneously accumulate in the outer mitochondrial membrane that promotes formation of homodimers, the minimal units required for the generation of higher-order oligomers that promote MOMP. These events are counteracted effectively by BCL-2 prosurvival homologs and do not require the input of any known BH3-only protein, p53, or Rb.

Ultimately, we can think of a possible scenario in which BAX indeed would have an inactive form and be able to transform into an active conformation via a relatively low-energy barrier and in which "direct activation" is not strictly necessary. The outer mitochondrial membrane would provide the right environment to support the active structure of BAX, thereby shifting the equilibrium toward the formation of oligomers and MOMP. This spontaneous activation of BAX or BAK is kept in check by prosurvival BCL-2 homologs. During stress, BH3-only proteins would then shift the balance toward MOMP by catalyzing the rate of BAX activation in the outer mitochondrial membrane and seem to do so apparently only by neutralizing their anti-apoptotic BCL-2 counterparts (Fig. 1).

Acknowledgments

We apologize to the many scientists in the field whose work we could not cite due to space constraints. Work in our laboratories is funded by the Deutsche Forschungsgemeinschaft (DFG) and the Austrian Science Fund (FWF) within the FOR2036 initiative "New insights into Bcl-2 family interactions."

References

Czabotar PE, Westphal D, Dewson G, Ma S, Hockings C, Fairlie WD, Lee EF, Yao S, Robin AY, Smith BJ, et al. 2013. Bax crystal structures reveal how BH3 domains activate Bax and nucleate its oligomerization to induce apoptosis. *Cell* **152:** 519–531.

Czabotar PE, Lessene G, Strasser A, Adams JM. 2014. Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. *Nat Rev Mol Cell Biol* **15:** 49–63.

Edlich F, Banerjee S, Suzuki M, Cleland MM, Arnoult D, Wang C, Neutzner A, Tjandra N, Youle RJ. 2011. Bcl-x(L) retrotranslocates Bax from the mitochondria into the cytosol. *Cell* **145:** 104–116.

Hockings C, Anwari K, Ninnis RL, Brouwer J, O'Hely M, Evangelista M, Hinds MG, Czabotar PE, Lee EF, Fairlie WD, et al. 2015. Bid chimeras indicate that most BH3-only proteins can directly activate Bak and Bax, and show no preference for Bak versus Bax. *Cell Death Dis* **6:** e1735.

Kuwana T, Bouchier-Hayes L, Chipuk JE, Bonzon C, Sullivan BA, Green DR, Newmeyer DD. 2005. BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell* **17:** 525–535.

Letai A, Bassik M, Walensky L, Sorcinelli M, Weiler S, Korsmeyer S. 2002. Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell* **2:** 183.

Llambi F, Moldoveanu T, Tait SW, Bouchier-Hayes L, Temirov J, McCormick LL, Dillon CP, Green DR. 2011. A unified model of mammalian BCL-2 protein family interactions at the mitochondria. *Mol Cell* **44:** 517–531.

Lovell JF, Billen LP, Bindner S, Shamas-Din A, Fradin C, Leber B, Andrews DW. 2008. Membrane binding by tBid initiates an ordered series of events culminating in membrane permeabilization by Bax. *Cell* **135:** 1074–1084.

O'Neill KL, Huang K, Zhang J, Chen Y, Luo X. 2016. Inactivation of prosurvival Bcl-2 proteins activates Bax/Bak through the outer mitochondrial membrane. *Genes Dev* (this issue). doi: 10.1101/gad.276725.115.

Senft D, Weber A, Saathoff F, Berking C, Heppt MV, Kammerbauer C, Rothenfusser S, Kellner S, Kurgyis Z, Besch R, et al. 2015. In non-transformed cells Bak activates upon loss of anti-apoptotic Bcl-XL and Mcl-1 but in the absence of active BH3-only proteins. *Cell Death Dis* **6:** e1996.

Subburaj Y, Cosentino K, Axmann M, Pedrueza-Villalmanzo E, Hermann E, Bleicken S, Spatz J, Garcia-Saez AJ. 2015. Bax monomers form dimer units in the membrane that further self-assemble into multiple oligomeric species. *Nat Commun* **6:** 8042.

Villunger A, Labi V, Bouillet P, Adams J, Strasser A. 2012. Can the analysis of BH3-only protein knockout mice clarify the issue of 'direct versus indirect' activation of Bax and Bak? *Cell Death Differ* **18:** 1545–1546.

Willis SN, Fletcher JI, Kaufmann T, van Delft MF, Chen L, Czabotar PE, Ierino H, Lee EF, Fairlie WD, Bouillet P, et al. 2007. Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. *Science* **315:** 856–859.

MOMP in the absence of BH3-only proteins

Ana J. García Sáez and Andreas Villunger

Genes Dev. 2016, **30:**
Access the most recent version at doi:10.1101/gad.281519.116