

# Interrogating the relevance of mitochondrial apoptosis for vertebrate development and postnatal tissue homeostasis

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**“Programmed cell death or ‘apoptosis’ is critical for organogenesis during embryonic development and tissue homeostasis in the adult. Its deregulation can contribute to a broad range of human pathologies, including neurodegeneration, cancer, or autoimmunity...”** These or similar phrases have become generic opening statements in many reviews and textbooks describing the physiological relevance of apoptotic cell death. However, while the role in disease has been documented beyond doubt, facilitating innovative drug discovery, we wonder whether the former is really true. What goes wrong in vertebrate development or in adult tissue when the main route to apoptotic cell death, controlled by the BCL2 family, is impaired? Such scenarios have been mimicked by deletion of one or more prodeath genes within the BCL2 family, and gene targeting studies in mice exploring the consequences have been manifold. Many of these studies were geared toward understanding the role of BCL2 family proteins and mitochondrial apoptosis in disease, whereas fewer focused in detail on their role during normal development or tissue homeostasis, perhaps also due to an irritating lack of phenotype. Looking at these studies, the relevance of classical programmed cell death by apoptosis for development appears rather limited. Together, these many studies suggest either highly selective and context-dependent contributions of mitochondrial apoptosis or significant redundancy with alternative cell death mechanisms, as summarized and discussed here.

BCL2, the founding member of the BCL2 family, was identified 30 years ago upon characterization of a frequently observed t14,18 chromosomal translocation in follicular lymphoma (Cleary et al. 1986; Tsujimoto and Croce

1986). After realizing that the presumed oncogenic potential of this gene lies within its anti-apoptotic property (Vaux et al. 1988), a number of related proteins were identified in protein–protein interaction screens, by sequence similarity, and/or by structural homology in humans as well as different vertebrate model organisms and viral pathogens. Some of these proteins exert inherent pro-survival function by blocking apoptosis, and some of them may even be converted into killers by limited proteolysis or upon alternative splicing of the encoding RNA or act exclusively as inducers of cell death (Tait and Green 2013; Czabotar et al. 2014). Orthologs of these proteins found in nematodes regulate apoptosis during embryonic development and upon exogenous stress in the germline, but molecular details do differ substantially from vertebrates, while, in insects, more distant BCL2 relatives seem to have no role in apoptosis regulation at all. In fact, it seems that “intrinsic” or “mitochondrial” BCL2-regulated apoptosis has been specially tuned to fit the needs of vertebrates (Bender et al. 2012). For more detailed information on the history of the discovery of the extended BCL2 family, we refer the interested reader to a recent comprehensive “time-line” article by Delbridge et al. (2016).

Next to anti-apoptotic BCL2, BCLXL, BCLW, BCLB, MCL1, and A1/BFL1, cell death-inducing family members include apical “BH3-only” proteins (e.g., BIM, PUMA, or BID) that act as preformed sentinels or are induced transcriptionally in response to stress as well as BAX-like proteins that control mitochondrial outer membrane (MOM) integrity. Once activated, BAX and related BAK can promote MOM permeabilization (MOMP) by pore formation, much like some bacterial toxins (Garcia-Saez et al. 2010) or phage-derived holins do (Pang et al. 2011), causing the release of “danger signals” from the intermitochondrial

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membrane space, including cytochrome c and inhibitor of apoptosis protein (IAP) antagonists such as SMAC/DIA-BLO (Tait and Green 2013). This leads to the activation and amplification of a proteolytic cascade, initiated and executed by endopeptidases of the caspase family. Along this route, caspases orchestrate largely immunologically silent cell death (and may even induce tolerance), contrasting their role in other, proinflammatory cell death paradigms (Martin et al. 2012). Although not all apoptotic cell death needs to be immunologically silent, it is fair to say that apoptosis occurring during vertebrate development indeed is. This is most likely also true in tissue homeostasis in the adult, where apoptosis aims to maintain steady state in the absence of injury or other types of stress (Martin et al. 2012).

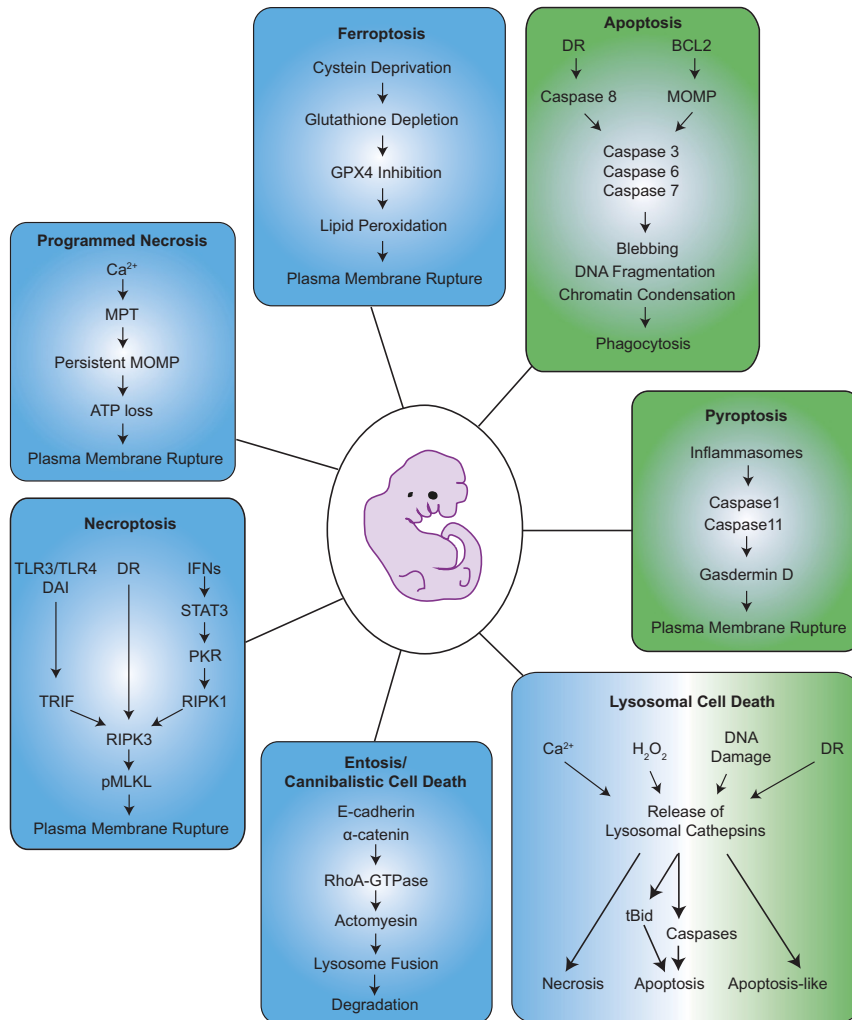
Numerous studies have explored the role of anti-apoptotic BCL2 proteins in mouse development, and the consequences of loss of function of these proteins—highlighting tissue and cell type-specific prosurvival function of these proteins—have been reviewed recently elsewhere (Sochalska et al. 2015). Clearly, these studies pinpoint that probably all cell types, even platelets, rely on apoptosis inhibition by different BCL2 family proteins during their development or in the adult. But what about the other side of the coin? What can we learn about physiology (rather than pathophysiology) when we block apoptosis? This review summarizes the consequences of impaired apoptotic cell death in vertebrate development and tissue homeostasis, largely based on mouse studies, and discusses possible explanations for the overall limited impact of apoptosis deficiency on these processes. We close with some possibly provocative thoughts about putative redundancies among the different cell death machineries described today (Fig. 1) and lines of research that might address these possibilities.

### Mitochondrial apoptosis: initiation, execution, and the enigmatic apoptotic pore

Intense research over the past decades has shaped a reasonably sharp picture of how prodeath BCL2 proteins initiate and execute mitochondrial apoptosis via MOM (Fig. 2). The early “rheostat model” proposed that the ratio of proapoptotic and anti-apoptotic proteins in a given cell known at that time—e.g., BCL2:BAX (or other combinations)—defines cellular destiny (Oltvai et al. 1993; Sedlak et al. 1995). This model had to be dismissed soon due to the discovery that BH3-only proteins can promote apoptosis by direct interaction with BCL2 prosurvival proteins, displacing BAX (Yang et al. 1995; Wang et al. 1996). The “neutralization” model of mitochondrial apoptosis hence suggested that BAX/BAK are constitutively active when all BCL2-like proteins present in a given cell are neutralized by direct engagement with BH3-only proteins that display different affinities for the former (Chen et al. 2005; Willis et al. 2005, 2007). This model had some competition based on findings that some BH3-only proteins, such as truncated BID (tBID) (Wang et al. 1996) or BIM, can trigger BAX/BAK conformational changes and activa-

tion by direct, albeit transient, interaction (Cheng et al. 2001; Letai et al. 2002; Kuwana et al. 2005). The ensuing “direct activation” model (Kim et al. 2006; Brunelle and Letai 2009) built on these parallel observations and gained much support by additional studies making use of full-length recombinant proteins to study permeabilization of artificial lipid vesicles in the absence or presence of mitochondria-specific lipids (in particular cardiolipin) but also whole mitochondria. These observations formed the basis of the “embedded together” model (Lovell et al. 2008), which incorporated the role of the mitochondrial membrane in the regulation of the interactions between Bcl-2 proteins in an advanced version of the “direct activation” model (Fig. 2; Bogner et al. 2010). Furthermore, the generation of “BH3 domain swap mutants,” where BH3 domains of different origin (e.g., those derived from BAD, PUMA, or NOXA) were grafted into the mouse *Bim* gene locus, provided long-sought-after *in vivo* evidence for this model (Merino et al. 2009). Previous nuclear magnetic resonance (NMR) (Walensky et al. 2006; Gavathiotis et al. 2008) and crystallography-based structural studies also suggested that a conformational change in BAX (and BAK) triggered by direct BH3-only protein binding is crucial to generate the minimum functional unit of these proteins (Czabotar et al. 2013; Brouwer et al. 2014), referred to as the “BH3 in groove” symmetric homodimer (involving helices  $\alpha 2$ – $\alpha 5$ ). Dimer formation in the MOM leads to the exposure of additional contact sites, likely involving helices 6 and 9 and allowing formation of higher-order oligomers between individual dimers that can span the MOM like a clamp (Bleicken et al. 2014). This culminates in the progressive assembly of a toroidal pore (a mix of proteins and lipids) that was visualized only recently in elegant studies using superresolution microscopy and stimulated emission depletion (STED) nanoscopy (Grosse et al. 2016; Salvador-Gallego et al. 2016). Of note, an auto-inhibited BAX dimer has been described recently to be present in the cytosol (Garner et al. 2016). This dimer conformation needs to be broken up to allow BAX activation, translocation, and accumulation in the MOM. However, this process is antagonized in healthy cells by continuous BCL2/BCLX-driven retrotranslocation of heterodimers back into the cytosol (Edlich et al. 2011; Todt et al. 2015), while retention at the MOM is facilitated by membrane-resident VDAC2 and BAK (Ma et al. 2014), adding additional layers of complexity to apoptosis regulation (Fig. 2).

However, to the delight of some but dismay of others, the requirement of BH3-only proteins for direct BAX/BAK activation needs to be reconsidered (Garcia Saez and Villunger 2016). First of all, in an attempt to reconcile all of these models, it became evident that BCL2 proteins can engage BH3-only proteins as well as activated BAX/BAK with high affinity (Llambi et al. 2011). Along that line, a recent study combining structural biology with mouse genetics provided the first evidence that impaired binding of BCLXL to BAK due to mutation of Glu75 of BAK (a mutant generated randomly during ENU mutagenesis in mice not affecting its structure or binding to direct activator BH3-only proteins and killing potential *in vitro*)



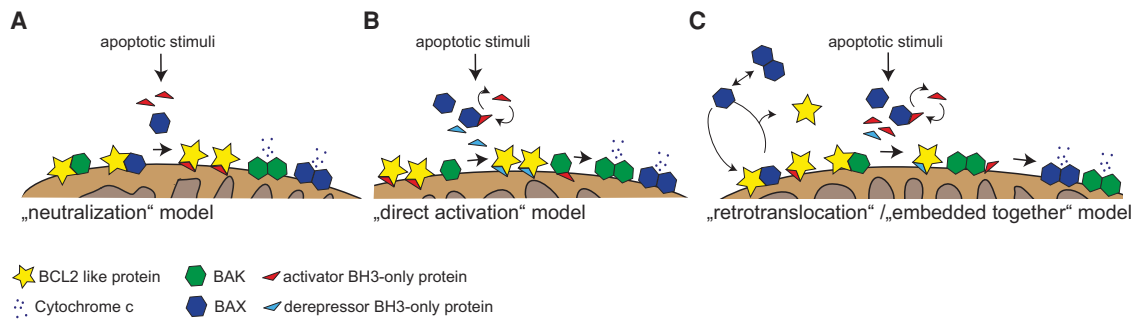
**Figure 1.** Signaling cascades implicated in developmental cell death and tissue homeostasis. Prevention of classic apoptosis pathways by genetic deletion of *Bax* and *Bak* (intrinsic) or impaired death receptor (DR) signaling (extrinsic) does not lead to major developmental abnormalities during embryogenesis or in surviving adults. Hence, additional cell death pathways have been implicated in the removal of excess cells during development. These pathways may involve caspases (green boxes) or not (blue boxes). Caspase-dependent cell death modalities involve apoptosis, ultimately engaging effector caspases 3, 6, and 7 and immunologically silent cell death that ultimately leads to corpse removal by phagocytes. Pyroptosis—a pathway activated by Caspases 1 and 11 in innate immune cells, mainly macrophages—leads to the cleavage of Gasdermin D and possibly other members of this family that ultimately triggers plasma membrane rupture. Lysosomal cell death involves lysosomal membrane permeabilization (LMP) and can be induced by several triggers, such as Ca<sup>2+</sup> overload, H<sub>2</sub>O<sub>2</sub>, extensive DNA damage, or possibly even DR signaling, leading to the release of cathepsins. These enzymes were reported to trigger either a necrotic, apoptotic, or apoptosis-like response, e.g., by involving the BCL2 family protein BID. Accordingly, caspase-dependent as well as caspase-independent forms of lysosomal cell death have been reported. Entosis is a cell death that is triggered by signals from neighboring cells expressing E-cadherins or α-catenin, and those cells are engulfed, followed by phagosome-to-lysosome fusion, leading to cell degradation. Necroptosis is characterized by the activation of RIPK3

and phosphorylation of MLKL. It requires engagement of cell surface receptors, such as toll-like receptor 4 (TLR4), TNFR family members, or the interferon receptor (IFN). Engagement of these or other intracellular receptors, including DAI or TLR3, under conditions where Caspase 8 is inhibited triggers necroptosis. Caspase 8 can cleave and thereby inhibit RIPK1/RIPK3, which are needed to activate MLKL's pore-forming potential, leading to plasma membrane rupture during necroptosis. Programmed necrosis is induced by excess of Ca<sup>2+</sup>, increased reactive oxygen species (ROS) levels, or heavy metals, causing persistent mitochondrial membrane permeabilization through the mitochondrial permeability transition (MPT) pore that spans both the inner and outer mitochondrial membrane. Finally, ferroptosis is triggered by deprivation of cysteine, leading to glutathione (GSH) depletion and inhibition of the detoxifying enzyme GPX4, thereby causing lipid peroxidation and plasma membrane rupture by so-far undefined mechanisms.

is sufficient to trigger spontaneous cell death upon MCL1 inhibition in mouse embryonic fibroblasts (MEFs). Importantly, loss of BAK–BCLXL interaction triggered a number of *in vivo* phenotypes associated usually with loss of *BclxL* *in vivo*, including T-cell loss and reduced platelet life span (Lee et al. 2016). This study also provides some evidence that BAK can act as an activator of BAX in some settings, an interesting idea proposed earlier (Ma et al. 2014). Overall, this suggests that sequestration of BAK by BCLXL is much more critical for keeping cells alive than preventing BAK from becoming directly activated by BH3-only proteins. These findings are consistent with other studies proposing a unifying or hierarchical model of BCL2 protein function (Llambi et al. 2011;

Chen et al. 2015) and also a recent report by Dai et al. (2015), who observed that drug sensitivity of hematopoietic cancers correlates well with the oligomeric state and the amount of BAK present in a cell rather than with the ability of BAK to engage direct activators, such as BIM or PUMA. Perhaps all of these interactions between BH3-only proteins and prosurvival BCL2 proteins serve only one higher purpose: facilitating direct interactions of BAX with BAK.

Furthermore, BAX or BAK can spontaneously accumulate at and permeabilize the MOM in the absence of any of the proteins previously reported to possess direct activation potential (i.e., BID, BIM, and PUMA as well as unrelated p53 and Rb) when all prosurvival proteins present



**Figure 2.** Models for the activation of BAX/BAK and apoptotic pore formation at the MOM. (A) The neutralization model proposes that BAX and BAK are sequestered in their active form by BCL2 prosurvival proteins and that apoptotic stimuli lead to the recruitment of BH3-only proteins by either post-translational stabilization or transcriptional induction. These BH3-only proteins then bind to BCL2-like proteins, thereby releasing BAX/BAK, which are intrinsically active. (B) The “direct activation” model implies that BAX or BAK needs to be directly engaged by “activator BH3-only” proteins in order to impose a conformational change needed for dimerization and subsequent oligomerization. In order to inhibit the initiation of apoptosis, BCL2 proteins sequester activator BH3-only proteins to prevent BAX/BAK activation. Binding of BCL2 to sensitizer BH3-only proteins (e.g., BAD, HRK, BMF) is needed to free activator BH3-only proteins (BIM, BID, and PUMA) to activate BAX or BAK. (C) The embedded together model implies that BCL2-like proteins sequester BH3-only proteins at the MOM, thereby inhibiting their interaction with and direct activation of BAK/BAX. In the MOM, BCL2-like proteins themselves also prevent dimerization and pore formation of BH3-only protein-activated/embedded BAX/BAK monomers. Additionally, the retrotranslocation concept proposes that BAX (and also BAK with slower kinetics) is in an equilibrium and is extracted from the MOM in a BCL2/BCLX-dependent manner and shuttled back into the cytoplasm, where it oscillates between an autoinhibitory dimer and a monomeric state. Only the latter is able to translocate to the mitochondria, where it needs to accumulate in order to form an active dimer that can then form the apoptotic pore upon oligomerization.

are effectively neutralized. Of note, literally all known BH3-only proteins can be rendered to become direct activators at least *in vitro*, depending on experimental conditions (Du et al. 2011; Chen et al. 2015). Hence, it is even more surprising that this autoactivation also happens in the absence of any known BH3-only protein ever reported to be involved in mitochondrial apoptosis or autophagy (O’Neill et al. 2016). While these studies do not completely exclude an accelerating or modulatory role of direct interaction of some BH3-only proteins with BAX or BAK in a given cellular- or stimulus-dependent context, it challenges the universal validity of the “direct activation/embedded together” models. It also points to the existence of so-far undefined membrane components, possibly even of lipid origin, that suffice to promote BAX or BAK dimerization and subsequent pore formation. As such, interrogation of protein–protein and lipid–protein interactions in the context of the BAX/BAK pore can be expected to yield new insights into the molecular biology of MOMP.

Along this line, despite the rather absolute apoptosis resistance phenotype of cells lacking BAX and BAK (see below), we need to keep in mind that the putative BAX-like family member BOK might be a mediator of mitochondrial apoptosis, as it can exert pore-forming properties in certain contexts. Work over the last few years has indicated that BOK functions may partly overlap with those of BAX/BAK but that there might also be unique roles to this still poorly understood family member. Regarding its proapoptotic functions, it remains to be clarified whether BOK can directly form pores in the MOM and, if confirmative, of what nature these pores are. For example, is the intermediate of the elusive BOK pore a dimer? BOK-induced membrane activity so far has been shown only in artificial membranes (liposomes), and proteasomal

degradation of BOK, regulated by members of the ERAD pathway, was also put forward recently as a major means of controlling (proapoptotic) BOK activity (Llambi et al. 2016), yet, in several tissues, BOK is found readily expressed (Ke et al. 2012). However, more in-depth biophysical analyses, preferably within a lipid membrane environment (as done for BAX) (for example, see Bleicken et al. 2014), are necessary to determine the protein–protein interactions, composition, size, and stability of pores. This would also allow one to determine whether members of the BCL-2 family can cooperate with BOK (e.g., tBID, BAX, and BAK) or inhibit its activity (e.g., MCL-1 and BFL-1/A1). Furthermore, solving the three-dimensional structure in the membrane-bound conformation is a prerequisite to advance our understanding of the molecular events regulating BOK activation.

### What have we learned about developmental cell death from the analysis of BH3-only knockout mouse mutants?

BH3-only proteins are considered to be stress-induced proteins and stimulus-dependent initiators of mitochondrial apoptosis that depend on the presence of BAX or BAK to execute their function (Wei et al. 2001). Despite remaining issues on how these proteins ultimately activate apoptosis, it is generally accepted that some BH3-only proteins are more potent killers than others: These include BIM, BID, and PUMA, which can bind to all prosurvival homologs with comparable affinity, while NOXA, BAD, BMF, DP5/HRK, and BIK/BLK, on the other hand, are believed to act mainly as auxiliary prodeath proteins by helping to sequester BCL2 and the like; however,

among those, NOXA recently has been assigned direct BAX/BAK-activating potential (Chen et al. 2015). Since such properties have also been assigned to BMF before (Du et al. 2011), a strict discrimination of two groups of BH3-only proteins appears difficult.

Substantial redundancy between BH3-only proteins has been observed in *in vitro* analyses and gene targeting studies in mice exploring the consequences of loss of one or a combination of BH3-only proteins (Table 1). Therefore, it became evident that *Bim* is probably the most critical member of the family, as single deletion of this gene has the most pronounced effects of all BH3-only protein genes studied in isolation (Pinon et al. 2008). Most strikingly, ablation of *Bim* causes the loss of approximately half of the progeny before embryonic day 10.5 (E10.5) in mouse development (Bouillet et al. 1999). The basis for this phenomenon and its partial penetrance is still unclear but may be related to the observation that FGF4-driven receptor tyrosine kinase signaling in trophoblast stem (TS) cells limits BIM protein levels, thereby setting the balance between TS cell proliferation and differentiation in early embryogenesis (Yang et al. 2006).

Adult *Bim*<sup>-/-</sup> animals show the most profound developmental defects within the hematopoietic system, displaying severe lymphoid hyperplasia. Increased secretion of immunoglobulins (Igs), including autoantibodies, by accumulating plasma cells leads to fatal kidney disease in ~50% of 1-yr-old *Bim*<sup>-/-</sup> mice (Bouillet et al. 1999). These effects were found ameliorated upon backcrossing onto a C57BL/6 genetic background, but cell death-resistance phenotypes of leukocytes and lymphadenopathy persisted. Importantly, loss of only one allele can prevent lymphopenia and polycystic kidney disease observed in mice lacking *Bcl-2*, while loss of both alleles are needed to prevent melanocytes from dying (Bouillet et al. 2001). Thus, BIM seems to be a crucial player in regulating apoptosis during kidney development, in hair follicle stem cells, and in the hematopoietic system. Concerning the latter, BIM is necessary for not only the deletion of autoreactive B and T lymphocytes during development (Bouillet et al. 2002; Enders et al. 2003; Villunger et al. 2004) but also the termination of T-cell- and B-cell-mediated immune responses (Hildeman et al. 2002; Pellegrini et al. 2003), affinity maturation (Fischer et al. 2007), or the survival of granulocytes (Villunger et al. 2003b). Moreover, it also acts as a gatekeeper, preventing cancer in response to oncogenic stress, as exemplified in the accelerated onset of c-MYC-driven lymphomas in the absence of a single allele of *Bim* (Egle et al. 2004; Frenzel et al. 2010).

Although, BIM-deficient cells show profound protection from a number of apoptotic stimuli, such as cytokine withdrawal and ionomycin or steroid treatment, they still remain sensitive to Fas ligation, etoposide treatment, or  $\gamma$  irradiation (Bouillet et al. 1999). These observations already suggested that apoptosis following DNA damage is regulated by other BH3-only proteins. Shortly thereafter, the p53 up-regulated modulators of apoptosis, PUMA (Nakano and Vousden 2001; Yu et al. 2001) and NOXA/PMAIP (Oda et al. 2000), two BH3-only proteins

induced by p53, were first described. NOXA seems to be less critical in this process, since its deletion does not impair apoptosis upon DNA damage in most cell types (Villunger et al. 2003a) but protected gastrointestinal epithelial cells from the consequence of high-dose  $\gamma$  irradiation *in vivo* (Shibue et al. 2003). Of note, keratinocytes and fibroblasts are significantly protected from UV-mediated cell death in its absence (Naik et al. 2007). Interestingly, NOXA might also play a role in immunity, as *Noxa*<sup>-/-</sup> mice were reported to show defects in the selection of high-affinity antigen receptors upon activation of T and B cells (Wensveen et al. 2010, 2012) and in emergency hematopoiesis (Wensveen et al. 2011). Loss of *Puma*, on the other hand, protects hematopoietic (stem) cells (Labi et al. 2010; Michalak et al. 2010), hepatocytes, and gastrointestinal epithelium (Qiu et al. 2008, 2011) effectively from genotoxic stress and  $\gamma$  irradiation (Happo et al. 2010), while combined loss of *Puma* and *Noxa* further increases radio resistance in some cell types, such as mouse oocytes (Michalak et al. 2008; Kerr et al. 2012). Interestingly, only the combined deletion of *Puma*, *Noxa*, and *Bim* provides protection to an extent comparable with p53 deficiency, at least in MYC-driven lymphoma cells (Happo et al. 2010), even though BIM is not a direct target of p53. The latter suggests the existence of a cross-talk between the DNA damage response and BIM, most likely involving activation of JNK signaling (Pinon et al. 2008).

Notably, PUMA-deficient lymphocytes are also refractory to certain p53-independent apoptotic stimuli such as dexamethasone or growth factor deprivation (Jeffers et al. 2003; Villunger et al. 2003a), and compound mutants lacking BIM and PUMA reveal synergistic functions of these proteins in cell death induction (Erlacher et al. 2006). However, no additional developmental defects were noted in *Bim*<sup>-/-</sup>*Puma*<sup>-/-</sup> mice when compared with *Bim* mutant mice (Erlacher et al. 2006), with the exception of a more pronounced lymphadenopathy and more severely compromised deletion of autoreactive T cells (Gray et al. 2012). More recently, it has been shown that *Puma*<sup>-/-</sup> mice display an increase in adipocyte size and increased serum leptin levels. Accordingly, food intake was reduced during a high-fat diet; however, no differences in body mass and glucose metabolism were observed, probably due to reduced physical activity compared with control mice (Litwak et al. 2016). However, it is difficult to put the observed phenotype in a developmental context, as the used mouse model had PUMA deleted in all somatic cells. Of note, PUMA has also been associated with glucose-induced cell death of pancreatic islets (McKenzie et al. 2010), maintaining the interest for this protein in diabetes- and obesity-associated research.

BID is a particular BH3-only protein because it is the only member of the subfamily that is intrinsically structured and needs to be cleaved by caspases, most commonly by caspase 8, for activation (Li et al. 1998; Luo et al. 1998). As such it connects the extrinsic and intrinsic apoptosis signaling pathways in certain cell types that rely on efficient neutralization of XIAP to generate above-threshold caspase activity upon death receptor (DR) ligation (Jost

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**Table 1.** Phenotypic characteristics of BH3-only protein loss-of-function mutants

Gene	Developmental and other cell death phenotypes	Reference
<i>Bim</i>	Approximately 50% of embryos die before E10; survivors show lymphoid hyperplasia; perturbed thymic development; leukocytes protected from a range of apoptotic stimuli; increased serum immunoglobulin (Ig) levels and autoantibodies; Ig deposit-driven kidney disease; impaired deletion of autoreactive T and B cells; defective termination of anti-viral immune responses; impaired affinity maturation during germinal center reaction; delayed duct formation; impaired late stage involution phenotype in the mammary glands	Bouillet et al. 1999, 2002; Hildeman et al. 2002; Enders et al. 2003; Pellegrini et al. 2003; Villunger et al. 2003b; Fischer et al. 2007; Maillieux et al. 2007; Schuler et al. 2016
<i>Bmf</i>	Normal embryonic development; lymphocytes and MEFs partially protected from glucocorticoid or histone deacetylase inhibition (HDACi)-induced apoptosis; mild B-cell hyperplasia; potential overlap with BIM in mammary gland involution	Labi et al. 2008; Hubner et al. 2010; Sakamoto et al. 2016; Schuler et al. 2016
<i>Bid</i>	Normal embryonic development; hepatocytes resist FasL-induced and TNF-induced hepatitis; delayed apoptosis upon FasL or TNF- $\alpha$ treatment in MEFs; pancreatic $\beta$ cells resist death receptor (DR) killing; reduced response to the DNA-damaging agents (controversial); neurons more protected from ischemic cell death (controversial)	Yin et al. 1999; Plesnila et al. 2001; Sax et al. 2002; Kamer et al. 2005; Zinkel et al. 2005; Kaufmann et al. 2007, 2009; McKenzie et al. 2008; Engel et al. 2010
<i>Bad</i>	Normal embryonic development; minor protection from apoptotic stimuli; modest reduction in B-cell proliferation in response to mitogens; decreased production of IgG upon immunization; prolonged platelet life span; reduced sensitivity to TNF-induced hepatitis (controversial); impaired glucose stimulated insulin secretion and glucokinase activity	Danial et al. 2003; Ranger et al. 2003; Kelly et al. 2010; Yan et al. 2013; Ottina et al. 2015
<i>Puma</i>	Normal embryonic development; hematopoietic and nonhematopoietic cell types highly resistant to genotoxic stress and $\gamma$ irradiation as well as growth factor deprivation; high-fat-diet-fed as well as chow diet-fed mice show decreased food intake and ambulatory capacity	Jeffers et al. 2003; Villunger et al. 2003a; Labi et al. 2010; Michalak et al. 2010; Litwak et al. 2016
<i>Noxa</i>	Normal embryonic development; minor protection from p53-dependent apoptotic stimuli; shifted T- and B-cell receptor repertoire upon antigenic challenge; impaired stress erythropoiesis; fibroblasts and keratinocytes resist UV radiation	Shibue et al. 2003; Villunger et al. 2003a; Naik et al. 2007; Wensveen et al. 2012, 2013a,b
<i>Hrk/Dp5</i>	Normal embryonic development; sensory neurons less sensitive to NGF withdrawal; protection from high-fat-diet-induced loss of glucose tolerance and increased pancreatic $\beta$ -cell mass	Coultas et al. 2007; Cunha et al. 2012
<i>Bik</i>	Normal embryonic development; no overt cell death phenotype	Coultas et al. 2004
<i>Bim/Puma</i>	Reduced survival of embryos, as in <i>Bim</i> <sup>-/-</sup> mice; lymphatic hyperplasia exceeds that observed in <i>Bim</i> <sup>-/-</sup> mice; increased resistance to apoptosis when compared with <i>Bim</i> mutants; no exacerbation of autoimmunity observed compared with <i>Bim</i> <sup>-/-</sup> mice despite increased impairment in the deletion of autoreactive thymocytes	Erlacher et al. 2006; Gray et al. 2012
<i>Bim/Bad</i>	Phenocopies <i>Bim</i> <sup>-/-</sup> mice with a slight increase in lymphocytes and increased protection of B cells and thymocytes from some apoptotic stimuli	Kelly et al. 2010
<i>Bim/Bid</i>	Phenocopies <i>Bim</i> <sup>-/-</sup> mice; reduced responsiveness to TNF-induced hepatitis when compared with single mutants	Willis et al. 2007; Kaufmann et al. 2009
<i>Bim/Bik</i>	Phenocopies <i>Bim</i> <sup>-/-</sup> mice regarding development; infertile males due to defective spermatogenesis (later than in <i>Bax</i> -deficient mice)	Coultas et al. 2005
<i>Bim/Bmf</i>	Born at reduced frequency, similar to <i>Bim</i> <sup>-/-</sup> mice; webbed feet; increased incidence of vaginal atresia (65%) and malocclusion of the incisors (25%); increased resistance of	Hubner et al. 2010; Labi et al. 2014; Woess et al. 2015; Sakamoto et al. 2016

Continued

Table 1. Continued

Gene	Developmental and other cell death phenotypes	Reference
	thymocytes and pre-B cells to dexamethasone, HDACi, and tunicamycin when compared with <i>Bim</i> <sup>-/-</sup> but not in mature T cells; enhanced B-cell lymphadenopathy; altered $\kappa/\lambda$ light chain Ig ratio; increased serum Ig levels; high incidence of glomerulonephritis and spontaneous tumor formation; impaired regression of ductal mammary epithelium during involution; restores B-cell development in the absence of BAFF signaling	
<i>Bmf/Bad</i>	No developmental abnormalities; B-cell lineage cellularity increased; reduced latency to spontaneous tumor formation	Baumgartner et al. 2013
<i>Bik/Noxa</i>	Normal embryonic development; cross with <i>E<math>\mu</math>-Myc</i> transgenic mice did not accelerate lymphoma development compared with wild type	Happo et al. 2012
<i>Puma/Noxa</i>	Normal embryonic development; slightly better protection of lymphocytes and MEFs from $\gamma$ irradiation in vitro and in vivo	Michalak et al. 2008
<i>Bim/Puma/Noxa</i>	No differences compared with BIM/PUMA double knockout (DKO) reported; triple-knockout (TKO) cells show protection from DNA damage similar to that seen in <i>p53</i> <sup>-/-</sup> mice	Happo et al. 2010
<i>Bim/Puma/Bid</i>	Limited similarity to BAX/BAK DKO mice/cells in some experimental settings; imperforate vaginal canal and persistent webbing reported	Ren et al. 2010
<i>Bim/Puma/Bid/Noxa</i>	No additional developmental deficiencies on top of BIM/PUMA/BID TKO reported; increased protection of small intestine from $\gamma$ irradiation, exceeding that of TKO mice and resembling BAX/BAK deficiency; protection from apoptosis comparable with that seen in BAX/BAK DKO in response to some but not all stimuli tested; frequency of surviving animals still higher than in BAX/BAK DKO mice	Chen et al. 2015

et al. 2009). As a consequence, hepatocytes or pancreatic  $\beta$  cells from BID-deficient mice are insensitive to Fas ligation in vivo (Yin et al. 1999). Neurons from *Bid*<sup>-/-</sup> mice were reported to better resist ischemic cell death that can involve caspase 8-activating ligands of the TNF family (Plesnila et al. 2001). However, the latter has been a matter of debate (Engel et al. 2010). Furthermore, BID has been reported to be a p53 target, and *Bid*<sup>-/-</sup> MEFs were suggested to be more resistant to DNA-damaging agents (Sax et al. 2002). Along this line, it is interesting to note that BID can be phosphorylated by ATM and has been reported to affect S-phase checkpoint stringency and mitochondrial reactive oxygen species (ROS) production (Kamer et al. 2005; Zinkel et al. 2005; Kaufmann et al. 2007), yet the role of BID in the DNA damage response remains disputed (Kaufmann et al. 2007). The combined deletion of BIM and BID did not cause any developmental abnormalities or phenotypes that exceed those observed in BIM mutant mice, with the notable exception that double-knockout (DKO) mice were protected from TNF-driven fulminant hepatitis more than single-mutant mice, placing BID and BIM downstream from TNFR signaling in hepatocytes (Kaufmann et al. 2009).

In an attempt to phenocopy BAX/BAK mutant mice (discussed below) in support of the direct activation model of BH3-only protein function, mice lacking BID, BIM, and PUMA were generated (Ren et al. 2010). However, ultimately, these triple-mutant (triple-knockout [TKO])

mice did not mimic combined loss of BAX and BAK, as indicated, e.g., by the fact that the rate of perinatal lethality and certain developmental phenotypes, such as persisting interdigital webbing and vaginal atresia, were clearly less pronounced and less penetrant than in BAX/BAK DKO mice (Ren et al. 2010). Furthermore, a follow-up publication by the very same group showed that TKO MEFs are less resistant to apoptosis induction than BAX/BAK DKO MEFs, and deletion of NOXA on top of BID, BIM, and PUMA (QKO) was required to generate comparable death resistance phenotypes in response to some but not all stimuli tested (Chen et al. 2015). The latter suggests that any of the remaining BH3-only proteins in TKO mice may become an activator of cell death in the right context.

Deletion of any of the other BH3-only proteins on their own failed to reveal key roles in development, and only minor roles in cell death signaling were observed. The synergistic potential of these "less potent" BH3-only proteins can be observed best when looking at loss-of-function studies on a BIM-deficient background. While BMF-deficient mice show only mild phenotypes, including B-cell-restricted lymphadenopathy and reduced sensitivity of lymphoid cells to glucocorticoids or histone deacetylase inhibitors (Labi et al. 2008; Grespi et al. 2010), the combined loss of BMF and BIM leads to pronounced developmental abnormalities, including webbed feet, vaginal atresia, and malocclusion of the incisors (Hubner et al.

2010; Labi et al. 2014). Additionally, increased lymphadenopathy, higher serum Ig levels, and a higher incidence of glomerulonephritis and malignant disease, leading to premature lethality, were observed when compared with BIM mutant mice (Labi et al. 2014; Woess et al. 2015). BMF also supports BIM in promoting mammary epithelial cell death during organ morphogenesis (Mailleux et al. 2007; Schmelzle et al. 2007) and the late stage of involution after the cessation of lactation, a classic example of developmental apoptosis (Sakamoto et al. 2016; Schuler et al. 2016).

Another prominent example for cell type-specific redundancy of BH3-only proteins is BIK, the first protein to be identified in this class (Boyd et al. 1995). BIK has been shown to reside at the endoplasmic reticulum (ER), where it might be involved in the transfer of  $Ca^{2+}$  to mitochondria. Although expressed at significant levels in several tissues, no overt phenotype or cell death deficit could be observed in mice lacking *Bik* (Coultas et al. 2004). However, BIK codeletion with BIM causes infertility in males due to perturbed spermatogenesis, resembling the phenotype of BAX-deficient mice, although the block in spermatogenesis occurs somewhat later in their development (Coultas et al. 2005). Notably, no additive cell death effects were observed when BIK and NOXA were codeleted (Happo et al. 2012).

Despite intensive research since its early discovery, the cell death function of the BH3-only protein BAD (Yang et al. 1995) remains somewhat elusive. BAD deficiency itself does not cause any aberrations in embryonic development, although *Bad*<sup>-/-</sup> mice were reported to show impaired B-cell function and develop diffuse large B-cell lymphoma with increased incidence (Ranger et al. 2003). Subsequent reports suggested a role in platelet life span (Kelly et al. 2010) and as regulator of glucose-induced insulin secretion and gluconeogenesis upon phosphorylation of its BH3 domain, precluding its binding to BCL2 (Danial et al. 2003, 2008; Gimenez-Cassina et al. 2014). Findings that BAD-deficient mice are less sensitive in a model of systemic TNF-mediated hepatitis (Yan et al. 2013) could not be confirmed in an independent study (Ottina et al. 2015). Of note, responses to TNF or glucose are strongly influenced by the genetic background of the mouse model used and, when poorly controlled for, can contribute to sometimes inconsistent findings (Leiter 2002; Vanden Berghe et al. 2015). Combined deletion of BAD plus BIM (Kelly et al. 2010) or BMF (Baumgartner et al. 2013), respectively, did not result in any particular developmental phenotypes and revealed only mildly increased cell death resistance upon cytokine withdrawal, B-cell-specific lymphadenopathy, and increased susceptibility to spontaneous as well as radiation-induced tumorigenesis.

Finally, deletion of *Dp5/Hrk*, a BH3-only family member that is expressed mainly in the central and peripheral nervous system (Imaizumi et al. 1997), does not impair neuronal development, although sensory neurons derived from these mice are less sensitive to nerve growth factor withdrawal *ex vivo* (Coultas et al. 2007). HRK-deficient mice were also reported to be protected from high-fat-diet-induced loss of glucose tolerance and displayed in-

creased pancreatic  $\beta$ -cell mass (Cunha et al. 2012); however, this phenotype awaits independent confirmation.

In summary, these studies point toward a large degree of redundancy among the BH3-only proteins in developmental cell death and substantial flexibility regarding their use and requirement in stress-induced apoptosis. Based on the reported phenotypes in single and compound BH3-only protein mouse mutants, the general relevance of mitochondrial cell death for organ development in the embryo and tissue homeostasis in the adult must be strongly questioned. Certainly, it remains possible that the combined deletion of all BH3-only proteins listed above may phenocopy BAX/BAK mutant mice, but, given the assumption that not all of these proteins have exclusive roles in apoptosis but also intersect, e.g., with the regulation of mitochondrial dynamics or the autophagy machinery, data interpretation will remain difficult.

### Defining the role of mitochondrial cell death in development by loss-of-function analysis in mice lacking BAK, BAX, BOK, or combinations thereof

BAX and BAK loss-of-function mutants have been generated early on but failed to reveal major developmental defects (Table 2). BAX mutant mice showed mild cell death resistance of neurons to trophic factor deprivation *in vivo*, an increase in neurons and lymphocytes, and, paradoxically, male sterility due to increased spermatogonia death, halting spermatogenesis (Knudson et al. 1995; Deckwerth et al. 1996; White et al. 1998; Fan et al. 2001). BAK mutant mice showed no discernable defects in development or mitochondrial or DR-mediated apoptosis. In striking contrast, mice lacking both genes showed significant developmental defects and a severe impairment in cell death in response to a broad range of apoptotic triggers. Most BAX/BAK DKO mice died perinatally, and only <10% of mice survived to adulthood (Lindsten et al. 2000). Of note, clear developmental phenotypes associated with defective developmental apoptosis, such as a persistent interdigitating mesenchyme and an imperforate vaginal canal, were observed. Furthermore, these mice also showed neurological deficits, as seen by circling behavior and frequent seizures upon stress. Unresponsiveness to auditory stimulation indicated deafness, and pups frequently died after a few days, often showing suckling defects and social isolation from littermates (Lindsten et al. 2000). The neurological defects were associated with excess numbers of undifferentiated cells in the periventricular zone, and follow-up studies documented massive lymphadenopathy in surviving or fetal liver-reconstituted mice (Lindsten et al. 2000; Rathmell et al. 2002), increased neuronal progenitor cell number *in situ* (Lindsten et al. 2003), and persisting fetal ocular vasculature and inner retinal cells, including rod photoreceptors, which normally die if they fail to migrate to the outer retina (Hahn et al. 2002, 2003). However, formation of other organs—including the kidney, heart, liver, lung, pancreas, or bladder—appeared to develop and function normally in the surviving mice (Lindsten et al. 2000; Lindsten and Thompson



**Table 2.** Phenotypic characteristics of *Bax/Bak/Bok* loss-of-function mutants

Gene	Developmental and other cell death phenotypes	Reference
<i>Bax</i>	Mild lymphoid hyperplasia; infertile males due to disordered seminiferous tubules; increase in number of several neuron types during embryogenesis; behavioral deficits affecting learning, socio-sexual behavior, and anxiety; greater locomotor activity but greater immobility times in forced swimming test; depression-like traits	Knudson et al. 1995; White et al. 1998; Fan et al. 2001; Sun et al. 2004; Jyotika et al. 2007; Lee et al. 2009; Luedke et al. 2013; Krahe et al. 2015
<i>Bak1</i>	Normal development; no abnormalities or age-related disorders	Lindsten et al. 2000
<i>Bok</i>	No apparent developmental phenotype; resistance to ER stress (controversial)	Ke et al. 2012; Carpio et al. 2015, 2016; Fernandez-Marrero et al. 2016; Llambi et al. 2016
<i>Bax/Bak1</i>	Perinatal death of >90% DKO mice; surviving mice show interdigital webbing; imperforation of the vagina; accumulation of excess cells in the CNS and hematopoietic systems; neurologic abnormalities include deafness; circling behavior; suckling defects; palate cleft; surviving fetal ocular vasculature; extra rod photoreceptors	Lindsten et al. 2000, 2003; Rathmell et al. 2002; Hahn et al. 2003, 2005; Lindsten and Thompson 2006
<i>Bax<sup>fl/fl</sup>-Bak1<sup>-/-</sup></i> , <i>CD19Cre</i> , or <i>MxCre</i>	Accumulation of B cells at all stages, but MZ B cells and B-1 cells are normal; defect in cell cycle progression upon stimulation with LPS and anti-IgM; cell death resistance	Takeuchi et al. 2005
<i>Bax<sup>fl/fl</sup>Bak1<sup>-/-</sup></i>	Reduced myocardial infarction area; apoptosis and necrosis resistance phenotypes	Whelan et al. 2012; Karch et al. 2013
<i>α-MHC-Cre</i> , <i>Albumin-Cre</i> , or <i>Nestin-Cre</i>	Protection from radiation damage	Ren et al. 2010
<i>Bax<sup>fl/fl</sup>Bak1<sup>-/-</sup></i> , <i>Villin-cre</i> , or <i>Tie2-Cre</i>	No improvement of survival after high-dose IR-induced gastrointestinal syndrome; protection from IR-induced bone marrow failure	Kirsch et al. 2010
<i>Bok/Bax</i> <i>Bok/Bak1</i>	No phenotype exacerbating <i>Bax</i> or <i>Bak</i> single deficiency, except increased oocyte numbers in <i>Bok/Bax</i> females	Ke et al. 2013
<i>Bok/Bak1/Bax</i>	Mildly more severe phenotype in leukocytes of mice reconstituted with TKO fetal liver compared with <i>Bax/Bak1</i> DKO controls; craniofacial defects (facial cleft)	Ke et al. 2015; Carpio et al. 2016

2006). This raises the possibility that the development of these organs may not be linked to developmental apoptosis or that alternative means to induce apoptosis or other forms of developmental cell death are engaged (Fig. 1).

One such cell death mediator might be BOK; however, apart from overexpression data, physiological evidence for a proapoptotic role of BOK is still meager. No developmental abnormalities or spontaneous phenotypes were reported for the first *Bok* gene-deficient mouse model generated, and hematopoietic cells derived from these mice were normally sensitive to classical apoptotic stresses (Ke et al. 2012). Along that line, *Bok<sup>-/-</sup>Bax<sup>-/-</sup>* and *Bok<sup>-/-</sup>Bak<sup>-/-</sup>* doubly deficient mice displayed no exacerbated phenotype over *Bax* or *Bak* single-deficient mice, respectively, with the exception of increased oocyte numbers in aged *Bok<sup>-/-</sup>Bax<sup>-/-</sup>* females (Ke et al. 2013). The latter finding may point toward a role of BOK in oocyte apoptosis that warrants further investigation. Recently, Ke et al. (2015) also reported a more pronounced phenotype in lymphocytes in the peripheral blood of mice reconstituted with *Bok<sup>-/-</sup>Bax<sup>-/-</sup>Bak<sup>-/-</sup>* TKO hematopoietic stem cells compared with *Bax<sup>-/-</sup>Bak<sup>-/-</sup>* DKO controls, indicating

that BOK may have redundant functions with BAX and BAK in this compartment. This is an interesting observation, taking into account the low BOK expression in mouse hematopoietic cells (Ke et al. 2012). Maybe not surprising, a comparison of DKO and TKO lymphocytes failed to reveal any differences in response to various apoptosis inducers, given that DKO cells were already fully protected. It is conceivable that more pronounced effects may be seen during development or in the adult organism in TKO tissues that are normally high in BOK expression, and a thorough analysis of embryogenesis in TKO mice or conditional deletion of all three genes in a given tissue is eagerly awaited.

#### Mitochondrial apoptosis and its role in early embryogenesis

Despite the fact that loss of individual BH3-only proteins or combinations thereof points toward rate-limiting roles in tissue homeostasis in adults, best documented in the hematopoietic system, the phenotypes observed are, at second thought, rather minor (Table 1). The same holds true

when considering that some *Bax*<sup>-/-</sup>*Bak*<sup>-/-</sup> mice can survive to adulthood (Lindsten and Thompson 2006), and chimeric mice carrying a reconstituted hematopoietic system lacking BAX/BAK and BOK develop “only” gradual lymphadenopathy with signs of autoimmunity but not cancer or inflammatory phenotypes due to excess leukocytes (Ke et al. 2015). Based on this and the fact that most BAX/BAK-deficient mice die perinatally only by largely unexplored causes, we need to conclude that cell death observed early in embryogenesis does not necessarily involve mitochondria or, alternatively, may be mediated by alternative factors that can promote MOMP. It is worth mentioning here that, in most vertebrates, cell death is not observed prior to gastrulation (Penalosa et al. 2006). However, in mammals, such as mice, cell death occurs already at the morula stage during compaction as well as during blastocoel formation and differentiation of the inner cell mass (ICM) in the early blastocyst (Zakeri et al. 2005; Busso et al. 2010). Why, then, is development not perturbed in the absence of BAX/BAK? One is tempted to argue here that an excess of cells of the ICM can be tolerated easily at that early stage, as we also introduce extra embryonic stem cells into the blastocyst in the process of generating gene-modified mice. Perhaps cell death in the blastocyst stage embryo is simply not critical for development or may not follow classic routes. Excess cells in BAX/BAK DKO blastocysts have not yet been demonstrated but might be cleared by other means; for example, cell cannibalization via entosis (Krishna and Overholtzer 2016) or nonprofessional phagocytosis by neighboring cells upon exposure of “eat me” signals (Arandjelovic and Ravichandran 2015). Certainly, a failure to clear dying cells from embryoid bodies—e.g., observed in the context of ATG5 or Beclin-1 deficiency—blocks cavitation (Qu et al. 2007), suggesting that lack of cell death and accumulation of cells at that stage should do the same. Clearly, this process does not seem perturbed in the absence of BAX/BAK. However, aficionados of alternative modes of cell death, such as necroptosis, may find it intriguing that general caspase inhibition by z-VAD-FMK or selective inhibition of caspase 8 halts the development of the preimplantation embryo (Zakeri et al. 2005; Busso et al. 2010). In fact, caspase inhibition even enhances rates of cell death normally observed in the ICM in vitro. However, whether this death occurs by necroptosis is yet unclear (Fig. 1).

Taken together, it seems that timed removal of excess cells of the ICM in the early preimplantation development of the embryo is either not mediated by BAX/BAK, compensated by alternative types of death, or simply well tolerated and balanced by other means (e.g., reduced proliferation rates or phagocytic clearance mechanisms). Interestingly, lack of *Bax/Bak* appears to delay the differentiation of embryonic stem cells upon retinoic acid treatment or embryoid body or teratoma formation by blocking CD95-mediated cell death of less differentiated cells (Wang et al. 2015). The notion that limited caspase activity—most likely generated by caspase 8 temporarily activating caspase 3 (Fujita et al. 2008)—seems required for proper blastocyst formation in vitro is certainly interesting but somewhat confounded by the fact that, in the ab-

sence of *Casp8*, embryogenesis halts only around E10.5 due to defective angiogenesis (Varfolomeev et al. 1998), while *Casp3*-deficient mice can come to term (Honarpour et al. 2000; Zheng et al. 2000), meaning that preimplantation development is not affected by lack of caspase activity. Whether BAX/BAK can generate such caspase activity in the absence of caspase 8 needs to be analyzed, but it is worth mentioning that caspase 6 can also cleave RIPK1 (van Raam et al. 2013) and could hence put a halt to necroptosis in the early embryo when caspase 8 is absent.

Following similar lines of thought, tube formation in the embryo is crucial to the normal development of many organs, including the mammary glands, kidneys, and lungs, the latter two developing normally in BAX/BAK DKO mice. Tube formation can be achieved by the closure of an epithelial sheet layer (e.g., neural tube and primitive gut) or by lumen formation involving cell clearance in a compact cell mass, also known as cavitation (female reproductive tract and exocrine glands). In mouse embryos, the first cavitation event happens shortly prior to gastrulation, converting the solid embryonic ectoderm into a hollow egg cylinder, thereby generating the proamniotic cavity, surrounded by a single epithelial cell layer (Coucouvanis and Martin 1995). The basic principles observed at that stage most likely reflect what is happening in organogenesis later many times. The cell death observed at that stage shows classic hallmarks of apoptosis (DNA laddering and pyknotic nuclei) and is induced by cues generated from the visceral endoderm, while the columnar epithelium is rescued from death by signals generated from its interaction of the basement membrane (i.e., integrin signaling) (Coucouvanis and Martin 1995; Murray and Edgar 2000). This type of cell death is hence highly reminiscent of what is often referred to in the cell death field as “anoikis,” which is induced upon loss of contact with the extracellular matrix or expulsion of cells from the epithelium (e.g., in the gastrointestinal tract). This death is blocked by BCL2 overexpression or loss of certain BH3-only proteins (Schmelzle et al. 2007) and hence is supposedly dependent on BAX/BAK. Again, this process must progress normally in their absence in the early embryo, as it occurs during lumen formation in many other organs during embryogenesis, e.g., in the kidneys, lungs, or heart. It deserves mentioning here that caspase 8 has been implicated in anoikis and lumen formation before (Frisch and Sreaton 2001). Although this has been deemed controversial, one may speculate that caspase 8 might well play a critical role in development, in particular when BAX and BAK are missing. Whether the surviving DKO mice have impaired duct formation in exocrine glands, such as the mammary gland, remains unexplored.

#### **Mitochondrial apoptosis as a regulator of tissue homeostasis in the adult**

Tissue-specific codeletion of *Bax* and *Bak*, usually achieved by conditional deletion of *Bax* on a *Bak*<sup>-/-</sup> background, raises the question of whether mitochondrial

apoptosis is critical for homeostasis in the adult. For example, *Bax/Bak* codeletion in the gastrointestinal tract using *Villin-Cre* does not cause spontaneous tissue hyperplasia, documenting that cell death of gastrointestinal epithelial cells can happen in the absence of mitochondrial apoptosis, and tissue homeostasis is effectively maintained in its absence (Kirsch et al. 2010). This is also supported by the fact that surviving DKO mice have not been reported to develop hyperplasia in the gastrointestinal tract. It remains possible, though, that shedding of cells from the tips of villi and their excretion together with feces are sufficient to maintain gut homeostasis.

Also, in tissues with a low proliferative index, like the brain (Ren et al. 2010), heart, and liver (Whelan et al. 2012; Karch et al. 2013), BAX and BAK seem dispensable for homeostasis, as their codeletion does not seem to trigger a hyperplastic phenotype in these organs (Table 2). This may be less of a surprise, as the promoters driving Cre expression to delete *Bax* on a *Bak*-deficient background usually stem from genes that associate with terminal differentiation. As the mitotic index and rate of post-mitotic cell death in these organs are known to be low, the observation time in these studies may not have been long enough to reveal developing organ deficits. Alternatively, mechanisms other than mitochondrial apoptosis may compensate to maintain organ homeostasis. It will certainly be of interest to monitor such conditional mouse mutants long term or expose them to challenges that associate with tissue regeneration that needs to be balanced by cell death induction; e.g., upon recovery from partial hepatectomy.

Taken together, all of these findings challenge textbook dogma about the importance of apoptosis in embryonic development or tissue homeostasis. As deletion of BAX and BAK effectively protects many cell types from death induced by diverse apoptosis-inducing agents and signaling cues but only causes modest changes in adult tissue homeostasis and embryogenesis, alternative explanations are needed.

As quickly touched on before, such an explanation might be redundancy with BOK. However, expression of *Bok* mRNA in tissue from BAX and BAK single or compound knockout mice as well as BOK protein in tissue from BAX or BAK single-knockout mice appears, on the whole, unaffected, arguing against compensatory effects, as do studies combining BAX or BAK deficiency with loss of BOK (Ke et al. 2012, 2013). The fact that mice reconstituted with *Bax/Bak/Bok* TKO hematopoiesis showed increased lymphocyte numbers in peripheral blood and a higher infiltration rate of lymphocytes into the gut may also be explained by loss of other processes, as published data also point toward nonapoptotic roles of BOK, such as in trophoblast proliferation (Ray et al. 2010). Even protective roles have been reported for BOK. It has been shown that *Bok* cells can be more sensitive to the Golgi/ER-impacting drug brefeldin A, presenting with a defective IRE1 $\alpha$ -mediated stress response and enhanced apoptosis (Echeverry et al. 2013). Likewise, D'Orsi et al. (2016) reported *Bok*-deficient neurons to be more sensitive to oxygen/glucose deprivation-induced or seizure-induced

neuronal injury. In essence, these findings point toward nonapoptotic or even protective roles of BOK at the ER. Certainly, it will be very interesting to see whether the *Bax/Bak/Bok* triple deficiency affects embryogenesis more severely than loss of BAK and BAX in tissues that do express discernable levels of BOK, such as the ovaries, uterus, or gastrointestinal tract (Ke et al. 2012). However, whether putative changes are related to impaired cell death due to lack of BOK needs to be thoroughly validated.

### Redundancies between cell death modalities as a means to control developmental cell death?

Assuming that the analysis of the above-mentioned TKO mice will not provide a universally satisfying answer to the question of whether mitochondrial apoptosis is strictly essential and uniquely required for embryogenesis and organ homeostasis, the follow-up question would be: What cell death mechanisms could contribute to the development of or compensate for *Bax/Bak/Bok* deficiency in tissue homeostasis? While it was easy to speculate why cells die when caspase activation is inhibited in the presence of MOMP, this will require entirely different thinking, looking at developmental cell death in the absence of BAX/BAK/BOK. In the first case, it is quite easy to accept that energy depletion, breakdown of membrane gradients, and solute influx in cells undergoing MOMP in a quantitative manner ultimately trigger cellular demise even in the absence of caspase activation, with features of programmed necrosis (Miyazaki et al. 2001; Tait and Green 2008). This was also confirmed by observations made in embryos surviving loss of *Apaf-1*, *Caspase 9*, or *Caspase 3* and managing to come to term (Honarpour et al. 2000; Zheng et al. 2000). These mice have cleared tissues usually removed by apoptotic mechanisms during development. Consistently, necrosis-like morphology of interdigital cells was observed in embryonic limb buds that were allowed to develop in culture in the presence of pharmacological caspase inhibitors (Chautan et al. 1999). This is in line with the notion that none of the reported mouse strains lacking caspases that are required for apoptosis exhibits soft-tissue syndactyly (Wang and Lenardo 2000). This suggests that, in some cell types and tissues, programmed necrosis can compensate for impaired mitochondrial apoptosis or that caspases can be engaged in a noncanonical manner during development, as suggested by observations that lymphocytes lacking *Apaf-1* or *Caspase 9* can still undergo BCL2-regulated apoptosis (Marsden et al. 2002).

Although necrotic cell death can clearly be programmed in response to certain types of stress, such as ischemia/reperfusion injury in the kidney or heart, Ca<sup>2+</sup> overload, or excitotoxicity in neurons, where necrosis plus secondary inflammatory cytokine-driven apoptosis frequently contributes to tissue damage (Linkermann et al. 2014), we do not have strong evidence that programmed necrosis contributes to organ development. This may be simply due to a lack of understanding of the underlying molecular machinery (Karch and

Molkentin 2015). The only gene confirmed so far to be rate-limiting in this process is cyclophilin D (*CypD*), a peptidylprolyl isomerase present in the mitochondrial matrix, which controls mitochondrial permeability transition (MPT). However, although mice lacking *CypD* show reduced programmed necrosis in ischemia models, embryogenesis is not perturbed (Schinzel et al. 2005).

Clearly, defects in MPT pore (MTP) opening do not impair apoptosis. Recently, a structural contribution of BAX and BAK that differs from their pore-forming potential driving MOMP has been proposed to be required for programmed necrosis upon MTP opening, e.g., in response to H<sub>2</sub>O<sub>2</sub>, Ca<sup>2+</sup> overload, or heavy-metal poisoning (Tischner et al. 2012; Karch et al. 2013). Most strikingly, MEFs and liver mitochondria isolated from *Bax/Bak* DKO mice showed clear deficits in MTP activity and codeletion of *Bax/Bak* in the heart (*αMHC-Cre*) protected from coronary artery ligation-induced myocardial infarction, an insult usually associated with rapid ATP depletion converting apoptotic death into necrosis involving MTP (Karch et al. 2013). Interestingly, however, conditional deletion of *Bax* on a *Bak/CypD* DKO background yielded comparable results and not a higher degree of protection, as anticipated (Whelan et al. 2012). Hence, these studies provide evidence that BAX/BAK contribute to MTP-driven programmed necrosis in some settings. Importantly, this activity does not require their oligomerization potential but rather their mitochondrial fusion potential, as fragmented mitochondria, like those found in BAX/BAK-deficient cells, have a deficit in MTP opening. Hence, the MTP-opening defect could be ameliorated by addition of an inhibitor of mitochondrial fission (Whelan et al. 2012). The investigators also conclude that BAX/BAK may actually form the outer constituent of the elusive MTP complex that is needed to promote solute entry and organ swelling during necrosis. However, assuming that this is indeed the case and that BAX/BAK can control apoptosis plus programmed necrosis at the same time, the rather mild phenotypes observed in BAX/BAK-deficient embryos would represent consequences of impairment of both cell death pathways, excluding a major role for MTP and programmed necrosis in embryogenesis.

Whether other proteolytic enzymes, such as cytosolic calpains or lysosomal cathepsins, may be needed to trigger death in the absence of BAX/BAK is more difficult to assess, as these enzymes are members of large families, some of which are essential for embryogenesis (Repnik and Turk 2010; Smith and Schnellmann 2012). Calcium-regulated calpains have been implicated repeatedly in cell death associated with neurodegenerative disorders, such as amyotrophic lateral sclerosis or multiple sclerosis, but not necessarily developmental cell death (Smith and Schnellmann 2012), although m-calpain mutant embryos fail to implant (Dutt et al. 2006). In contrast, induction of lysosomal cathepsins and their subsequent release have been reported to precede interdigital cell death in avian limb buds, and up-regulation of cathepsin D has also been observed in the limb buds of mouse embryos. Simultaneous pharmacological inhibition of caspases and cathepsins delayed mesodermal cell death in chicken

limbs more potently than caspase inhibition alone (Zuzarte-Luis et al. 2007). Furthermore, a critical role of cathepsins B and L has been reported in mammary gland remodeling during involution (Kreuzaler et al. 2011), although its physiological role remains disputed (Sakamoto et al. 2016). Regardless, these observations again support the idea that mitochondrial apoptosis is not solely responsible for developmentally programmed cell death in vertebrates but works in conjunction with, or can be compensated for by, other cell killing mechanisms.

Based on available data today, components of the DR pathway of apoptosis are equally unlikely to compensate for mitochondrial cell death during embryonic development, although unrestrained necroptosis causes embryonic lethality in animals lacking FADD, FLIP, or Caspase 8. This excess cell death that destroys the vasculature of the embryo, leading to developmental arrest around E10.5 (Varfolomeev et al. 1998; Yeh et al. 1998, 2000), can be fully rescued by codeletion of RIPK3 or MLKL, the key driver and effector of necroptosis, respectively. FADD/MLKL-deficient and Caspase 8/MLKL-deficient mice, which lack literally all DR-mediated apoptosis as well as programmed necroptosis are born at a normal Mendelian frequency and show no developmental abnormalities, as do mice lacking FADD plus RIPK3 or Caspase 8 plus RIPK3 (Kaiser et al. 2011; Oberst et al. 2011; Alvarez-Diaz et al. 2016; Zhang et al. 2016). Tissue homeostasis also appears unaffected in these mice, with one notable exception, the hematopoietic compartment, which gradually expands and provokes fatal autoimmunity in aged mice (Alvarez-Diaz et al. 2016; Zhang et al. 2016). As such, all of these mutants mimic the pathology noted in *lpr* mutant mice that lack functional FAS/CD95 but develop normally otherwise (Watanabe-Fukunaga et al. 1992). Synergy between mitochondrial and DR-mediated apoptosis has been reported before as being critical for immune response termination (Hughes et al. 2008; Weant et al. 2008). Whether they cooperate during development needs to be investigated.

The generation of BAX/BAK DKO mice that lack MLKL or RIPK3 may reveal unexpected insight regarding the potential redundancy between these cell death pathways in embryogenesis. However, as MLKL-deficient (Murphy et al. 2013) or RIPK3-deficient (Newton et al. 2004) mice do not show developmental phenotypes, one would need to speculate that BAX/BAK can compensate well for their absence in cell death initiation during embryogenesis. However, this is unlikely, as BCL2 overexpression or loss of BAX/BAK fails to block MLKL-driven cell death, and activation of BAX/BAK does not trigger necrosome formation (Pasparakis and Vandenabeele 2015). Whether BAX/BAK-deficient embryos show increased rates of inflammation due to nonapoptotic cell death—e.g., via necrosis that may then activate MLKL-driven necroptosis—remains to be sorted out. Clearly, simultaneous deletion of RIPK3 mediated necroptosis, requiring MLKL for execution, and *CypD*, which is required for regulated necrosis by the MTP, does not impair embryogenesis and tissue homeostasis (Linkermann et al. 2013). Whether simultaneous inhibition of several of these cell death

pathways—e.g., in a BAX/BAK/MLKL/CYPD/CASP8 compound mutant mouse—will halt embryogenesis in its tracks needs to be investigated in the future by those who can afford the involved mouse work. But what about other pore-forming complexes that can kill cells, such as Caspase 1/11-activated Gasdermin D (Ding et al. 2016; Liu et al. 2016)? Is this killing machinery really exclusive for innate immune cells? What about ferroptosis? This cell death process is regulated by the antioxidant enzyme glutathione peroxidase 4 (GPX4), which depends on glutathione to prevent toxic iron-dependent lipid peroxidation and cell death (Yang and Stockwell 2016). Of note, ferroptosis inhibition can ameliorate developmental defects noted in the absence of MDM2 when the canonical effector functions of p53 are impaired (Jiang et al. 2015). Hence, under certain conditions, this type of cell death can become activated during embryogenesis.

However, in times of CRISPR/CAS9-mediated gene editing of mouse embryos, those with the necessary resources at hand may find these intriguing possibilities to explore. Regardless of the phenotype of such “multi-CRISPR” embryos, establishing a direct link to absence of cell death of any kind will be challenging. Clearly, signals emanating from dying cells serve as a cue to control proliferation, migration, or differentiation during development, and separating cause from consequence and phenotype will be difficult. Do we need cell death execution at all to clear superfluous cells? Perhaps the exposure of certain engulfment signals suffices to clear cells during organogenesis. In the end, we may learn yet again that nature finds its way.

### Conclusions and perspectives

Given that work on BCL2 family proteins has been overwhelming in its quantity in the past decades and that apoptosis has made it into every cell biology and embryology textbook as a key regulator of development and tissue homeostasis, it is surprising how little we actually do know about its actual relevance for these processes. One is tempted to say that (cell) death is not an essential part of life after all, which might sound comforting but ultimately must be wrong. We all know it. Of course, this may in part be due to the fact that cell death events during vertebrate and in particular mammalian development are difficult to follow and study. Regardless, keeping in mind that there are still huge gaps in our knowledge of what controls cavitation and duct and organ formation during embryonic development and how homeostasis is maintained in the adult in the absence of mitochondrial apoptosis, particularly outside the hematopoietic system, we can anticipate that research along these lines will still be rewarding. We may find not only novel regulators of these developmental processes but also valuable new insights for human disease.

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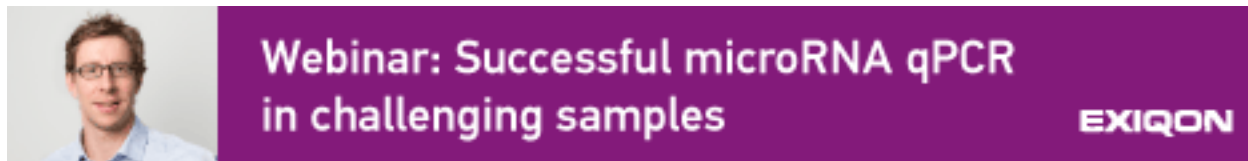
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