An interconnected hierarchical model of cell death regulation by the BCL-2 family

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Multidomain pro-apoptotic BAX and BAK, once activated, permeabilize mitochondria to trigger apoptosis, whereas anti-apoptotic BCL-2 members preserve mitochondrial integrity. The BH3-only molecules (BH3s) promote apoptosis by either activating BAX–BAK or inactivating anti-apoptotic members. Here, we present biochemical and genetic evidence that NOXA is a bona fide activator BH3. Using combinatorial gain-of-function and loss-of-function approaches in BID, BIM, PUMA and NOXA directly induce stepwise, bimodal activation of BAX–BAK. BCL-2, BCL-XL and MCL-1 inhibit both modes of BAX–BAK activation by sequestering activator BH3s and ‘BH3-exposed’ monomers of BAX–BAK, respectively. Furthermore, autoactivation of BAX and BAK can occur independently of activator BH3s through downregulation of BCL-2, BCL-XL and MCL-1. Our studies lay a foundation for targeting the BCL-2 family for treating diseases with dysregulated apoptosis.

Central players of the mitochondrial-dependent apoptotic program are the BCL-2 family proteins3–5, consisting of: multidomain anti-apoptotic BCL-2, BCL-XL and MCL-1; multidomain pro-apoptotic BAX and BAK; and pro-apoptotic BH3-only molecules (BH3s). Multidomain members contain all four BCL-2 homology domains (BH1–4) whereas BH3s share sequence homology only within the BH3 domain. Multiple apoptotic stimuli culminate in mitochondrial outer membrane permeabilization (MOMP), resulting in the release of cytochrome c into the cytosol to activate caspases4,5. The decision of a given cell to undergo MOMP is determined by the interplays among these three BCL-2 subfamilies1–3,6,7. BAX and BAK are the essential effectors responsible for MOMP whereas BCL-2, BCL-XL and MCL-1 preserve mitochondrial integrity6,9. BH3s are death sentinels that relay upstream apoptotic signals to initiate apoptosis by either activating BAX–BAK directly (‘activator’ BH3s) or inactivating BCL-2, BCL-XL and MCL-1 (‘inactivator’ or ‘sensitizer’ BH3s; refs 6,9–15). BH3s execute their function through binding of their BH3 domains into the hydrophobic binding groove of multidomain pro-apoptotic or anti-apoptotic members16–22. BID, BIM and PUMA are ‘activator BH3s’ that directly interact with BAX–BAK to induce the stepwise structural reorganization and oligomerization of BAX–BAK (ref. 7). One important intermediate step of BAX–BAK activation driven by activator BH3s is to expose the BH3 domain of BAX–BAK such that the ‘BH3-exposed’ BAX–BAK monomer can bind to the hydrophobic dimerization pocket of another BAX–BAK molecule, initiating homo-dimerization and subsequent homo-oligomerization23,24. Anti-apoptotic BCL-2 members (BCL-2s) inhibit apoptosis principally through sequestering tBID–BIM–PUMA from activating BAX–BAK, providing front-line protection against apoptotic insults5,9. Additionally, anti-apoptotic BCL-2s can also sequester partially activated, BH3-exposed, monomeric BAX–BAK to prevent their homo-oligomerization23,7 serving as a fail-safe mechanism. The ability of anti-apoptotic BCL-2s to sequester tBID–BIM–PUMA is further modulated by ‘inactivator’ BH3s (BAD and NOXA) through high-affinity, competitive binding. Specifically, BAD and NOXA displace sequestered tBID–BIM–PUMA from BCL-2–BCL-XL and MCL-1, respectively, to activate BAX–BAK indirectly6,10–13.
Two non-mutually exclusive models have been proposed concerning how BH3s activate BAX–BAK (refs 2,3). The direct activation model states that the ‘activator’ subgroup of BH3s can directly induce the conformational changes of BAX–BAK (refs 6,8,9,14,25,26). The indirect model proposes that BAX and BAK are kept in check by the anti-apoptotic BCL-2 proteins and activation of BAX–BAK occurs by default as long as all of the anti-apoptotic BCL-2s are neutralized by BH3s (ref. 27). The generation of Bid−/−Bim−/−Puma−/− triple knockout (TKO) mice provides in vivo evidence supporting the direct activation model15. However, the observation that double deficiency of Bax and Bak incure more severe embryonic lethality than triple deficiency of Bid, Bim and Puma (refs 15,28) suggests that either additional activators of BAX–BAK exist in other cell types, or BAX–BAK can be activated through an alternative mechanism that is entirely independent of BH3s. Alternatively, non-apoptotic functions of BAX–BAK, such as regulation of mitochondrial fission/fusion or endoplasmic reticulum (ER) calcium homeostasis29,30, may account for the more severe embryonic lethality of Bax−/−Bak−/− mice.

Here, we demonstrate that NOXA is a bona fide activator of BAX and BAK that directly interacts with BAX–BAK to drive the homo-oligomerization of BAX–BAK even in the absence of BID, BIM and PUMA. Owing to the unique high expression of NOXA in mouse embryonic fibroblasts (MEFs), Bid−/−Bim−/−Puma−/− MEFs succumbed to diverse intrinsic apoptotic signals whereas Bid−/−Bim−/−Puma−/−Noxa−/− MEFs were completely resistant to growth factor deprivation or ER stress. Surprisingly, BAX and BAK could be ‘autoactivated’ by DNA damage in transformed Bid−/−Bim−/−Puma−/−Noxa−/− MEFs through downregulation of BCL-2, BCL-X1 and MCL-1. Liberation of a small fraction of ‘BH3-exposed’ BAK/BAX monomers from the anti-apoptotic BCL-2s is sufficient to induce a ‘feed-forward’ amplification loop for the initiation of mitochondrial apoptosis. Interestingly, BCL-X1 is superior to BCL-2 and MCL-1 in preventing DNA damage-induced apoptosis owing to its dual inhibition of BAX and BAK as well as higher protein stability. On the basis of these results, we propose an interconnected hierarchical model that accommodates and explains how the intricate interplays between BH3s, multidomain anti-apoptotic and multidomain pro-apoptotic BCL-2 members dictate the cellular decision of survival versus death.

RESULTS

NOXA directly activates BAX and BAK independently of BID, BIM and PUMA

Genetic loss-of-function studies indicate that BID, BIM and PUMA are required for the activation of BAX and BAK in neurons and T cells15. However, it remains unclear whether BID, BIM and PUMA represent the full repertoire of activator BH3s in all cell types, and whether BAX and BAK can be activated to induce apoptosis in the absence of activator BH3s. To address these questions, we first examined fibroblasts generated from Bid−/−Bim−/−Puma−/− TKO mouse embryos. Although TKO MEFs were significantly more resistant to apoptosis than wild-type MEFs, they eventually succumbed to various apoptotic stimuli (Supplementary Fig. 1). These data suggest the existence of yet unidentified activator(s) of BAX and BAK in fibroblasts. During the process of searching for additional activator(s) of BAX–BAK, we found that recombinant NOXA protein generated using wheat germ extract (WGE) but not reticulocyte lysates was capable of inducing cytochrome c release from mitochondria isolated from wild-type but not Bax−/−Bak−/− cells in a dose-dependent manner (Fig. 1a,b). The cytochrome c-releasing activity of NOXA was abrogated by BH3 mutations (Fig. 1a), supporting the importance of the BH3 domain in activating BAX–BAK. We have previously demonstrated that in vitro-transcribed, -translated (IVTT) mouse NOXA protein using reticulocyte lysates failed to induce cytochrome c efflux from mitochondria6. As we encountered difficulty in generating IVTT human NOXA protein generated using reticulocyte lysates, we exploited WGE. Both human and mouse NOXA generated using WGE exhibited comparable cytochrome c-releasing activity (Fig. 1c). Notably, the BAD protein could not induce cytochrome c release irrespective of the IVTT system employed (Fig. 1c). These data suggest that either WGE activates or reticulocyte lysates inhibit the cytochrome c-releasing activity of NOXA. The NOXA protein generated using reticulocyte lysates could not induce cytochrome c release even in the presence of WGE (Supplementary Fig. 2a). In contrast, the cytochrome c-releasing activity of the NOXA protein generated using WGE was abrogated by reticulocyte lysates (Supplementary Fig. 2a), supporting the theory that an inhibitory factor or activity is present in reticulocyte lysates. Interestingly, the inhibitory effect of reticulocyte lysates seems to be specific for NOXA (Supplementary Fig. 2a,b).

We next examined whether NOXA can interact and activate BAX–BAK directly. Indeed, direct interaction between NOXA and BAX or BAK was detected by GST-pulldown assays (Fig. 1d). Furthermore, wild-type NOXA but not BH3 mutant NOXA induced the formation of BAX or BAK homo-oligomers (Fig. 1e). More importantly, NOXA induced cytochrome c efflux in Bid−/−Bim−/−Puma−/− TKO mitochondria (Fig. 1f). Overexpression of mouse NOXA protein did not induce robust apoptosis possibly owing to its labile nature. Supporting this notion, overexpression of mutant NOXA with substitutions of arginine for all lysine residues that stabilized the NOXA protein significantly enhanced the death-inducing activity of mouse NOXA (Fig. 1g). Interestingly, human NOXA protein was more stable than mouse NOXA protein and consequently induced more apoptosis (Fig. 1g). To further validate that NOXA is a bona fide activator BH3, liposome permeabilization assays were performed (Fig. 1h,i). As previously reported31,32, recombinant BAX protein was unable to induce liposomal release of fluorophore unless it was activated by tBID (Fig. 1h). By analogy, wild-type NOXA but not BH3 mutant NOXA could induce BAX-dependent liposome permeabilization (Fig. 1h,i). In contrast, BAD failed to activate BAX to permeabilize liposomes (Fig. 1j,k). These data strongly support that NOXA can directly activate BAX to induce MOMP.

Noxa deficiency further protects Bid−/−Bim−/−Puma−/− MEFs and small intestine from apoptosis

We reasoned that differential expressions of NOXA in various cell types might contribute to the different apoptotic phenotypes of Bid−/−Bim−/−Puma−/− neurons, T cells and MEFs. Quantitative PCR with reverse transcription (qRT–PCR) demonstrated that NOXA was highly expressed in MEFs compared with neurons and lymphocytes (Fig. 2a), which is consistent with previous
Figure 1 NOXA directly activates BAX and BAK independently of BID, BIM and PUMA. (a) Mitochondria isolated from wild-type or Bax\(^{-/-}\)Bak\(^{-/-}\)MEFs were incubated with the indicated IVTT proteins generated using reticulocyte lysates or wheat germ extract (WGE) at 30°C for 30 min, after which the release of cytochrome c was quantified by ELISA assays (mean ± s.d., \(n = 3\) independent experiments). (b) Isolated wild-type mitochondria were incubated with the indicated amounts of IVTT mouse NOXA protein (WGE) and the release of cytochrome c was quantified (mean ± s.d., \(n = 3\) independent experiments). (c) Isolated wild-type mitochondria were incubated with the indicated IVTT proteins (WGE) and the release of cytochrome c was quantified (mean ± s.d., \(n = 3\) independent experiments). (d) Radiolabelled IVTT NOXA or tBID protein was incubated with GST, GST-BAX\(_{AC}\) or GST-BAK\(_{AC}\) protein immobilized on glutathione beads. The precipitates and input were analysed by NuPAGE and autoradiography. (e) Isolated wild-type mitochondria were incubated with the indicated IVTT proteins for 30 min then treated with BMH crosslinker. The BAX and BAK homo-oligomers were detected by anti-BAX and anti-BAK immunoblots, respectively. (f) Mitochondria isolated from wild-type or Bid\(^{-/-}\)Bim\(^{-/-}\)Puma\(^{-/-}\) MEFs were incubated with IVTT NOXA (WGE) at 30°C for the indicated times and the release of cytochrome c was quantified (mean ± s.d., \(n = 3\) independent experiments). (g) Bid\(^{-/-}\)Bim\(^{-/-}\)Puma\(^{-/-}\) MEFs were infected with the indicated retrovirus. Cell death was quantified by annexin-V staining at 30 h (mean ± s.d., \(n = 3\) independent experiments). The expression of the indicated proteins was detected by an anti-HA immunoblot. (h,i) ANTS/DPX (fluorophore/quencher)-encapsulated liposomes were incubated with recombinant BAX protein plus the indicated IVTT proteins generated using WGE. The release of entrapped fluorophore monitored with time is shown in h (mean ± s.d., \(n = 3\) independent experiments). The release of entrapped fluorophore at 60 min is shown in i (mean ± s.d., \(n = 3\) independent experiments). The expression of the indicated proteins was detected by an anti-HA immunoblot. (h,i) ANTS/DPX-capaculated liposomes were incubated with recombinant BAX protein plus the indicated IVTT proteins generated using WGE. The release of entrapped fluorophore monitored with time is shown in h (mean ± s.d., \(n = 3\) independent experiments). The release of entrapped fluorophore at 60 min is shown in i (mean ± s.d., \(n = 3\) independent experiments). The expression of the indicated proteins was detected by an anti-HA immunoblot. (h,i) ANTS/DPX-capaculated liposomes were incubated with recombinant BAX protein plus the indicated IVTT proteins generated using WGE. The release of entrapped fluorophore monitored with time is shown in h (mean ± s.d., \(n = 3\) independent experiments). The release of entrapped fluorophore at 60 min is shown in i (mean ± s.d., \(n = 3\) independent experiments). The expression of the indicated proteins was detected by an anti-HA immunoblot. (h,i) ANTS/DPX-capaculated liposomes were incubated with recombinant BAX protein plus the indicated IVTT proteins generated using WGE. The release of entrapped fluorophore monitored with time is shown in h (mean ± s.d., \(n = 3\) independent experiments). The release of entrapped fluorophore at 60 min is shown in i (mean ± s.d., \(n = 3\) independent experiments). The expression of the indicated proteins was detected by an anti-HA immunoblot.
observations that deficiency of Noxa protects transformed MEFs but not lymphocytes from DNA damage-induced apoptosis\textsuperscript{33-35}. To determine whether NOXA is the missing activator of BAX and BAK in Bid\textsuperscript{−/−} Bim\textsuperscript{−/−} Puma\textsuperscript{−/−} MEFs, we generated Bid\textsuperscript{−/−} Bim\textsuperscript{−/−} Puma\textsuperscript{−/−} Noxa\textsuperscript{−/−} quadruple knockout (QKO) MEFs. Primary Bid\textsuperscript{−/−} Bim\textsuperscript{−/−} Puma\textsuperscript{−/−} Noxa\textsuperscript{−/−} MEFs were more resistant than Bid\textsuperscript{−/−} Bim\textsuperscript{−/−} Puma\textsuperscript{−/−} MEFs to apoptosis triggered by serum or glucose deprivation, and ER stress (tunicamycin or thapsigargin; Fig. 2b). Remarkably, the protection conferred by quadruple deficiency of Bid, Bim, Puma and Noxa was comparable to that of double deficiency of Bax and Bak (Fig. 2b). The reason why deletion of Noxa further protects Bid\textsuperscript{−/−} Bim\textsuperscript{−/−} Puma\textsuperscript{−/−} MEFs is not due to compensatory induction of Noxa (Supplementary Fig. 2c). Together, these data indicate that NOXA, along with BID, BIM and PUMA, is an activator of BAX-BAK in MEFs. As intermediate expression of Noxa was reported to confer protection against ionizing radiation\textsuperscript{14}, we next examined whether deficiency of Noxa further protects Bid\textsuperscript{−/−} Bim\textsuperscript{−/−} Puma\textsuperscript{−/−} small intestine from irradiation. Similarly to Bid\textsuperscript{−/−} Bim\textsuperscript{−/−} Puma\textsuperscript{−/−} mice, Bid\textsuperscript{−/−} Bim\textsuperscript{−/−} Puma\textsuperscript{−/−} Noxa\textsuperscript{−/−} mice were born at a less-than-expected Mendelian ratio and exhibited the same developmental defects including persistent interdigital webs and imperforate vagina (Supplementary Fig. 2d,e and Supplementary Table 1). Although triple deletion of Bid, Bim and Puma provided profound inhibition of irradiation-induced apoptosis in small intestine, only quadruple deletion of Bid, Bim, Puma and Noxa or double deletion of Bax and Bak completely blocked apoptosis (Fig. 2c,d). Consistent with the lower NOXA expression in lymphocytes (Fig. 2a) and the absence of BAX–BAK activation detected in Bid\textsuperscript{−/−} Bim\textsuperscript{−/−} Puma\textsuperscript{−/−} T cells as reported previously\textsuperscript{15}, Bid\textsuperscript{−/−} Bim\textsuperscript{−/−} Puma\textsuperscript{−/−} T cells were as resistant as Bid\textsuperscript{−/−} Bim\textsuperscript{−/−} Puma\textsuperscript{−/−} Noxa\textsuperscript{−/−} T cells to various apoptotic signals including cytokine withdrawal, glucocorticoid, and genotoxic stress (Fig. 2e). Of note, Noxa expression is lower both at the basal level and in response to genotoxic stress in T cells than MEFs (Supplementary Fig. 2f). Total body irradiation-induced apoptosis in spleen was greatly reduced in both Bid\textsuperscript{−/−} Bim\textsuperscript{−/−} Puma\textsuperscript{−/−} and Bid\textsuperscript{−/−} Bim\textsuperscript{−/−} Puma\textsuperscript{−/−} Noxa\textsuperscript{−/−} mice (Supplementary Fig. 3a). Triple deletion of Bid, Bim and Puma or quadruple deletion of Bid, Bim, Puma and Noxa almost completely rescued splenic CD4\textsuperscript{+} T-cells from irradiation (Supplementary Fig. 3b). Collectively, these data suggest that although NOXA can directly activate BAX and BAK, Noxa deficiency confers resistance to apoptosis only in tissues or cell types that express sufficient NOXA for the activation of BAX- and BAK-dependent death program.

**DNA damage activates BAX- and BAK-dependent mitochondrial apoptosis in transformed Bid\textsuperscript{−/−} Bim\textsuperscript{−/−} Puma\textsuperscript{−/−} Noxa\textsuperscript{−/−} MEFs**

To characterize DNA damage-induced cell death in transformed Bid\textsuperscript{−/−} Bim\textsuperscript{−/−} Puma\textsuperscript{−/−} Noxa\textsuperscript{−/−} MEFs, we first examined cytochrome c-initiated caspase activation. In SV40-transformed wild-type MEFs, etoposide induced cytochrome c efflux from the mitochondria to the cytosol within 15 h (Fig. 4a), which coincides with the peak of caspase activation (Fig. 4b). In contrast, cytochrome c translocation was not detected in Bid\textsuperscript{−/−} Bim\textsuperscript{−/−} Puma\textsuperscript{−/−} Noxa\textsuperscript{−/−} cells until 24 h and was to a much lesser extent than wild-type cells (Fig. 4a). Accordingly, lower caspase activity was detected in Bid\textsuperscript{−/−} Bim\textsuperscript{−/−} Puma\textsuperscript{−/−} Noxa\textsuperscript{−/−} cells than wild-type cells (Fig. 4b). Cleavage of PARP and caspase-3 was detected in QKO cells on etoposide but not ER stress (Fig. 4c). In support of the involvement of the cytochrome c–APAF-1–caspase-9 axis in DNA damage-induced apoptosis in QKO cells, knockdown of cytochrome c, Apaf-1 or caspase-9 protected QKO cells from etoposide-induced apoptosis (Fig. 4d and Supplementary Fig. 4a). We next determined whether BAX and BAK are activated in QKO cells on DNA damage. Indeed, both BAX and BAK homo-oligomers were detected in QKO cells on etoposide at a later time point (Fig. 4e). Consequently, knockdown of either Bax or Bak partially inhibited whereas knockdown of both Bax and Bak near completely blocked etoposide-induced apoptosis in QKO cells (Fig. 4f and Supplementary Fig. 4a). Together, these data indicate that DNA damage can activate BAX–BAK in transformed Bid\textsuperscript{−/−} Bim\textsuperscript{−/−} Puma\textsuperscript{−/−} Noxa\textsuperscript{−/−} MEFs. The low amplitude of cytochrome c efflux and caspase activation suggest that this mode of BAX and BAK activation is less efficient than that involving BID, BIM, PUMA and NOXA.

**BAX and BAK can be autoactivated in the absence of activator BH3s through downregulation of BCL-2, BCL-X\textsubscript{L} and MCL-1**

To elucidate how DNA damage activates BAX–BAK in QKO cells, we first investigated whether other known BH3s are involved. Knockdown of Bad, Bmf, Bik or Hrk failed to protect QKO cells from etoposide-induced apoptosis (Fig. 5a and Supplementary Fig. 4b–d). Moreover, overexpression of BAD, BMF, BIK or HRK induced minimal apoptosis in QKO cells (Fig. 5b), supporting the proposed hierarchy of BH3s where activator BH3s function downstream of inactivator BH3s (ref. 6). Consistent with the notion that BID, BIM, PUMA and NOXA are activator BH3s, overexpression of these BH3s effectively induced...
spontaneous apoptosis in QKO cells (Fig. 5b). The inability of BH3s other than BID, BIM, PUMA and NOXA to kill QKO cells raises a possibility that an alternative, BH3-independent mechanism may be involved in the activation of BAX–BAK in QKO cells on DNA damage.

Although most BAX and BAK proteins do not interact with anti-apoptotic BCL-2s in viable cells, those complexed with anti-apoptotic BCL-2s probably have already exposed their BH3 domains that bind to the hydrophobic dimerization groove of...
Figure 3 Quadruple deficiency of Bid, Bim, Puma and Noxa abrogates apoptosis in transformed MEFs triggered by growth factor deprivation and ER stress but not genotoxic stress. (a–g) E1A/Ras-transformed wild-type Bid<sup>−/−</sup> Bim<sup>−/−</sup> Puma<sup>−/−</sup> TKO, Bid<sup>−/−</sup> Bim<sup>−/−</sup> Puma<sup>−/−</sup> Noxa<sup>−/−</sup> QKO, or Bax<sup>−/−</sup> Bak<sup>−/−</sup> DKO MEFs were untreated, or cultured in the presence of tunicamycin, thapsigargin or etoposide, or irradiated with ultraviolet-C (UV; g). Cell death was quantified by annexin-V staining at the indicated times (mean ± s.d., n=3 independent experiments). (h) SV40-transformed wild-type, Bid<sup>−/−</sup> Bim<sup>−/−</sup> Puma<sup>−/−</sup> TKO, Bid<sup>−/−</sup> Bim<sup>−/−</sup> Puma<sup>−/−</sup> Noxa<sup>−/−</sup> QKO, or Bax<sup>−/−</sup> Bak<sup>−/−</sup> DKO MEFs were untreated, or cultured in the presence of tunicamycin, thapsigargin or etoposide, or irradiated with ultraviolet-C. Cell death was quantified by annexin-V staining at the indicated times (mean ± s.d., n=3 independent experiments). ∗, P < 0.05; ∗∗, P < 0.01; ∗∗∗, P < 0.001 (Student’s t-test).

anti-apoptotic BCL-2s (refs 3,7). Foreseeably, if anti-apoptotic BCL-2s are downregulated by DNA damage in QKO MEFs, the ‘BH3-exposed’ monomers of BAX–BAK will probably be liberated to activate other native molecules of BAX–BAK, initiating ‘autoactivation’. Indeed, BCL-2, BCL-X<sub>L</sub> and MCL-1 were decreased in QKO MEFs on etoposide treatment (Fig. 5c). In contrast, no concurrent downregulation of anti-apoptotic BCL-2s was detected in QKO MEFs on ER stress (Fig. 5c). Significantly, proteasome inhibition mitigated DNA damage-induced decrease of anti-apoptotic BCL-2s, and consequently protected QKO cells from etoposide-induced apoptosis (Fig. 5d,e). Of note, etoposide-induced downregulation of BCL-2 and BCL-X<sub>L</sub> was also observed in wild-type MEFs but not in T cells (Supplementary Fig. 5a,b). Interestingly, ultraviolet but not ionizing radiation could induce downregulation of anti-apoptotic BCL-2s in
**Figure 4** DNA damage activates BAX- and BAK-dependent mitochondrial apoptosis in transformed Bid<sup>−/−</sup>Bim<sup>−/−</sup>Puma<sup>−/−</sup>Noxa<sup>−/−</sup> QKO MEFs. (a) SV40-transformed wild-type or Bid<sup>−/−</sup>Bim<sup>−/−</sup>Puma<sup>−/−</sup>Noxa<sup>−/−</sup> QKO MEFs, untreated or treated with etoposide for the indicated times, were subjected to subcellular fractionation. Cytosolic and mitochondrial fractions were analysed by anti-cytochrome c, anti-LDH and anti-VDAC1 immunoblots. (b) SV40-transformed wild-type or Bid<sup>−/−</sup>Bim<sup>−/−</sup>Puma<sup>−/−</sup>Noxa<sup>−/−</sup> QKO MEFs, untreated or treated with etoposide or tunicamycin (TC), were analysed by anti-PARP, anti-actin immunoblots. (c) SV40-transformed wild-type, Bid<sup>−/−</sup>Bim<sup>−/−</sup>Puma<sup>−/−</sup> TKO, or Bid<sup>−/−</sup>Bim<sup>−/−</sup>Puma<sup>−/−</sup>Noxa<sup>−/−</sup> QKO MEFs, untreated or treated with etoposide (Etop) or tunicamycin (TC), were analysed by anti-cytochrome c, anti-LDH and anti-VDAC1 immunoblots. Cytosolic and mitochondrial fractions were subjected to subcellular fractionation. Cytosolic and mitochondrial fractions were analysed for caspase-3/7 activities (mean ± s.d., n = 3 independent experiments). (d) SV40-transformed Bid<sup>−/−</sup>Bim<sup>−/−</sup>Puma<sup>−/−</sup>Noxa<sup>−/−</sup> QKO MEFs were infected with retrovirus expressing shRNA against luciferase or Apaf-1, or transfected with scrambled siRNA (Scr siRNA) or siRNA against cytochrome c or caspase-9. After 48 h, cells were untreated or treated with etoposide for 36 h. Cell death was quantified by annexin-V staining (mean ± s.d., n = 3 independent experiments). (e) Mitochondria isolated from SV40-transformed wild-type or Bid<sup>−/−</sup>Bim<sup>−/−</sup>Puma<sup>−/−</sup>Noxa<sup>−/−</sup> QKO MEFs untreated or treated with etoposide for 15 h (WT) or 36 h (QKO) were subjected to BMH crosslinking. The BAX and BAK homo-oligomers were detected by anti-BAX and anti-BAK immunoblots, respectively. (f) SV40-transformed Bid<sup>−/−</sup>Bim<sup>−/−</sup>Puma<sup>−/−</sup>Noxa<sup>−/−</sup> QKO MEFs were transfected with scrambled siRNA (Scr siRNA) or siRNA against Bax and/or Bak. After 48 h, cells were untreated or treated with etoposide for 36 h. Cell death was quantified by annexin-V staining (mean ± s.d., n = 3 independent experiments). *P < 0.05; **P < 0.001 (Student’s t-test). Unprocessed original scans of blots are shown in Supplementary Fig. 7.
Figure 5 BAX and BAK can be autoactivated by DNA damage independently of activators BID, BIM, PUMA and NOXA through downregulation of BCL-2, BCL-X, and MCL-1. (a) SV40-transformed Bid+/− Bim+/− Puma+/− Noxa+/− QKO MEFs were infected with retrovirus expressing shRNA against luciferase, Bad, Bmf or Bik. After 48 h, cells were untreated or treated with etoposide for 36 h. Cell death was quantified by annexin-V staining (mean ± s.d., n = 3 independent experiments). (b) SV40-transformed wild-typeBid+/− Bim+/− Puma+/− TKO, Bid+/− Bim+/− Puma+/− Noxa+/− QKO, or Bax+/− Bak−/− MEFs were infected with retrovirus expressing GFP or the indicated BH3-only proteins to induce spontaneous apoptosis. NOXA denotes human NOXA. Cell death was quantified by annexin-V staining at 30 h (mean ± s.d., n = 3 independent experiments). (c) SV40-transformed Bid+/− Bim+/− Puma+/− Noxa+/− QKO MEFs, untreated or treated with etoposide, tunicamycin (TC) or thapsigargin (TG), were subjected to immunoblot analysis using the indicated antibodies. (d) SV40-transformed Bid+/− Bim+/− Puma+/− Noxa+/− QKO MEFs were untreated or treated with etoposide and/or MG132 for 18 h, and subjected to immunoblot analysis using the indicated antibodies. (e) SV40-transformed Bid+/− Bim+/− Puma+/− Noxa+/− QKO MEFs were untreated or treated with etoposide and/or MG132 for 36 h. Cell death was quantified by annexin-V staining (mean ± s.d., n = 3 independent experiments). (f) SV40-transformed wild-type Bid+/− Bim+/− Puma+/− TKO, Bid+/− Bim+/− Puma+/− Noxa+/− QKO, or Bax+/− Bak−/− MEFs were transfected with scrambled siRNA (Scr siRNA) or siRNA against Bcl-2, Bcl-x, and/or Mcl-1 to induce spontaneous apoptosis. After 2 days, cell death was quantified by annexin-V staining (mean ± s.d., n = 3 independent experiments). (g) SV40-transformed Bid+/− Bim+/− Puma+/− Noxa+/− QKO MEFs were untreated or treated with etoposide in the presence of the pan-caspase inhibitor Q-VD-OPh to preserve cell integrity on apoptosis induction. After 24 h, cells were permeabilized with digitonin and subjected to limited trypsin proteolysis. The BAK cleavage products were detected by an anti-BAK (G23) immunoblot. (h) SV40-transformed Bid+/− Bim+/− Puma+/− Noxa+/− QKO MEFs transfected with scrambled siRNA or siRNA against Bcl-x, and Mcl-1 were subjected to limited trypsin proteolysis. The BAK cleavage products were detected by an anti-BAK (G23) immunoblot. ∗∗, P < 0.01; ∗∗∗, P < 0.001 (Student’s t-test). Unprocessed original scans of blots are shown in Supplementary Fig. 7. QKO cells (Supplementary Fig. 5c). Consequently, QKO cells were almost as resistant as Bax−/− Bak−/− cells to ionizing radiation but succumbed to ultraviolet (Supplementary Fig. 5d and Fig. 3h).

To further confirm that loss of anti-apoptotic BCL-2s can lead to autoactivation of BAX–BAK and apoptosis, short interfering RNA (siRNA)-mediated knockdown of anti-apoptotic BCL-2s was performed. Concurrent knockdown of Bcl-2, Bcl-xL and Mcl-1 induced significant apoptosis after 2 days in wild-type, TKO and QKO cells but not in Bax−/− Bak−/− cells in the absence of any death stimulus (Fig. 5f and Supplementary Fig. 4a). Interestingly, knockdown of both Bcl-xL and Mcl-1 was sufficient to induce spontaneous apoptosis. Of note, knockdown of Bcl-2, Bcl-xL or Mcl-1 did not sensitize QKO cells to overexpression of BAD, BMF, BIK or HRK (Supplementary Fig. 5e). Given that the basal levels of activator BH3s are low in healthy cells and most of the activator BH3s are induced by intrinsic death signals, deficiency of activator BH3s seems to provide more protection against apoptosis triggered by intrinsic death signals than knockdown of anti-apoptotic BCL-2s. It is noteworthy that overexpression of NOXA induces more apoptosis than knockdown of Mcl-1 in MEFs (Fig. 5f), supporting that NOXA does not simply induce apoptosis by inactivating MCL-1.

Liberation of the ‘BH3-exposed’ BAK monomers from anti-apoptotics initiates a feed-forward amplification loop for the activation of mitochondrial apoptosis

It was reported that the BH3 domain of BAK is inaccessible in viable cells and cleavage of BAK at the BH3 domain by limited proteolysis can reflect the exposure of the BH3 domain in BAK.
Limited proteolysis of BAK also revealed another feature of BAK activation, that is, exposure of the α1 helix or BH4 domain, leading to the generation of a 19 kDa mitochondrially targeted C-terminal fragment p19 (Fig. 5g,h). These data suggest that liberation of the ‘BH3-exposed’ BAK from anti-apoptotic BCL-2s may induce a ‘feed-forward’ mechanism to activate other native molecules of BAX–BAK. Accordingly, even a small fraction of ‘BH3-exposed’ BAX–BAK monomers that are set free from the anti-apoptotic BCL-2s is sufficient to initiate the mitochondrial apoptotic program. Collectively, these data support a model in which BAX and BAK can undergo autoactivation on DNA damage through downregulation of anti-apoptotic BCL-2s in transformed Bid<sup>−/−</sup>Bim<sup>−/−</sup>Puma<sup>−/−</sup>Noxa<sup>−/−</sup> MEFs.

Figure 6 BCL-X<sub>L</sub> is superior to BCL-2 and MCL-1 in preventing DNA damage-induced apoptosis owing to its dual inhibition of BAX and BAK as well as higher protein stability. (a) SV40-transformed Bid<sup>−/−</sup>Bim<sup>−/−</sup>Puma<sup>−/−</sup>Noxa<sup>−/−</sup> QKO MEFs stably expressing GFP, HA–BCL-2, HA–BCL-X<sub>L</sub> or HA–MCL-1 were subjected to anti-HA immunoprecipitation in 0.2% NP-40 or 1% CHAPS lysis buffer. The input (5%) and immunoprecipitates were analysed for HA–BCL-2, HA–BCL-X<sub>L</sub> or HA–MCL-1 by an anti-HA immunoblot with or without etoposide treatment for 12 h. (b) SV40-transformed Bid<sup>−/−</sup>Bim<sup>−/−</sup>Puma<sup>−/−</sup>Noxa<sup>−/−</sup> QKO MEFs stably expressing GFP, HA–BCL-2, HA–BCL-X<sub>L</sub> or HA–MCL-1 were untreated or treated with etoposide. Cell death was quantified by annexin-V staining at the indicated times (mean ± s.d., n=3 independent experiments). The expression of HA–BCL-2, HA–BCL-X<sub>L</sub> or HA–MCL-1 was detected by an anti-HA immunoblot with or without etoposide treatment for 12 h. (c) SV40-transformed Bid<sup>−/−</sup>Bim<sup>−/−</sup>Puma<sup>−/−</sup>Noxa<sup>−/−</sup> QKO MEFs stably expressing GFP, BCL-2 or BCL-X<sub>L</sub> were transfected with scrambled siRNA (Scr siRNA) or siRNA against Bax or Bak. After 48 h, cells were untreated or treated with etoposide for 36 h. Cell death was quantified by annexin-V staining (mean ± s.d., n=3 independent experiments). (d) SV40-transformed wild-type MEFs stably expressing HA-tagged BCL-2 or BCL-X<sub>L</sub> were untreated or treated with etoposide. Cell death was quantified by annexin-V staining at the indicated times (mean ± s.d., n=3 independent experiments). The expression of HA-tagged BCL-2 and BCL-X<sub>L</sub> was detected by an anti-HA immunoblot. (e) SV40-transformed wild-type or Bid<sup>−/−</sup>Bim<sup>−/−</sup>Puma<sup>−/−</sup>Noxa<sup>−/−</sup> QKO MEFs stably expressing GFP, wild-type BCL-X<sub>L</sub>, BCL-X<sub>L</sub> mutant 1 (F131V/D133A) or BCL-X<sub>L</sub>, mutant 8 (G138E/R139L/140N) were untreated or treated with etoposide. Cell death was quantified by annexin-V staining at the indicated times (mean ± s.d., n=3 independent experiments). The expression of BCL-X<sub>L</sub> was analysed by an anti-BCL-X<sub>L</sub> immunoblot. *, P<0.05; **, P<0.01; ***, P<0.001 (Student’s t-test). Unprocessed original scans of blots are shown in Supplementary Fig. 7.
BCL-X<sub>L</sub> is superior to BCL-2 and MCL-1 in preventing DNA damage-induced apoptosis

The loss-of-function studies of anti-apoptotic BCL-2s suggest that BCL-2 may be less important than BCL-X<sub>L</sub> and MCL-1 in gauging cellular survival (Fig. 5f). This could be due to the fact that BCL-2 interacts only with BAX but not BAK (refs 37,38). In QKO cells, co-immunoprecipitation assays demonstrated that BAX interacted with BCL-2, BCL-X<sub>L</sub> and MCL-1 whereas BAK interacted only with BCL-X<sub>L</sub> and MCL-1 but not BCL-2 (Fig. 6a). The same interaction profiles were also observed in WT and TKO MEFs (Supplementary Fig. 5f), which is consistent with previously published results<sup>27,38</sup>. Notably, the same results were obtained using either CHAPS or NP-40 buffer except that more heterodimers were detected in the presence of NP-40 than CHAPS (Fig. 6a). Consistent with the notion that most endogenous BAX–BAK does not interact with anti-apoptotic BCL-2s in viable cells<sup>6,7</sup>, only a portion of BAX–BAK was co-precipitated with anti-apoptotic BCL-2s (Fig. 6a).

We next investigated whether anti-apoptotic BCL-2s might exhibit differential activity against DNA damage in QKO cells. Indeed, overexpression of BCL-2 provided less protection to QKO cells against etoposide-induced apoptosis than BCL-X<sub>L</sub> (Fig. 6b). Surprisingly, MCL-1 also provided less protection than BCL-X<sub>L</sub> even though both interact with BAX and BAK. The lesser protection conferred by MCL-1 is probably due to its labile nature, which is supported by the observation that overexpressed MCL-1 was rapidly degraded by etoposide within 12 h (Fig. 6b). To further assess whether the differential activity of BCL-2 versus BCL-X<sub>L</sub> in regulating apoptosis is due to the inability of BCL-2 to inhibit BAK, we silenced Bax or Bak in QKO cells overexpressing BCL-2 or BCL-X<sub>L</sub>. Knockdown of Bak but not Bax further protected BCL-2-overexpressing QKO cells from etoposide-induced apoptosis (Fig. 6c), suggesting that etoposide is capable of activating BAK in BCL-2-overexpressing cells. In contrast, knockdown of either Bax or Bak had no effect on BCL-X<sub>L</sub> in protecting QKO cells against etoposide (Fig. 6c). Even in wild-type cells, overexpression of BCL-2 only delays whereas overexpression of BCL-X<sub>L</sub> completely blocks etoposide-induced apoptosis (Fig. 6d). The delayed death kinetics observed in BCL-2-overexpressing cells further supports the lower efficiency of BAK autoactivation in triggering mitochondrial apoptosis. It is noteworthy that transformed Bax<sup>−/−</sup>Bak<sup>−/−</sup> MEFs still succumb to DNA damage-induced programmed necrotic death between 2–3 days that cannot be inhibited by overexpression of BCL-X<sub>L</sub> (ref. 39). In aggregate, BCL-X<sub>L</sub> is superior to BCL-2 and MCL-1 in preventing DNA damage-induced apoptosis owing to its dual inhibition of BAX and BAK as well as higher protein stability.

Binding to BAX and BAK is required for BCL-X<sub>L</sub> to inhibit apoptosis in QKO cells

To further illustrate that BCL-X<sub>L</sub> inhibits etoposide-induced apoptosis in QKO cells through sequestering BAX–BAK but not BH3s, we employed reported, distinct BCL-X<sub>L</sub> mutants that differentially interact with BH3s or BAX–BAK (refs 9,40). The BCL-X<sub>L</sub> mutant 1 (F131V/D133A) known to interact with BH3s but not BAX–BAK protected wild-type but not QKO cells from etoposide-induced apoptosis (Fig. 6e). Conversely, the BCL-X<sub>L</sub> mutant 8 (G138E/R139L/I140N) known to interact with neither BH3s nor BAX–BAK failed to inhibit apoptosis in both wild-type and QKO cells (Fig. 6e). Together, these data suggest that BCL-X<sub>L</sub> protects wild-type cells through sequestration of either activator BH3s or multidomain BAX–BAK. In the absence of activator BH3s, BCL-X<sub>L</sub> binds to BAX–BAK to inhibit apoptosis. In summary, our data support a model in which anti-apoptotic BCL-2s keep both activator BH3s and the ‘BH3-exposed’ BAX–BAK monomers in check in viable cells. In response to intrinsic death signals, BID, BIM, PUMA and NOXA are activated through transcriptional regulation and/or post-translational modifications, triggering the homo-oligomerization of BAX–BAK to initiate apoptosis. In addition, etoposide and ultraviolet decrease anti-apoptotic BCL-2s in transformed MEFs, resulting in autoactivation of BAX–BAK even in the absence of BID, BIM, PUMA and NOXA (Supplementary Fig. 6).

**DISCUSSION**

Our results support an interconnected hierarchical model in which the BCL-2 family proteins regulate mitochondrial apoptosis (Fig. 7). BID, BIM, PUMA and NOXA activate BAX–BAK directly to induce stepwise, bimodal activation of BAX–BAK. Activator BH3s convert inactive BAX–BAK monomers to ‘BH3-exposed’ BAX–BAK monomers, initiating the activation of BAX–BAK. Subsequently, activator BH3s prevent anti-apoptotic BCL-2s from sequestering ‘BH3-exposed’ monomers of BAX–BAK, leading to homo-oligomerization of BAX–BAK. The anti-apoptotic BCL-2s inhibit both modes of BAX–BAK activation. First, they sequester activator BH3s to prevent the initiation of BAX–BAK activation, providing front-line protection<sup>6,9</sup>. Second, the anti-apoptotic BCL-2s can also sequester ‘BH3-exposed’ BAX–BAK monomers to prevent the homo-oligomerization of BAX–BAK, serving as a fail-safe mechanism or the second line of defence. The interaction
between activator BH3s and anti-apoptotic BCL-2s confers mutual inhibition because it not only prevents activator BH3s from activating BAX–BAK but also refrains the anti-apoptotics from sequestering the ‘BH3-exposed’ BAX–BAK monomers. Conceivably, BID, BIM and PUMA can prevent BCL-2, BCL-X\(_l\) and MCL-1 from sequestering BAX–BAK whereas NOXA can only inhibit MCL-1. This difference may contribute to the lower death-inducing activity of NOXA in comparison with BID, BIM and PUMA (Fig. 5b). Consequently, Noxa deficiency confers resistance to apoptosis only in tissues or cell types that express sufficient NOXA for the activation of BAX–BAK. The remaining BH3s, including BAD, BMF, BIK and HRK, cannot activate BAX–BAK directly and seem to promote apoptosis by preventing BCL-2 and BCL-X\(_l\) from sequestering activator BH3s. NOXA is unique among all BH3s in the sense that it can prevent MCL-1 from sequestering BID, BIM and PUMA owing to its high binding affinity to MCL-1. The observation that deficiency of BID, BIM, PUMA and NOXA abrogates apoptosis triggered by overexpression of BAD, BMF, BIK or HRK (DP5) supports the BH3 hierarchy entailing upstream ‘inactivator’ BH3s and downstream ‘activator’ BH3s (ref. 6).

The interaction between activator BH3s and multidomain BAX–BAK has been debated for decades owing to low binding affinity\(^{27}\). Recent biophysical demonstrations of BID, BIM or PUMA bound to BAX or BAK have helped resolve this controversy\(^{18-22}\). It is now recognized that the binding of activator BH3s to BAX–BAK has to be transient and dynamic to induce stepwise, bimodal activation of BAX–BAK (refs 14,18,19,22,24,41). The interaction between activator BH3s and BAX–BAK should be ‘hit-and-run’ because the same binding pocket of BAX–BAK is utilized for homo-oligomerization. Hence, the low binding affinity between activator BH3s and BAX–BAK renders a simple solution. In contrast, BH3s bind tightly to the anti-apoptotic BCL-2s. By analogy, the anti-apoptotic BCL-2s function like ‘decoy’ death receptors that form inert stable complexes with BH3s.

As BID, BIM and PUMA also bind and inactivate all of the anti-apoptotic BCL-2s, it was proposed that BAX and BAK can be autoactivated once they are released from the anti-apoptotic BCL-2s by BID, BIM and PUMA (indirect activation model)\(^{27}\). The identification of NOXA as an activator of BAX–BAK argues against the indirect activation model because NOXA exhibits high binding affinity only to MCL-1 (refs 6,11-13). Nonetheless, unequivocal evidence of indirect activation of BAX–BAK has not been revealed until our generation\(^{18-22}\). One plausible explanation is that different activation models can coexist within the BCL-2 family. The BAX–BAK homodimer directly targets BAK and induce BAK-dependent liposome permeabilization in one report but not another\(^{19,47}\). Other plausible explanations include different BH3 peptide preparations result in different alpha helicity. Alternatively, different BH3 peptides with different BH3 domain structures or different BH3 peptide-related BH3-like domains may bind to the BH3 domain of BAX–BAK and may not engage the BH3 binding pocket of BAX–BAK directly. However, the BH3 peptides also induce apoptosis only in tissues or cell types that express sufficient BH3 peptides for the activation of BAX–BAK. Consequently, BH3 peptides can recapitulate full-length proteins in activating BAX–BAK. For example, whereas full-length PUMA protein has potent cytochrome c-releasing activity\(^6\), conflicting results have been reported with regard to the PUMA BH3 peptide\(^{12,13,21,45,46}\).

Here, we show that full-length NOXA protein is capable of permeabilizing wild-type mitochondria and liposomes in the presence of BAX. As for PUMA BH3 peptide, inconsistent results have been obtained from NOXA BH3 peptide. NOXA BH3 peptide was shown to bind to BAK and induce BAK-dependent liposome permeabilization in one report but not another\(^{19,47}\). It is possible that different BH3 peptide preparations result in different alpha helicity. Alternatively, regions other than the BH3 domain may enhance the interaction between PUMA–NOXA and BAX–BAK directly or indirectly.

The identification of NOXA as a missing activator of BAX–BAK and the creation of Bid\(^{-/-}\)/Bim\(^{-/-}\)/Puma\(^{-/-}\)/Noxa\(^{-/-}\) QKO cells provide an unprecedented opportunity to solve the puzzle of the BCL-2 family signalling network. Our studies comparing cells deficient for upstream apoptotic initiators (activator BH3s) with those deficient for downstream apoptotic effectors (BAX and BAK) have helped resolve previously elusive, even contradictory, observations concerning the BCL-2 family proteins. Targeting the BCL-2 family to promote apoptosis holds great promise for cancer therapy\(^{18-43}\).}

**METHODS**

Methods and any associated references are available in the online version of the paper.

**ACKNOWLEDGEMENTS**

We apologize to all of the investigators whose research could not be appropriately cited owing to space limitation. We thank H.-F. Chen and S. Han for technical assistance. This work was supported by grants to E.H.C. from the NIH (R01CA125562) and the American Cancer Society (118518-RSG-10-030-01-CRCG), and to E.G. from the NIH (R01CA178394). This work was also supported by the NIH P30CA008748.

**AUTHOR CONTRIBUTIONS**

H.-C.C. designed and conducted experiments, and analysed data. E.H.C. designed research, analysed data and supervised the project. H.-C.T., M.K., Y.H., H.K., A.I.-Y., Y.T.G. and D.E.R. conducted experiments. J.J.H. and E.G. supervised some experiments. H.-C.T., D.R., P.M.C., S.T. and C.-P.L. generated essential reagents. This work was supported by grants to E.H.C. from the NIH P30CA008748.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

Published online at http://dx.doi.org/10.1038/ncb3236

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1. Gross, A., McDonnell, J. M. & Korsmeyer, S. J. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev. 13*, 1899–1911 (1999).

2. Youle, R. J. & Strasser, A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat. Rev. Mol. Cell Biol. 9*, 47–59 (2008).

**Note:** Supplementary Information is available in the online version of the paper.
3. Czabotar, P. E., Lessene, G., Strasser, A. & Adams, J. M. Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. Nat. Rev. Mol. Cell Biol. 15, 49–63 (2014).

8. Wang, X. The expanding role of mitochondria in apoptosis. Genes Dev. 15, 2922–2933 (2001).

10. Letai, A. BH3 domains other than Bim and Bid can directly activate Bax/Bak. Cell Death Differ. 17, 965–974 (2010).

18. Czabotar, P. E. BH3 domains of BH3-only proteins differentially regulate Bax- and Bak-mediated mitochondrial apoptosis. Mol. Cell 8, 705–711 (2001).

21. Westphal, D., Kluck, R. M. & Dewson, G. Building blocks of the apoptotic pore: how Bak and Bax are activated and oligomerize during apoptosis. Cell Death Differ. 21, 196–205 (2014).

29. Lindsten, T. et al. The combined functions of proapoptotic Bcl-2 family members Bak and Bax are essential for normal development of multiple tissues. Mol. Cell 6, 1389–1399 (2000).

34. Shibue, T. et al. Integral role of Noxa in p53-mediated apoptotic response. Genes Dev. 17, 2233–2238 (2003).

37. Naik, E., Michalak, E. M., Villunger, A., Adams, J. M. & Strasser, A. Ultraviolet radiation triggers apoptosis of fibroblasts and skin keratinocytes mainly via the BH3-only protein Noxa. J. Cell Biol. 176, 415–424 (2007).

39. Tu, H. C. et al. The p53-cathepsin axis cooperates with ROS to activate programmed necrotic death upon DNA damage. Proc. Natl Acad. Sci. USA 106, 1093–1098 (2009).

45. Cheng, E. H., Levine, B., Boise, L. H., Thompson, C. B. & Hardwick, J. M. Bax-independent inhibition of apoptosis by Bcl-xL. Nature 379, 554–556 (1996).

50. Davids, M. S. & Letai, A. Targeting the B-cell lymphoma/leukemia 2 family in cancer. Cancer Cell 13, 393–403 (2005).

53. Anderson, M. A., Huang, D. & Roberts, A. Targeting BCL2 for the treatment of lymphoid malignancies. Semin. Hematol. 51, 219–227 (2014).
Institutional Animal Care and Use Committee at Memorial Sloan Kettering.

Animal experiments were performed in accordance with

\[ 5 \times 10^5 \] Bax KO mice were intraperitoneal injection into Bax KO mice. Bax KO mice were confirmed by DNA sequencing. The production of retroviruses was described previously. The BH3 mutant of NOXA contains L27E mutations. All of the constructs were generated by PCR-based site-directed mutagenesis. The NOXA mutants were described previously.

METHODS

Generation of knockout mice. Bid \(-/-\), Bim \(-/-\), Puma \(-/-\), Noxa \(-/-\) mice were generated using pSuper-Retro-Puro or pSuperior-Retro-Puro according to the manufacturer's instructions. For the generation of MEFs, two wild-type E13.5 embryos were reverse-transfected using Lipofectamine RNAiMAX (Life Technologies) to a pool of siRNAs. Mouse embryonic fibroblasts (MEFs) were generated from E13.5 embryos and immortalized by transfection of pSV40 and pSV2-neom. MEFs were purchased from Dharmacon: 5' AGAtt-3' and one male (C57BL/6J). MEFs were infected with retrovirus expressing E1A or E1B and analyzed by flow cytometry. Flow cytometry was performed using either a FACSCalibur (BD Biosciences) or a BD LSRFortessa (BD Biosciences). Data were analyzed using CellQuest Pro (BD Biosciences).

Cell culture, viability assay, caspase-3/7 activity assay and TUNEL assay. Cell culture, viability assay, caspase-3/7 activity assay and TUNEL assay. The NOXA mutants were described previously. Quantification of cytochrome c release was performed using colorimetric ELISA assays (MTCO, R&D Systems). To obtain the percentage of cytochrome c release, the amount of cytochrome c present in mitochondrial supernatant was divided by total cytochrome c present in both mitochondrial supernatant and pellet. Crosslinking of BAX or BAK was performed as described previously. Briefly, 5 or 10 mM BMH (Pierce) in dimethylsulfoxide was added to mitochondria for 30 min at room temperature. Approximately 2 μg of GST, GST-BAKAC (amino acids 1-183), or GST–BAXAC (amino acids 1-171) proteins were immobilized on glutathione–agarose beads (Sigma), followed by incubation with IVTT [35S]methionine-labelled BAX-only proteins in buffer (0.2% Triton X-100, 140 mM NaCl, 5 mM MgCl2, 50 mM Tris, pH 8.0). The bound complexes were washed by 10% NP40 (Life Technologies) and analyzed by autoradiography.

Antibodies, immunoblot analysis, immunoprecipitation and limited trypsin proteolysis. Antibodies, immunoblot analysis, immunoprecipitation and limited trypsin proteolysis. Cells were lysed in RIPA buffer. Protein concentration was determined by the BCA kit (Pierce). Twenty-five to fifty micrograms of protein was resolved by 10% PAGE (Life Technologies), transferred onto PVDF membranes and immunoblotted. Antibody detection was achieved using the enhanced chemiluminescence method (Western Lighting, PerkinElmer) and LAS-3000 imaging system (FUJIFILM). Antibodies used for immunoblot analysis are follows: anti-BAX (NT, Upstate), anti-BAX (G23, Santa Cruz Biotechnology), anti-BAX (N-20, Santa Cruz Biotechnology), anti-HA (12CA5, anti-BCL-2 (no. 140-1177, Rockland), anti-VDAC1 (ab16814, Abcam), anti-cleaved caspase-3 (no. 9661, Cell Signaling Technology), anti-PARP (no. 9542, Cell Signaling Technology), anti-BAD (C-20, Santa Cruz), anti-APO-1 (AB16941, Upstate Biotechnology), anti-caspase-9 (no. 9504, Cell Signaling Technology), and anti-actin (Chemicon). The dilution for all of the primary antibodies for immunoblot analysis was 1:1,000. For co-immunoprecipitation, cells were lysed in 0.2% NP40, 140 mM KCl, 5 mM MgCl2, 1 mM EGTA, 20 mM HEPES at pH 7.5 or 1% CHAPS buffer (1% CHAPS, 142.5 mM KCl, 2 mM CaCl2, 20 mM Tris-Cl, pH 7.4) supplemented with complete protease inhibitors (Roche). Cell lysates were immunoprecipitated with anti-HA antibody (12CA5) and analysed by 10% PAGE (Life Technologies) and immunoblots using the indicated antibodies. To assess BAK cleavage by limited trypsin proteolysis, 5 cells (5 x 10⁶) were suspended in 10 μl of PBS containing 0.02% digitonin and 30 μg/ml trypsin on ice for 30 min. Following quenching of protease activity with 100 μl of PBS containing 0.02% digitonin and 30 μg/ml trypsin on ice for 30 min. Following quenching of protease activity with 100 μg/ml soybean trypsin inhibitor (Sigma), samples were analysed by anti-BAX (G23) immunoblots.

Reverse transcription and quantitative PCR. Reverse transcription and quantitative PCR. Total RNA was extracted from cells using TRIzol (Life Technologies) according to the manufacture's instructions. Reverse transcription was performed with oligo-dT plus random decamer primers (Ambion) using Superscript II (Life Technologies). Quantitative PCR was performed with SYBR green master mix (Applied Biosystems) in duplicates using the indicated primer sequence.
gene-specific primers. Quantitative PCR was performed on a Viia 7 Real-Time PCR System (Applied Biosystems). Data were analysed as described previously by normalization against GAPDH or 18S rRNA (ref. 55). The primers for quantitative PCR are as follows:

- **Noxa**, 5'-CCAGATTGGGGACCTTAGTCTCC-3' and 5'-AGTTGAGCACACTCGTCCTTCAAG-3';
- **Bmf**, 5'-CTTGTGGGGTGAGGCTTCAAG-3';
- **Bik**, 5'-GAGAGACGTGGACCTCATGGAG-3' and 5'-TGAGGCTTCGAATCAAGCTCCTG-3'; and
- **Hrk**, 5'-GTACTGCTGCAAGGAGAGGAG-3'.

**Liposomal release assay.** Liposomes were prepared, and release assays were performed as previously described32,56. Liposomal composition reflects a mixture of the following molar percentages of lipids (Avanti Polar Lipids): phosphatidylcholine, 48%; phosphatidylethanolamine, 28%; phosphatidylinositol, 10%; dioleoyl phosphatidylserine, 10%; and tetraoleoyl cardiolipin, 4%. Aliquots of mixed lipids (1 mg total) are stored in glass at −20 °C under nitrogen, and before use, resuspended in liposome assay buffer (10 mM HEPES, 200 mM KCl, and 1 mM MgCl₂ (pH 7)) containing 12.5 mM fluorescent dye ANTS (8-aminonaphthalene-1,3,6-trisulphonic acid, disodium salt) and 45 mM quencher DPX (p-xylene-bis-pyridinium bromide). The resulting slurry is vortexed for 10 min and freeze-thawed five times in liquid nitrogen and a 40 °C water bath, respectively. The solution is then passed through an Avanti Mini-Extruder Set (no. 610000) equipped with a 100 nm filter, followed by passage through a Sepharose column (GE Healthcare) to remove residual ANTS/DPX. The liposomes are brought up to a volume of 3 ml to produce a final liposome stock. For the liposomal release assay, recombinant BAX (400 nM) is combined with IVTT tBID, wild-type NOXA or BH3 mutant NOXA at the indicated protein/ligand ratios in a 96-well black flat-bottom plate (Costar) and then 10 μl of liposomes is added to a final volume of 100 μl in liposome assay buffer. Liposomal release is quantified on the basis of the increase in fluorescence that occurs when the ANTS fluorophore is separated from the DPX quencher on release from the liposomes into the supernatant. Fluorescence intensity (λex = 355 nm and λem = 520 nm) is measured over time at 32 °C using a Tecan Infinite M1000 plate reader. Fluorescence measurements are recorded each minute until the release measurements plateau, at which point the liposomes are quenched with 0.2% Triton X-100 (100% release). Maximal ligand-induced BAX-mediated release was determined by treating liposomes with a mixture of tBID and BAX (400 nM). The percentage release of ANTS/DPX was calculated according to the equation 

\[
\frac{T_F - F_0}{F_{100} - F_0} \times 100
\]

where \(F_0\) and \(F_{100}\) are baseline and maximal fluorescence, respectively.

54. Takeuchi, O. et al. Essential role of BAX, BAK in B cell homeostasis and prevention of autoimmune disease. Proc. Natl Acad. Sci. USA 102, 11272–11277 (2005).
55. Takeda, S. et al. Proteolysis of MLL family proteins is essential for taspase1-orchestrated cell cycle progression. Genes Dev. 20, 2397–2409 (2006).
56. Yethon, J. A., Epand, R. F., Leber, B., Epand, R. M. & Andrews, D. W. Interaction with a membrane surface triggers a reversible conformational change in Bax normally associated with induction of apoptosis. J. Biol. Chem. 278, 48935–48941 (2003).
Supplementary Figure 1  Triple deletion of Bid, Bim, and Puma fails to completely block intrinsic apoptosis in mouse embryonic fibroblasts. E1A/Ras-transformed wild-type (WT), Bid<sup>−/−</sup>Bim<sup>−/−</sup>, Bim<sup>−/−</sup>Puma<sup>−/−</sup>, or Bid<sup>−/−</sup>Bim<sup>−/−</sup>Puma<sup>−/−</sup> MEFs were untreated, or cultured in the absence of serum or glucose, or in the presence of tunicamycin, thapsigargin or etoposide. Cell death was quantified by annexin-V staining at the indicated times (mean ± s.d., n = 3 independent experiments). *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Student’s t-test).
**Supplementary Figure 2** (a) Reticulocyte lysates abrogate the cytochrome c releasing activity of NOXA. Isolated wild-type mitochondria were incubated with IVTT mouse NOXA generated using wheat germ extract (WGE) in the absence or presence of reticulocyte lysates (R) or IVTT mouse NOXA generated using reticulocyte lysates (R) in the absence or presence of wheat germ extract (WGE) at 30 °C for 30 min, after which the release of cytochrome c was quantified by ELISA assays (mean ± s.d., n = 3 independent experiments). (b) Isolated wild-type mitochondria were incubated with IVTT mouse tBID generated using wheat germ extract (WGE) in the absence or presence of reticulocyte lysates (R) at 30 °C for 30 min, after which the release of cytochrome c was quantified by ELISA assays (mean ± s.d., n = 3 independent experiments). (c) The expression of Noxa mRNA is comparable in wild-type and Bid−/−Bim−/−Puma−/− MEFs. The mRNA levels of Noxa in primary wild-type or Bid−/−Bim−/−Puma−/− MEFs were assessed by qRT-PCR. Data were normalized against GAPDH (mean ± s.d., n = 3 independent experiments). (d) Bid−/−Bim−/−Puma−/− TKO and Bid−/−Bim−/−Puma−/−Noxa−/− QKO mice display persistence of interdigital webs. Ventral views of paws from WT, Bid−/−Bim−/−Puma−/− TKO, and Bid−/−Bim−/−Puma−/−Noxa−/− QKO mice at 6 to 8 weeks of age. Representative images from at least 20 mice are shown. (e) Bid−/−Bim−/−Puma−/−Noxa−/− QKO mice fail to develop external vaginal introituses. Photographs of vaginal openings from WT, Bid−/−Bim−/−Puma−/− TKO, and Bid−/−Bim−/−Puma−/−Noxa−/− QKO mice at 6 to 8 weeks of age. Arrows point to external vaginal region. Representative images from at least 6 mice are shown. (f) Noxa expression is lower both at the basal level and in response to genotoxic stress in T-cells than transformed mouse embryonic fibroblasts. The mRNA levels of Noxa in the indicated cells untreated or treated with etoposide for 6 h were assessed by qRT-PCR. Data were normalized against 18S rRNA (mean ± s.d., n = 3 independent experiments). **, P < 0.01 (Student’s t-test).
Supplementary Figure 3: Total body irradiation-induced apoptosis in spleen was greatly reduced in both Bid−/−Bim−/−Puma−/− and Bid−/−Bim−/−Puma−/−Noxa−/− mice. (a) Apoptosis in the spleens of wild-type, Bid−/−Bim−/−Puma−/− TKO, or Bid−/−Bim−/−Puma−/−Noxa−/− QKO mice at 8 to 17 weeks of age at 4 h after 18 Gy total body irradiation was assessed by TUNEL staining. Representative light microscopy images from two independent experiments are shown (brown, magnification 200x). Scale bars, 50 μm. (b) Wild-type, Bid−/−Bim−/−Puma−/− TKO, or Bid−/−Bim−/−Puma−/−Noxa−/− QKO mice at 8 to 10 weeks of age were unirradiated (n = 3 for each genotype) or irradiated with 5 Gy total body irradiation (n = 3 for each genotype). 3 days later, total numbers of splenocytes and CD4+ splenocytes were assessed. The percentage of survival was defined as the numbers of viable cells from irradiated mice divided by those from unirradiated ones with the same genotypes (mean ± s.d., n = 3 for each genotype). **, P < 0.01 (Student’s t-test).
Supplementary Figure 4 (a) Immunoblot analyses of siRNA- or shRNA-mediated knockdown. SV40-transformed Bid<sup>−/−</sup>Bim<sup>−/−</sup>Puma<sup>−/−</sup>Noxa<sup>−/−</sup> MEFs infected with retrovirus expressing shRNA against the indicated genes, or transfected with the indicated siRNA, were harvested at 72 h later and analyzed with immunoblots using the indicated antibodies. SV40-transformed Bid<sup>−/−</sup>Bim<sup>−/−</sup>Puma<sup>−/−</sup>Noxa<sup>−/−</sup> MEFs were sequentially infected with retrovirus expressing HA-tagged BIK or BMF and retrovirus expressing shRNA against luciferase, Bik, or Bmf. The expression of HA-tagged BIK or BMF was assessed by an anti-HA immunoblot. (b) SV40-transformed Bid<sup>−/−</sup>Bim<sup>−/−</sup>Puma<sup>−/−</sup>Noxa<sup>−/−</sup> MEFs infected with retrovirus expressing shRNA against luciferase, Bik, or Bmf, were harvested at 72 h later. The mRNA levels of Bmf or Bik were assessed by qRT-PCR. Data were normalized against GAPDH (mean ± s.d., n = 3 independent experiments). (c) SV40-transformed Bid<sup>−/−</sup>Bim<sup>−/−</sup>Puma<sup>−/−</sup>Noxa<sup>−/−</sup> MEFs transfected with scrambled siRNA (siScr) or siRNA against Hrk were harvested at 72 h later. The mRNA levels of Hrk were assessed by qRT-PCR. Data were normalized against GAPDH (mean ± s.d., n = 3 independent experiments). (d) SV40-transformed Bid<sup>−/−</sup>Bim<sup>−/−</sup>Puma<sup>−/−</sup>Noxa<sup>−/−</sup> MEFs transfected with scrambled siRNA (siScr) or siRNA against Hrk were untreated or treated with etoposide for 2 days. Cell death was quantified by annexin-V staining (mean ± s.d., n = 3 independent experiments). **, P < 0.01; ***, P < 0.001 (Student’s t-test).
Supplementary Figure 5  (a) SV40-transformed wild-type MEFs, untreated or treated with etoposide, tunicamycin (TC), or thapsigargin (TG), were subjected to immunoblot analysis using the indicated antibodies. (b) CD4+ T cells purified from the spleens of two wild-type mice were untreated or treated with etoposide for 18 h, and subjected to immunoblot analysis using the indicated antibodies. (c) SV40-transformed Bid−/−Bim−/−Puma−/−Noxa−/− QKO MEFs, untreated or treated with etoposide, or irradiated with 20 Gy γ-irradiation or UV-C, were subjected to immunoblot analysis using the indicated antibodies. (d) SV40-transformed wild-type, Bid−/−Bim−/− Puma−/− TKO, Bid−/−Bim−/−Puma−/−Noxa−/− QKO, or Bax−/−Bak−/− DKO MEFs were untreated or irradiated with 20 Gy γ-irradiation. Cell death was quantified by annexin-V staining at the indicated times (mean ± s.d., n = 3 independent experiments). (e) Knockdown of Bcl-2, Bcl-xL, or Mcl-1 does not sensitize QKO cells to overexpression of BAD, BMF, BIK or HRK. SV40-transformed Bid−/−Bim−/−Puma−/−Noxa−/− QKO MEFs were transfected with scrambled siRNA (siScr) or siRNA against Bcl-2, Bcl-xL, or Mcl-1. After 2 days, cells were infected with retrovirus expressing GFP or the indicated BH3-only proteins. Cell death was quantified by annexin-V staining at 30 h (mean ± s.d., n = 3 independent experiments). (f) SV40-transformed wild-type or Bid−/−Bim−/−Puma−/− QKO MEFs stably expressing GFP, HA-BCL-2, HA-BCL-XL or HA-MCL-1 were subjected to anti-HA immunoprecipitation in 0.2% NP-40 lysis buffer. The input (5%) and immunoprecipitates were analyzed by anti-BAX, anti-BAK, and anti-HA immunoblots. **, P < 0.01; ***, P < 0.001 (Student’s t-test).
Supplementary Figure 6 A schematic depicts activation of BAX and BAK upon DNA damage.
Supplementary Figure 7  Full scans of immunoblots. In some experiments, membranes were cut prior to probing each strip with a separate antibody.
| Genotype                        | Persistent Interdigital Webs | Imperforate Vagina |
|--------------------------------|-------------------------------|---------------------|
| $\text{Bid}^{-/-} \text{Bim}^{-/-} \text{Puma}^{+/-}$ | 100% (27/27)                  | 75% (6/8)           |
| $\text{Bid}^{-/-} \text{Bim}^{-/-} \text{Puma}^{-/-} \text{Noxa}^{+/-}$ | 100% (21/21)                  | 83% (10/12)         |

**Supplementary Table 1** $\text{Bid}^{-/-} \text{Bim}^{-/-} \text{Puma}^{+/-}$ and $\text{Bid}^{-/-} \text{Bim}^{-/-} \text{Puma}^{-/-} \text{Noxa}^{+/-}$ mice display persistent interdigital webs and imperforate vagina.