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Editorial

Can the analysis of BH3-only protein knockout mice clarify the issue of 'direct *versus* indirect' activation of Bax and Bak?

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A recent study by Ren *et al.*[1] contributes to the ongoing debate about how interactions between factions of the Bcl-2 protein family provoke apoptosis, but the data presented do not, in our view, support the overall conclusion that 'Bid, Bim and Puma are essential for activation of the Bax- and Bak-dependent cell death program'.

It is generally accepted that, in response to diverse cellular stresses, the Bcl-2 distant cousins termed 'BH3-only proteins', for example, Bim, Bid or Puma, initiate the apoptotic process and that the pivotal step (mitochondrial outer membrane permeabilization) requires the proapoptotic Bcl-2 family members Bak or Bax,[2] but how the BH3-only proteins provoke activation of Bax and Bak remains controversial. The 'direct activation' model[3] posits that Bim, Bid and possibly Puma serve as direct 'activators'. In healthy cells, prosurvival Bcl-2 proteins sequester them, but cytotoxic stimuli upregulate or activate 'sensitizer BH3-only proteins' (Bad, Bik, Hrk, Noxa and Bmf) whose binding to the prosurvival Bcl-2 proteins liberates the 'activators' to transiently engage and activate Bax/Bak. Conversely, the 'indirect model'[4] postulates that in healthy cells a small proportion of Bax and Bak is primed to elicit cell death but sequestered by prosurvival Bcl-2 proteins, and that BH3-only proteins must engage all prosurvival proteins in a given cell to unleash Bax/Bak for death duty. This can either be achieved by Bim, Puma or Bid, which can bind all their prosurvival relatives, or by combinations of BH3-only proteins that bind complementary subsets (e.g., Bad, binding Bcl-2, Bcl-x_L and Bcl-w, plus Noxa, binding Mcl-1 and A1[4,5]). Although biochemical studies have provided conflicting results, the indirect activation model was supported by the observation that Bax/Bak double-deficient (DKO) mice[2] have much more severe developmental and apoptotic defects than mice lacking Bim and Bid,[4] the two most widely accepted 'direct activators' within the Bcl-2 family.

Ren *et al.*[1] generated *Bim/Bid/Puma* triple-deficient (TKO) mice to resolve whether Puma also functions as a 'direct activator' and to clarify the mechanisms of Bax/Bak activation. They report that triple deficiency for Bim, Bid and Puma mirrors Bax/Bak double deficiency and argue that this provides proof for the 'direct activation' model. This is, however, incorrect. First, there are substantial differences in phenotype between *Bim/Bid/Puma* TKO[1] and *Bax/Bak* DKO mice.[2] Although *Bax/Bak* DKO mice die perinatally with severe brain abnormalities, no such profound neurological defects and associated perinatal lethality were reported for the *Bim/Bid/Puma* TKO mice. Furthermore, although some interdigital webbing persisted in *Bim/Bid/Puma* TKO mice,[1] it appears less extensive than in *Bax/Bak* DKO mice[2] or in $Bim^{-/-}Bmf^{-/-}$ mice[6] (AV, PB and VL, unpublished), in which webs persist despite the presence of both Bid and Puma. Moreover, the defect in vaginal development in *Bim/Bid/Puma* TKO mice shows incomplete penetrance,[1] but occurs in 100% of Bax/Bak DKO mice.[2] Thus, in a significant portion of *Bim/Bid/Puma* TKO mice, the physiological cell death driven by Bax and/or Bak continues to some extent in multiple tissues.

The reported *in vitro* cell survival assays also fail to unambiguously demonstrate that all induction of apoptosis requires Bid, Bim or Puma. A proportion of the TKO lymphoid cells still died in response to DNA damage or glucocorticoids,[1] whereas Bax/Bak DKO cells are fully refractory.[2] This difference may indicate that these death stimuli activate additional (i.e., non 'direct activator') BH3-only proteins that collectively can neutralize the prosurvival Bcl-2 proteins in these cells, thereby leading to Bax/Bak activation, consistent with the 'indirect model'. Moreover, the role of Bid in the lymphocyte death probably is negligible: although this study failed to provide data on survival of Bim/Puma DKO lymphocytes, previous studies[7,8] have shown that their combined loss renders multiple hematopoietic cell types as resistant to the apoptotic stimuli studied as reported for the Bim/Bid/Puma TKO cells.[1]

Thus, the phenotype of the TKO mice is less profound than that of Bax/Bak DKO animals and does not prove the direct activation model. As the TKO mice lack the three BH3-only proteins that can neutralize all the prosurvival family members,[5] the observed apoptotic deficiencies are also

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compatible with the 'indirect model'. Nevertheless, increasing *in vitro* findings, for example,[9,10] suggest that certain BH3 domains can directly activate Bax, and a recent *in vivo* study using gene-targeted mice in which the BH3 region of Bim has been subtly altered argues that aspects of both models may well hold.[11] Most of the seemingly conflicting published results can be reconciled if Bax and Bak can be activated in multiple ways: in some circumstances by Bid, Bim or Puma, but also, albeit perhaps less efficiently, by certain other BH3-only proteins,[12] or by mechanisms independent of BH3-only proteins,[3] such as by Bax phosphorylation, heat-induced conformational change, or spontaneous activation after the neutralization or degradation of the restraining prosurvival Bcl-2 proteins, as seen in platelets. As small molecules that target prosurvival Bcl-2 proteins are showing great clinical promise, it will be essential to understand these mechanisms to achieve optimal killing of tumor cells.

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