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News and Commentary

Pro-apoptotic BH3-only Bcl-2 family members in vertebrate model organisms suitable for genetic experimentation

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Genetic and biochemical studies in mammals and *C. elegans* indicate that pro-apoptotic BH3-only members of the Bcl-2 protein family are essential initiators of developmentally programmed cell death and stress-induced apoptosis.[1] Loss-of-function mutations in *egl-1*, the only BH3-only gene so far discovered in *C. elegans* prevent all the programmed deaths of somatic cells that occur during worm development.[2] EGL-1 triggers cell death by binding to and antagonizing the *C. elegans* Bcl-2 homolog CED-9, which conveys survival by preventing the adaptor protein CED-4 from activating the caspase CED-3.[3] At least 8 BH3-only genes have been identified in mice and humans: *bik/blk/nbk, bad, hrk/dp5, bid, bim/bod, noxa, puma/bbc3* and *bmf*.[1] Gene targeting experiments in mice have shown that different BH3-only proteins have specific physiological roles. For example, Bim is essential for apoptosis of lymphocytes deprived of cytokines[4] or that express autoreactive antigen receptors,[5] whereas Bid is activated by death receptor signaling in hepatocytes.[6]

Additional insight into the developmental roles of BH3-only proteins could emerge from studies of their counterparts in other model vertebrates. Well-studied examples of programmed cell death in non-mammalian vertebrates include apoptosis in the developing zebrafish embryo (*Danio rerio*),[7] morphogenesis in frogs (*Xenopus laevis*)[8,9] and neural development in the chick (*Gallus gallus*). These classic developmental systems have recently become much more tractable for genetic analysis through new powerful techniques such as modified antisense oligonucleotides or (in zebrafish) through identification of existing mutants using radiation hybrid maps. Homologs of pro-survival and Bax/Bak-like Bcl-2 family members from these organisms have been described: Bcl-x_L, Mcl-1 and Bax in zebrafish,[10–12] Bcl-x_L and Bax in *Xenopus*[13,14] and Bcl-2 and Bok in chicks.[15,16] Of the BH3-only genes, however, only Bad has been reported in non-mammalian vertebrates, with an EST in zebrafish and a protein reactive to an anti-Bad antibody in chicks.[12,17] We show here that a number of the mammalian BH3-only proteins have clear homologs in the model vertebrates.

To identify orthologs of mammalian BH3-only genes, we trawled the non-redundant and EST (non-mouse/non-human) Genbank databases, dynamically translated into all 6 reading frames (tblastn).[18] Table 1 shows that, of all the mammalian BH3-only genes, only *bik/nbk/blk* (see below), *hrk/dp5* and *puma/bbc3* could not be found in non-mammalian species. Because Hrk/DP5 and Puma/Bbc3 are only expressed at a very low level until cells receive certain cytotoxic stimuli,[19–22] our failure to detect orthologs of them probably simply reflects insufficient EST's having been generated from tissues of non-mammalian organisms exposed to the relevant stimuli.

Blk appears to be the mouse homolog of human Bik/Nbk, because we found no sequences in the human EST or genomic databases more similar to Blk than Bik/Nbk or vice versa. While most human BH3-only proteins (e.g. Bid, Bmf) are 60–99% identical to their mammalian orthologs, the sequence identity between human and other mammalian Bik/Nbk/Blk proteins was considerably lower (rat 42.9, mouse 41.7, bovine 40.3%). This poor conservation may explain why we found no Bik/Nbk/Blk orthologs in non-mammalian vertebrates.

The Bid gene proved the most highly represented gene in the database, with orthologs in two species of fish (*D. rerio* and *O. latipes*), eel (*A. japonica*), frog (*X. laevis*), chick (*G. gallus*) as well as many mammals (mouse, rat, rabbit (not shown), cow, pig, and humans). Bad, Bmf and Noxa could all be found as far back as zebrafish, while the Bim_{EL} isotype was represented in frogs (Table 1).

A curious feature of murine Noxa is the presence of two BH3 regions, whereas Noxa from human, cow, swine, frog and fish (like all other known BH3-only proteins) contains a single BH3 domain (Figure 1).[23] Detailed inspection of the human and murine Noxa proteins and genomic sequences suggests that the two BH3 regions in mouse may have arisen by a tandem duplication and fusion of the entire ancestral *noxa* gene (two exons and intervening intron) such that it remains in frame with itself, effectively producing a fusion protein comprised of two nearly identical *noxa* open reading frames. It will be interesting to see if this duplication occurred only in the mouse or in a common ancestor of all present day rodents, and to learn whether it affects Noxa function.

The pro-apoptotic activity of BH3-only proteins needs to be tightly regulated.[1] As mentioned above, some BH3-only genes are subject to stringent transcriptional control, and it will be interesting to see whether their regulation has been conserved. Mammalian *noxa*[23] and *puma/bbc-3*[20–22] are both induced by the p53 tumor suppressor protein and therefore may be critical initiators of DNA damage-induced apoptosis. Moreover, *hrk/dp5* and *bim* can be transcriptionally induced by AP-1,[24,25] and *bim* also appears to be regulated by the Forkhead-related transcription factor FKHR-L1.[26] It will be informative to see whether the relevant transcription factor binding sites are conserved

Table 1 BH3-only genes in diverse vertebrates. The orthologs of known mammalian BH3-only genes were found by tblastn screens of the non-redundant and EST (non-mouse/non-human) genbank databases. Genbank accession numbers for genes and ESTs are supplied below

	Species							
	Fish[a]	Frog[b]	Aves[c]	Mouse[d]	Rat[e]	Bovine[f]	Swine[g]	Human[h]
Bmf	✔	✔		✔				✔
Bim/Bod		✔		✔	✔			✔
Bid	✔	✔	✔	✔	✔	✔	✔	✔
Bad	✔			✔	✔	✔	✔	✔
Bik/Blk/Nbk				✔	✔	✔		✔
Hrk/DP5				✔	✔			✔
Noxa	✔	✔		✔		✔	✔	✔
Puma/bbc3				✔		✔		✔

[a]*D. rerio* (Bmf BI891121; Bid BM036570; Bad BI868155, BI984603, AF231017, AI332008, AW594979, AW595186; Noxa BM403263, BE557856), *O. latipes* (Bid BJ012077), *A. japonica* (Bid C24490). [b]*X. laevis* (Bmf BJ095470, BJ032144, BE131862, BJ099303; Bim AF209718; Bid AW641666, BF048324, BF049428; Noxa BG364855, BG407194), *S. tropicalis* (Bmf AL655017). [c]*G. gallus* (Bid BM439781, BM486711, AL586805, BG710723, BG712566, BG712567). [d]*M. musculus* (Bmf [34]; Bim [38]; Bid : [39]; Bad [40]; Blk [41]; Hrk/DP5 [19]; Noxa [23]; Puma/Bbc3 [20–22]). [e]*R. norvegicus* (Bim [42]; Bid AF259503; Bad AF003523; Blk NM_053704, AF372501; Hrk/DP5 [19]). [f]*B. taurus* (Bid BF652109, BM286424, BM286634, BM364680, AV597074, AV603029, AW353187, BF603154, BI540539; Bad BE666665, BE753767, BF043486, BF075900; Bik/Blk AW484752; Noxa BF075905; Puma/Bbc3 BF890319, BE665333). [g]*S. scrofa* (Bid AW312919, AW417419, BG895060, BM484509; Bad AW619074, BF710370, BM484664, BF441436; Noxa AF319660). [h]*H. sapiens* (Bmf [34]; Bim [38]; Bid [39]; Bad [40]; Bik/Blk [43,44]; Hrk/DP5 [45]; Noxa [23]; Puma/Bbc3 [20–22])

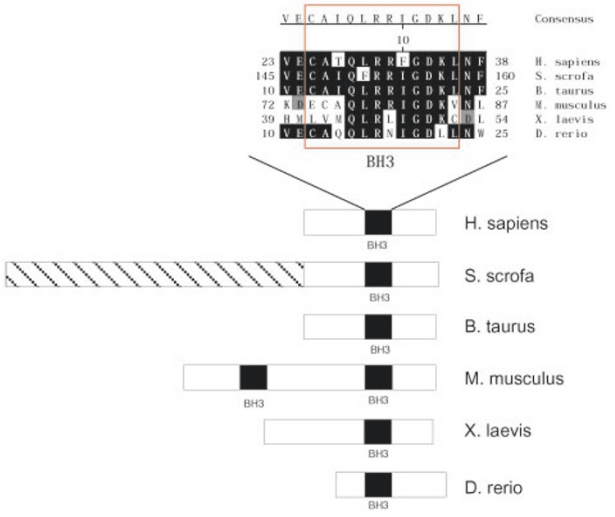

Figure 1 Comparison of *noxa* genes from multiple species. Schematic representation of alignments of Noxa proteins and of Noxa BH3 region sequences (boxed in red), performed with the clustal alignment program. Residues identical to the consensus are highlighted black and functionally related residues grey. The hatched portion of *S. scrofa* Noxa is suspected to be a cloning artifact. Genbank entries and references for each species are listed in Table 1

between the mammalian genes and their orthologs from lower vertebrates as their genomes are solved.

The pro-apoptotic activity of mammalian BH3-only proteins can also be regulated post-translationally through phosphorylation, sequestration or proteolysis.[1] For example, the full length Bid found in healthy cells has low activity, but its cleavage by caspase-8 following ligation of cell surface death receptors (e.g. Fas/APO-1/CD95) liberates a p15 BH3 domain-containing fragment[27,28] with an exposed myristoylation site[29] that has enhanced targeting to mitochondrial membranes and increased apoptotic potency. Interestingly, sequence alignment of all the Bid species reveals conservation not only of the BH3-region but also of the caspase-8 cleavage site (LQTD) (Figure 2). Due to the flexibility in the consensus sequence recognized by myristoyl transferases,[30] we cannot unambiguously deduce from the sequences alone whether the myristoylation site is also conserved. Bid is also reportedly proteolytically processed by calpain and granzymeB and it has been postulated that Bid cleavage by granzyme B plays a critical role in target cell killing by cytotoxic T cells.[31,32] Outside of mammals, however, the cleavage site for each of these proteases appears to be poorly conserved, perhaps indicating that processing by these enzymes is not a critical feature of Bid.

Bad is sequestered by 14-3-3 scaffold proteins after its phosphorylation by AKT/PKB and perhaps other kinases. Sequence alignments showed that not only the BH3-region but also both Akt/PKB phosphorylation sites of Bad (S115 and S136 in mouse) are conserved through to zebrafish, as is the serine reportedly phosphorylated at residue 170 and that within the BH3 itself (S155) (Figure 2).

Bim and Bmf are regulated by sequestration to the cytoskeleton: Bim is carried to the microtubular dynein motor complex by dynein light chain DLC-1[33] and Bmf to the actin-based myosin V motor complex by DLC-2.[34] Significantly, both the BH3 domain and the dynein light chain binding motif of Bmf are conserved in all species (Figure 2).

Collectively, these observations indicate that not only the killing activity but also the post-translational control mechanisms of BH3-only proteins probably are evolutionarily conserved.

The data reported here indicate that at least half of the mammalian BH3-only genes have orthologs in model organisms suitable for genetic analysis, and others will almost certainly be discovered as more sequences are deposited, preparing the way for the study of their roles in development. It is noteworthy that experiments with Bim-deficient mice have implicated inappropriate activation of the apoptotic program by BH3-only proteins in degenerative disease.[35] Hence, dysregulation of expression of the BH3-only orthologs described here could provide useful animal models of degenerative disease. Mutagenesis screens in zebrafish have already produced many mutant phenotypes

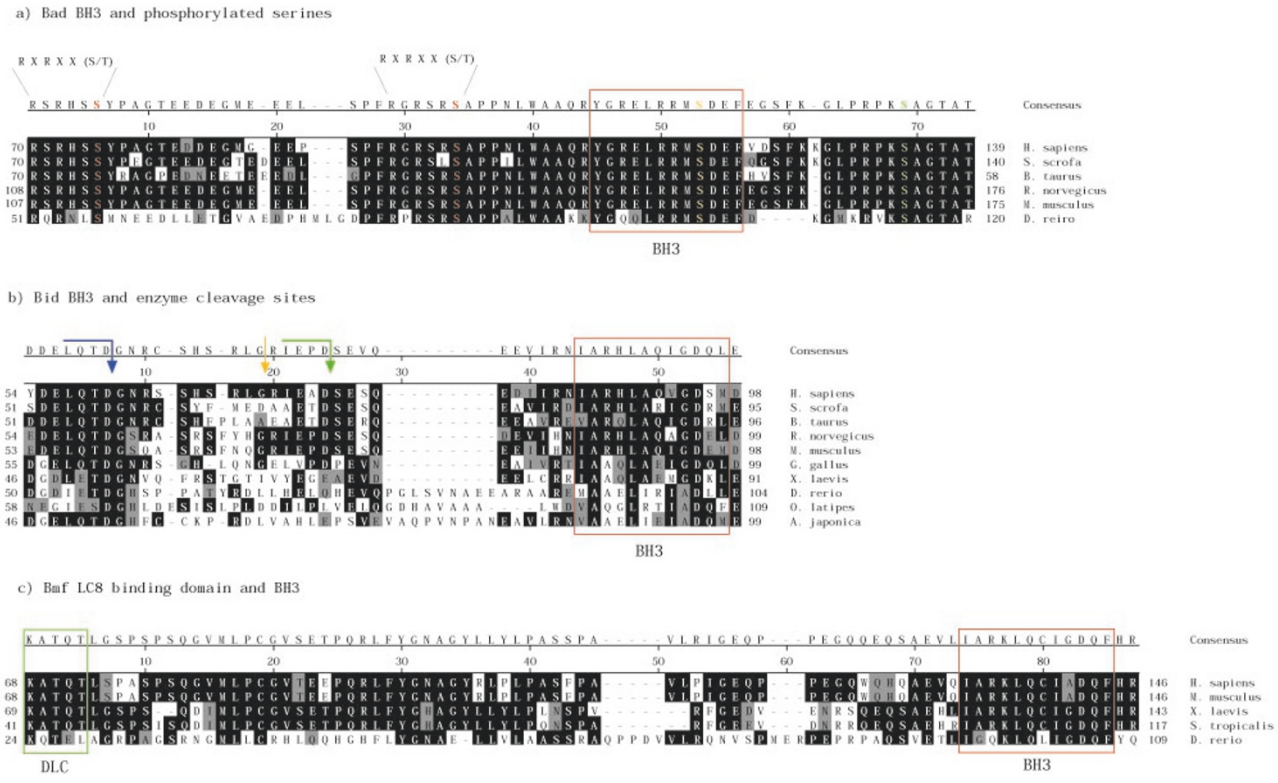

Figure 2 Alignments of selected BH3-only genes. BH3 regions (boxed red) for Bad, Bid and Bmf proteins from each species identified were aligned using the clustal alignment program. Residues identical to the consensus are highlighted black and functionally related residues grey. Akt/PKB phosphorylation site serines in Bad are shown in red and the consensus Akt/PKB sequence (RXRXX(S/T)) is indicated above each site. The phosphorylated serine 155 in the Bad BH3 is in yellow, phosphorylated serine 170 is in green. The blue arrow in Bid indicates the caspase-8 cleavage site (LQTD), the green arrow the granzyme B cleavage site (IEPD) and the yellow arrow the calpain cleavage site. The dynein light chain binding motif (DLC) of Bmf is boxed in green. Genbank entries and references for each species are listed in Table 1

resembling those of human diseases and can be used as model organisms for the study of cancer.[36,37] Thus, the identification of BH3-only genes in model vertebrates provides a valuable genetic tool with which to examine their roles in both development and disease.

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