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Functional characterization of the *Bcl-2* gene family in the zebrafish

E Kratz[1], PM Eimon[1], K Mukhyala[2], H Stern[3], J Zha[3], A Strasser[4], R Hart[2] and A Ashkenazi*[,1]

[1] Department of Molecular Oncology, Genentech Inc., South San Francisco, CA, USA
[2] Department of Bioinformatics, Genentech Inc., South San Francisco, CA, USA
[3] Department of Pathology, Genentech Inc., South San Francisco, CA, USA
[4] The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia
* Corresponding author: A Ashkenazi, Department of Molecular Oncology, Genentech Inc., 1 DNA Way, MS 42, South San Francisco, CA 94080, USA. Tel: 650-225-1853; Fax: 650-467-8195; E-mail: aa@gene.com

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Abstract

Members of the Bcl-2 protein family control the intrinsic apoptosis pathway. To evaluate the importance of this family in vertebrate development, we investigated it in the zebrafish (*Danio rerio*). We found that the zebrafish genome encodes structural and functional homologs of most mammalian *Bcl-2* family members, including multi-Bcl-2-homology (BH) domain proteins and BH3-only proteins. Apoptosis induction by γ-irradiation required *zBax1* and *zPuma*, and could be prevented by overexpression of homologs of prosurvival *Bcl-2* family members. Surprisingly, zebrafish *Bax2* (*zBax2*) was homologous to mammalian *Bax* by sequence and synteny, yet demonstrated functional conservation with human *Bak*. Morpholino knockdown of both *zMcl-1a* and *zMcl-1b* revealed their critical role in early embryonic zebrafish development, and in the modulation of apoptosis activation through the extrinsic pathway. These data indicate substantial functional similarity between zebrafish and mammalian *Bcl-2* family members, and establish the zebrafish as a relevant model for studying the intrinsic apoptosis pathway.

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Abbreviations: BH, Bcl-2-homology; DL, death ligand; MO, morpholino

Introduction

Members of the *Bcl-2* gene family are critical mediators of the delicate balance between survival and apoptosis in eucaryotic cells.[1] Dysregulation of this signaling pathway can result in a myriad of diseases, including cancer, autoimmune diseases and degenerative disorders.[2,3] The *Bcl-2* family consists of three classes of proteins: prosurvival (including Bcl-2, Bcl-x_L, Mcl-1, A1, Boo/DIVA, Bcl-B, and Bcl-w), multidomain proapoptotic (including Bax, Bak, and Bok), and proapoptotic BH3 only (Bid, Bad, Bmf, Bik, Puma, Noxa, Hrk, and BNip3).[4] In unstimulated cells, interactions between prosurvival and proapoptotic multidomain family members prevent the latter from oligomerizing and initiating the apoptotic program.[5] Upon apoptotic stimulation, BH3-only proteins relieve the inhibition of multidomain proapoptotic proteins, freeing them to initiate apoptosis.[6,7] The subsequent release of proapoptotic factors from the mitochondria results in the stimulation of caspase-9, and cleavage and activation of effector caspases such as caspase-3, -6, and -7.

Previous experiments demonstrate that several members of the *Bcl-2* family are required for normal development; however, the function of this protein family during development is largely unknown. Simultaneous knockout of *Bax* and *Bak* results in drastically decreased perinatal survival, with fewer than 10% of the double-knockout mice surviving to adulthood. These mice are born in normal Mendelian ratios, but are neglected by the mother and likely die from failure to nurse,[8] perhaps as a result of massive inappropriate neuronal accumulation. *Bcl-x* knockouts reveal a critical role for *Bcl-x_L* in the survival of developing neuronal and hematopoietic cell types,[9] whereas the *Bcl-2* knockout has only moderate effects in adult mice.[10–12] Knockout of *Mcl-1* results in preimplantation lethality.[13] While these experiments clearly indicate important roles for *Bcl-2* family members in early development, in each of these knockouts neither the initiating apoptotic signal nor the activated BH3-only proteins are known.

We have therefore characterized the complement of Bcl-2 family proteins in zebrafish. This genetically tractable model organism offers the unique opportunity to elucidate the role of the *Bcl-2* genes during embryogenesis *ex utero*. While the existence of several *Bcl-2* family members in zebrafish has been reported,[14,15] little is known about the functional homology between zebrafish and mammalian *Bcl-2* family members. Here, we report that the zebrafish genome contains full-length, functional homologs of most *Bcl-2* family members. Overexpression of homologs of most mammalian multidomain or BH3-only proapoptotic genes results in apoptosis. Coexpression of homologs of mammalian prosurvival *Bcl-2* family members can rescue this death. Furthermore, we show that DNA damage induced by γ-irradiation leads to a massive, *p53*-dependent upregulation of *zPuma*. Induction of the apoptotic response to γ-irradiation is dependent on zebrafish homologs of mammalian *Bax*, and ectopic expression of any zebrafish homolog of mammalian prosurvival *Bcl-2* family member is sufficient to prevent irradiation-induced apoptosis. In addition, we show that endogenous *zMcl-1a* and *zMcl-1b*, but not the *Bcl-2* homolog *zBlp2*, play an important role in maintaining the health of early zebrafish embryos. Importantly, we also demonstrate that *zMcl-1a* and *zMcl-1b* are specifically required to protect

embryos from death ligand (DL)-induced apoptosis early in development, indicating that the intrinsic pathway confers sensitivity to death receptor-mediated apoptosis. Thus, the zebrafish provides a promising model to investigate the function of *Bcl-2* family members in nontransformed vertebrate tissues and during normal embryogenesis.

Results

Zebrafish genome contains homologs of most mammalian *Bcl-2* family members

We have identified and functionally characterized the complement of full-length zebrafish *Bcl-2* family members, including homologs of mammalian prosurvival *Bcl-x$_L$*, *Bcl-2*, *Mcl-1*, and *Boo/DIVA*; proapoptotic *Bax* and *Bok*; and BH3-only genes *Bid*, *Bad*, *Bmf*, *Noxa*, *Puma*, and *Bik*. Previous reports have described zebrafish homologs to a subset of mammalian *Bcl-2* family genes, including *Bcl-2*, *Bcl-x$_L$*, *Mcl-1*, *NR13*, and *Bad*.[16–19] Furthermore, ESTs bearing homology to proapoptotic *Bcl-2* family members *Bax*, *BNip3*, *Bmf*, *Noxa*, and *Bid* have been identified.[14,15] Here, we present the first comprehensive evaluation of this gene family in zebrafish.

Because of genomic duplication in the teleost lineage,[20] several mammalian *Bcl-2* family genes have two copies in zebrafish, including *Mcl-1*, *Bmf*, *Bok*, and *Bax* (Table 1). Regions sharing conserved synteny with human *Mcl-1* chromosome locus 1q21.2 are present in two zebrafish chromosomes; thus, despite being in separate chromosomal locations, both zebrafish copies of *Mcl-1* have a conserved syntenic relationship with the human counterpart. Similarly, both *zBok1* and *zBok2* share conserved synteny with human *Bok* despite the zebrafish genes mapping to different chromosomes. In contrast, *zBmf2*, but not *zBmf1*, has conserved synteny with its mammalian homolog; *zBax2*, but not *zBax1*, shares conserved synteny with human *Bax*. *zBlp1*, *zNR13*, *zBad*, *zBik*, and *zPuma* all share a conserved syntenic relationship with their human counterparts (Table 1).

Overall, the multidomain *Bcl-2* family members bear the highest degree of homology to their mammalian counterparts (Figure 1a). Alignment of the amino-acid sequence reveals that each of the zebrafish homologs of mammalian prosurvival *Bcl-2* genes cluster most closely with their mammalian homologs, as do zBok1, zBok2, zBax1, and zBax2.

Zebrafish homologs of Noxa, Bad, Bmf, and Bid all cluster with their mammalian counterparts, yet they share only distant identity (Figure 1a). While the overall amino-acid sequence of zebrafish BH3-only genes has diverged significantly from mammalian counterparts, the critical BH3 domain retains a higher degree of homology (Figure 1b), highlighting the functional importance of this domain.

Zebrafish *Bik* and *Puma* are the most divergent of the zebrafish BH3-only genes. However, both share conserved synteny with the human sequence (Table 1), and in the case of zPuma, the critical BH3 domain aligns most closely with the mammalian homolog (Figure 1b). In contrast, the BH3 domain of zBik bears a striking resemblance to the corresponding domain of human and mouse Bid, rather than mammalian Bik (Figure 1b). However, as zBik lacks a predicted caspase cleavage site and is syntenic with human *Bik*, it is clearly the zebrafish ortholog of human *Bik*.

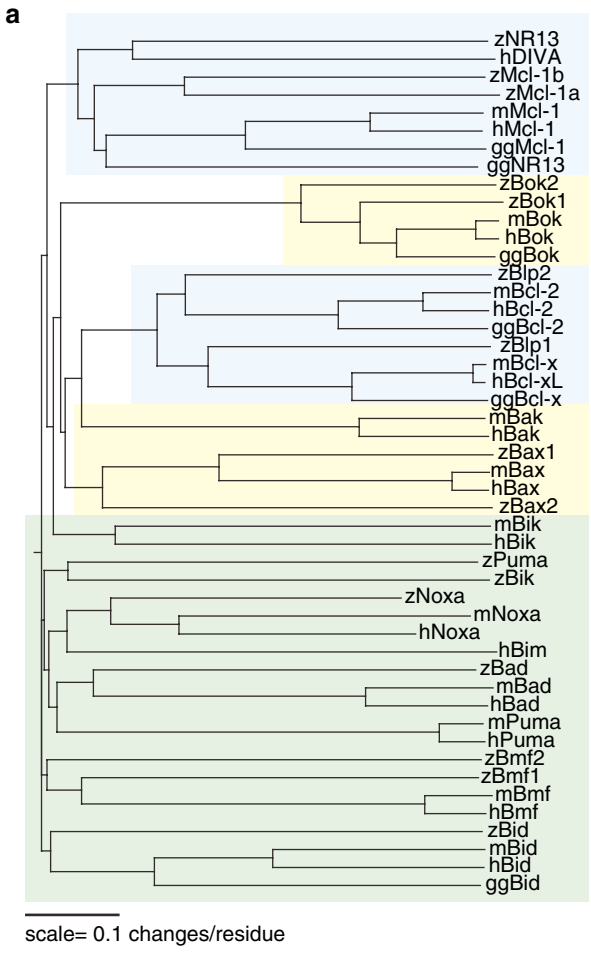
While we initially identified zebrafish *Bcl-2* family genes on the basis of Bcl-2-homology (BH) domain structure, percent homology, and synteny with human homologs, we examined additional sequence features to confirm homolog identity when possible. Alignments between zBad and its human

Table 1 *Bcl-2* family is strongly conserved between zebrafish and human species

Zebrafish gene	Closest mammalian homolog	Percent homology	Accession number	Fish chromosome	Human chromosome	Conserved synteny?
Prosurvival						
zBlp1	Bcl-xL	67	AF317837	5	20q11.21	Yes
zBlp2	Bcl-2	57	NM_001030253.1	24	18q21.33	No
zMcl-1a	Mcl-1	43	NM_131599	19	1q21.2	Yes
zMcl-1b	Mcl-1	38	NM_194394.2	16	1q21.2	Yes
zNR13	Boo/DIVA	30	AF441285	18	15q21.2	Yes
Proapoptotic BH3 only						
zBad	Bad	46 (79)	BC097099.1	7	11q13.1	Yes
zBid	Bid	35 (66)	DQ860155	18	22q11.21	No
zBik	Bik	30 (66)	DQ860154	4	22q13.2	Yes
zBmf1	Bmf	38 (66)	DQ860153	23	15q15.1	No
zBmf2	Bmf	46 (72)	DQ860156	20	15q15.1	Yes
zNoxa	Noxa	38 (66)	DQ860152	19	18q21.32	No
zPuma	Puma	27 (66)	DQ860151	16	19q13.32	Yes
zBim*	Bim	(86)		13	2q13	ND
Proapoptotic multidomain						
zBax1	Bax	73	NM_131562.2	3	19q13.33	No
zBax2	Bax	41	DQ860150	3	19q13.33	Yes
zBok1	Bok	81	NM_001003612.1	6	2q37.3	Yes
zBok2	Bok	74	DQ860157	2	2q37.3	Yes

ND, not determined. Percent homology was determined for the full-length amino-acid sequence. Homology over the BH3 domain alone is given in parentheses for BH3-only proteins. **zBim* is likely a pseudogene (Mukhyala *et al*., manuscript in preparation)

ortholog revealed that residues Ser112 and Ser136 are highly conserved: phosphorylation of these serines in mammalian Bad facilitates binding to 14-3-3, regulating its proapoptotic activity.[21] Similarly to human Bid,[22] zBid has a putative caspase-8 cleavage site, ostensibly allowing generation of a tBid-like fragment. In addition, zBmf1 and zBmf2 contain putative dynein light-chain-binding domains: mammalian Bmf is regulated via dynein light-chain-mediated sequestration to the actin cytoskeleton until activation.[23] These findings suggest that several *Bcl-2* family members may be regulated by similar mechanisms in zebrafish and mammals. Zebrafish homologs of *Bcl-B*, *Bcl-w*, *A1*, and *Hrk* have yet to be identified.



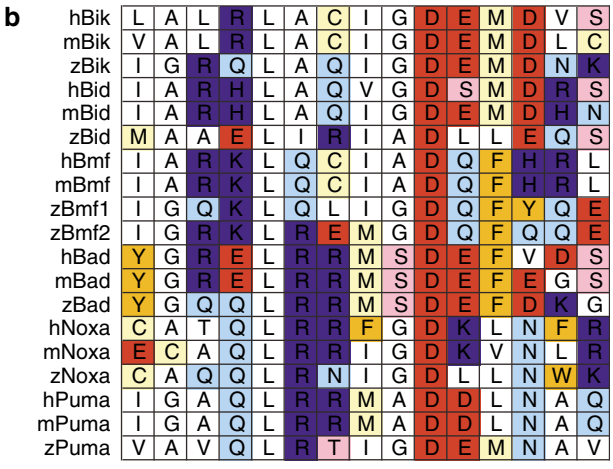


Figure 1 Homologs of the majority of mammalian *Bcl-2* family members are present in zebrafish. (**a**) Zebrafish *Bcl-2* family members are aligned with their human (h), mouse (m), and (where available) chicken (gg) homologs. Prosurvival genes are highlighted in blue, multidomain proapoptotic genes are highlighted in yellow, and BH3-only genes are highlighted in green. (**b**) The BH3 domains of human, mouse, and zebrafish BH3-only proteins are grouped according to gene

Zebrafish *Bcl-2* family members are expressed throughout early embryonic development and in adult tissues

To assess whether any zebrafish *Bcl-2* family members are present during embryonic development, we analyzed their expression by stage-specific RT-PCR (Figure 2a). We detected maternal expression of all of the *Bcl-2* family members, except *zBik* and *zBok1* (1000-cell stage). After the initiation of zygotic transcription, the levels of *zBid*, *zBik*, *zBad*, *zBmf1*, *zNoxa*, *zBax1*, *zMcl-1a*, *zMcl-1b*, *zBlp1*, and *zNR13* were fairly constant. Conversely, the transcription of *zBax2*, *zBmf2*, and *zBlp2* decreased dramatically after the 1000-cell stage before increasing again later in development.

Next, we asked whether the expression of *Bcl-2* family members is tissue specific in adult zebrafish (Figure 2b). The zebrafish ovary was especially rich in *Bcl-2* family members, expressing all genes. In contrast, the liver only weakly expressed *zBid*, *zBax1*, *zMcl-1a*, and *zMcl-1b*. Several of

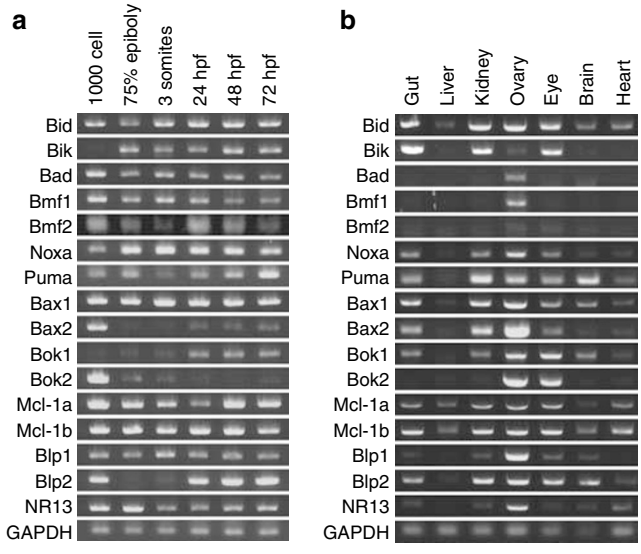


Figure 2 Homologs of mammalian *Bcl-2* family members are expressed in zebrafish. (**a**) Stage-specific RT-PCR reveals that most zebrafish *Bcl-2* family members are expressed at readily detectable levels from 1000-cell stage up until 72 h.p.f. Exceptions are *zBik* and *zBok1*, which are absent maternally and are turned on later in development; *zBax2* and *zBlp2*, which are present maternally and later in development; and *zBok2*, which is strongly expressed maternally but is largely absent later in embryonic development. *GAPDH* is included as a control for the amount of input RNA. (**b**) Zebrafish *Bcl-2* family members are expressed specifically in adult tissues. The ovary is especially rich in *Bcl-2* family members, whereas brain and liver express selected *Bcl-2* family genes at much lower levels. *GAPDH* is included as a control for the amount of input RNA

the zebrafish BH3-only genes, including *zBik*, *zBad*, *zPuma*, and *zBmf1*, showed a remarkably specific expression pattern at the RNA level: for example, *zBik* was highly expressed in the gut, kidney, and eye, but was absent in the liver, brain, and heart, whereas *zPuma* was enriched in brain tissue.

Ectopic expression of zebrafish homologs of mammalian proapoptotic *Bcl-2* family members induces apoptosis

We next sought to determine whether the structural conservation between mammalian proapoptotic *Bcl-2* family members and corresponding zebrafish homologs translated into conservation of *in vivo* function. Injection of mRNA encoding zebrafish homologs of mammalian proapoptotic BH3-only or multi-BH domain *Bcl-2* family members triggered dose-dependent cell death, characterized by disintegration of the blastomeres and yolk cell (Figure 3a and b). Exceptions were *zBad*, *zBok1*, and *zBok2*, which did not induce significant apoptosis in early embryos even at high mRNA doses.

To confirm that these genes induced cell death by engaging the apoptosis pathway, we injected embryos with a minimum lethal dose of each mRNA and analyzed proteolytic activation of effector caspase-3 by immunohistochemistry (Figure 3c). Compared to untreated and mock-injected embryos, overexpression of the death-inducing *Bcl-2* family proteins resulted in a dramatic increase in activated caspase-3. Again, the exceptions were *zBad*, *zBok1*, and *zBok2*, which did not trigger readily detectable caspase-3 activation upon ectopic expression (Figure 3c, and data not shown). It is unclear why these homologs of mammalian proapoptotic genes did not induce death when overexpressed in zebrafish embryos; perhaps the encoded proteins require a specific cellular context (e.g. specific post-translational modification, such as dephosphorylation in the case of Bad) to engage the apoptotic program.

Ectopic expression of zebrafish homologs of mammalian prosurvival *Bcl-2* family members inhibits intrinsic pathway-mediated apoptosis

Apoptosis induced by overexpression of a minimum lethal dose of *zBid*, *zBmf1*, *zBmf2*, *zPuma*, *zNoxa*, or *zBax1* could be rescued by coexpression of *zMcl-1a*, *zMcl-1b*, *zBlp1*, or *zBlp2* (Figure 3d). These data suggest that upon

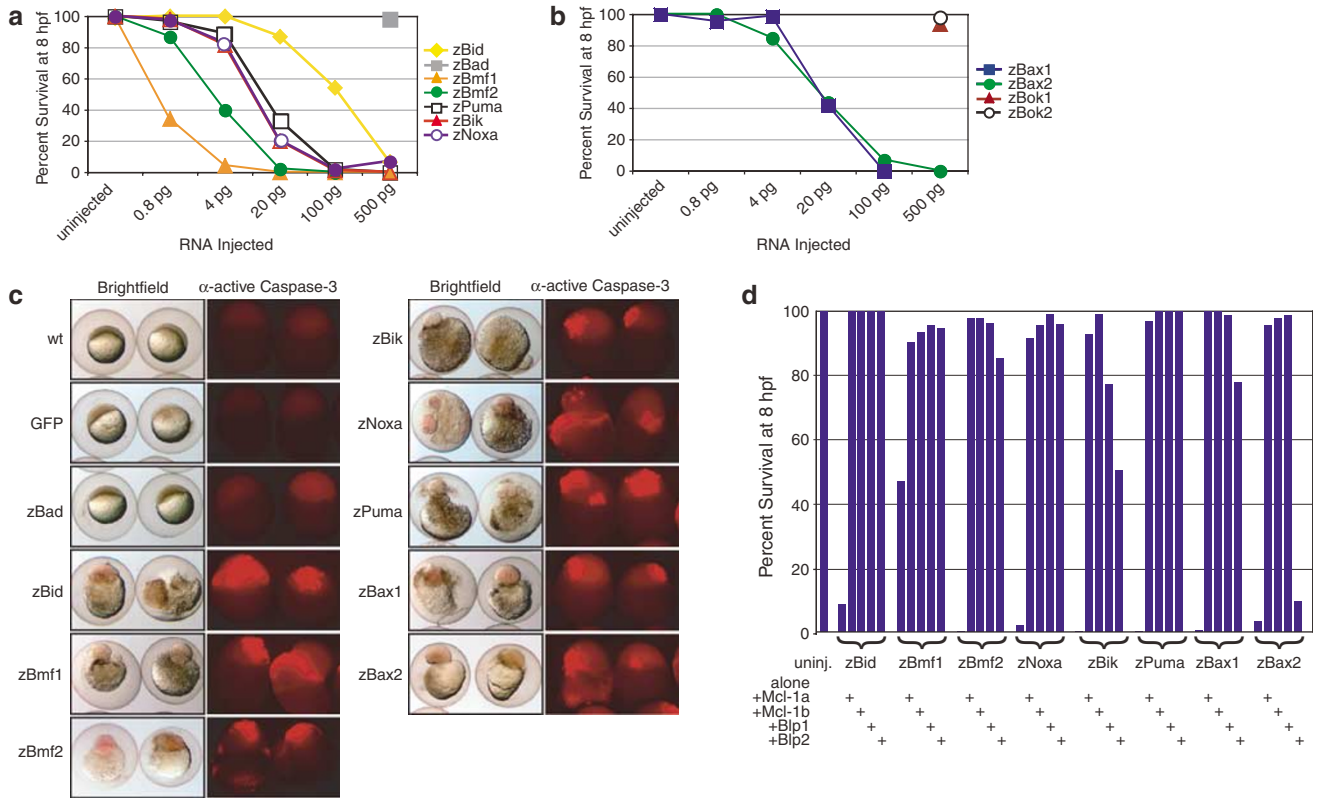


Figure 3 *zBcl-2* family members are functional in embryonic zebrafish. Ectopic expression of BH3-only (**a**) and multi-BH domain (**b**) proapoptotic *zBcl-2* family members induces death in a dose-dependent manner. Data are compiled from a minimum of two independent experiments per data point. (**c**) This death is defined by embryonic morphology at 8 h.p.f. (left panels), and by caspase-3 activation, which was detected by whole-mount immunohistochemistry at 3–5 h.p.f. (right panels). Embryos were injected with 500 pg *GFP* or *zBad* mRNA, or a minimally lethal dose of each proapoptotic mRNA. Each panel is representative of a minimum of two independent experiments. (**d**) Coexpression of prosurvival *zBcl-2* family members inhibits death induced by overexpression of proapoptotic zBcl-2 proteins. Coexpression of *zBlp2* cannot inhibit death induced by *zBax2* overexpression, and can only weakly inhibit apoptosis induced by enforced expression of *zBik*. Five hundred picograms of mRNA of each prosurvival *Bcl-2* family member and a minimally lethal dose of mRNA of each proapoptotic *Bcl-2* family member were injected. Data are compiled from a minimum of two independent experiments per data point

overexpression in zebrafish embryos, the proapoptotic genes induced cell death via a mechanism that resembles the mammalian apoptotic pathway. In contrast to *zBlp1*, *zMcl-1a*, or *zMcl-1b*, overexpressed *zBlp2* failed to inhibit apoptosis triggered by ectopic expression of *zBax2* (Figure 3d). These data suggest that zBax2 does not interact with the zebrafish homolog of mammalian Bcl-2. Similarly, human Bak interacts preferentially with Bcl-x_L and Mcl-1, but not with Bcl-2.[24] These results imply that zBax2, although orthologous to human *Bax*, has evolved similar selectivity to human Bak. Furthermore, *zBlp2* could inhibit apoptosis triggered by overexpression of *zBik* only poorly, indicating that zBik interacts only weakly with zBlp2. Together, these findings suggest functional as well as structural conservation between mammalian and zebrafish *Bcl-2* family genes, and support the existence of selective interactions between certain prosurvival and proapoptotic family members.

γ-Irradiation engages the intrinsic apoptosis pathway in zebrafish

To investigate the role of *Bcl-2* family members in the response to DNA damage, a well-established activator of the intrinsic apoptotic pathway, we subjected embryos to γ-irradiation. Exposure to γ-irradiation induced a substantial increase in activated caspase-3, which was dependent on *p53* (Figure 4a).[25] Ectopic expression of prosurvival *Bcl-2* family members, such as *zMcl-1a*, *zMcl-1b*, *zBlp1*, or *zBlp2*, protected embryos from γ-irradiation-induced apoptosis (Figure 4b). Thus, *Bcl-2* family genes control activation of the intrinsic pathway in response to γ-irradiation in zebrafish as well as in mammals.[26]

In most mammalian cell types, *Bax* and/or *Bak* are necessary and sufficient to trigger apoptosis activation through the intrinsic pathway.[27] To determine whether *zBax1* and *zBax2* are functionally redundant in zebrafish, we injected morpholino oligonucleotides (MOs) directed against *zBax1* and *zBax2* individually or together and then subjected embryos to γ-irradiation (Figure 4c). Knockdown of *zBax1* was sufficient to ameliorate the effects of γ-irradiation in four out of five experiments ($n = \sim 30$ embryos per condition in each experiment, Supplementary Figure 1). In the fifth experiment, knockdown of both *zBax1* and *zBax2* was required to abrogate γ-irradiation-induced caspase-3 activation (data not shown). Some caspase-3 activity remained detectable in the irradiated embryos injected with *zBax1* and *zBax2* MOs: this is probably a consequence of either incomplete knockdown or the function of residual maternal proteins present in the embryo (see Figure 2a). These data suggest that zBax1 plays a more important role than zBax2 in mediating activation of the apoptotic program in response to γ-irradiation, although both proteins support this function.

Next, we examined which BH3-only protein(s) is (are) critical for γ-irradiation-induced apoptosis in zebrafish embryos. MO knockdown of *zBik*, *zBmf1*, *zBid*, or *zNoxa* did not diminish caspase-3 activation in response to γ-irradiation. In contrast, knockdown of *zPuma* attenuated caspase-3 activation as effectively as did knockdown of *p53* (Figure 4d), suggesting that this BH3-only protein is the primary initiator of this apoptotic response. Furthermore, quantitative RT-PCR analysis revealed that while γ-irradiation induced a three- to four-fold upregulation in *zNoxa*, it augmented *zPuma* expression almost 100-fold (Figure 4e). No other BH3-only gene showed increased transcription in response to γ-irradiation (data not shown). These data support previous findings in mammalian cells, indicating that Puma is the principal initiator of γ-irradiation-induced apoptosis.[26,28] Upregulation of *zPuma* was *p53*-dependent, reinforcing the importance of *p53* and its transcriptional link to *zPuma* for apoptosis induction in response to DNA damage. Activation of *zPuma* may in turn lead to the activation of zBax1 and zBax2, an effect that we were able to antagonize by overexpression of *zBlp1*, *zBlp2*, and *zMcl-1a* and *zMcl-1b*.

Knockdown of *zMcl-1a* and *zMcl-1b*, but not *zBlp2*, decreases viability of zebrafish embryos

Having established that the Bcl-2 protein family is present and functional in zebrafish, we examined whether prosurvival family members play similar roles in zebrafish embryogenesis as they do in mice. To this end, we used MOs against *zMcl-1a*, *zMcl-1b*, and *zBlp2*. Knockdown of *zBlp2* had no obvious effect on early zebrafish development. The gross morphology of embryos injected with *zBlp2* MO was normal, and caspase-3 activity showed no detectable increase (data not shown). These data are reminiscent of the *Bcl-2*-knockout mice, which lack an embryonic phenotype,[10] and suggest that the *Bcl-2* homolog is dispensable for normal early embryonic development in vertebrates.

Knockdown of either *zMcl-1a* or *zMcl-1b* alone did not increase embryonic mortality (Figure 5a). In contrast, knockdown of both *zMcl-1a* and *zMcl-1b* resulted in a variable but significant decrease in viability by 8 hpf, suggesting that together the two zMcl-1 proteins are critical for normal embryonic development. These results agree with the knockout of mouse *Mcl-1*, which causes preimplantation lethality.[13]

It is formally possible that the lethality caused by combined knockdown of *zMcl-1a* and *zMcl-1b* is not specific to these genes; rather, knockdown of any two pro-survival genes could affect early zebrafish viability. To examine this possibility, we assessed the effect of pair-wise knockdown of different prosurvival *Bcl-2* family members on embryonic viability (Figure 5a). Combined knockdown of *zMcl-1a* and *zMcl-1b* significantly affected the survival of injected embryos, while knockdown of *zMcl-1a* plus *zBlp2* or *zMcl-1b* plus *zBlp2* had little or no effect. Thus, *zMcl-1a* and *zMcl-1b* specifically are critical to normal early zebrafish development. Together, these results suggest that prosurvival *Bcl-2* family members and particularly *Mcl-1* have conserved functions during early development in vertebrates.

Knockdown of *zMcl-1a* and *zMcl-1b*, but not *zBlp2*, sensitizes zebrafish embryos to apoptosis induction through the extrinsic pathway

In certain mammalian cell types, apoptosis induction through the extrinsic pathway requires augmentation of the cell death signal through the intrinsic apoptotic pathway.[29] *Apo2L/TRAIL*

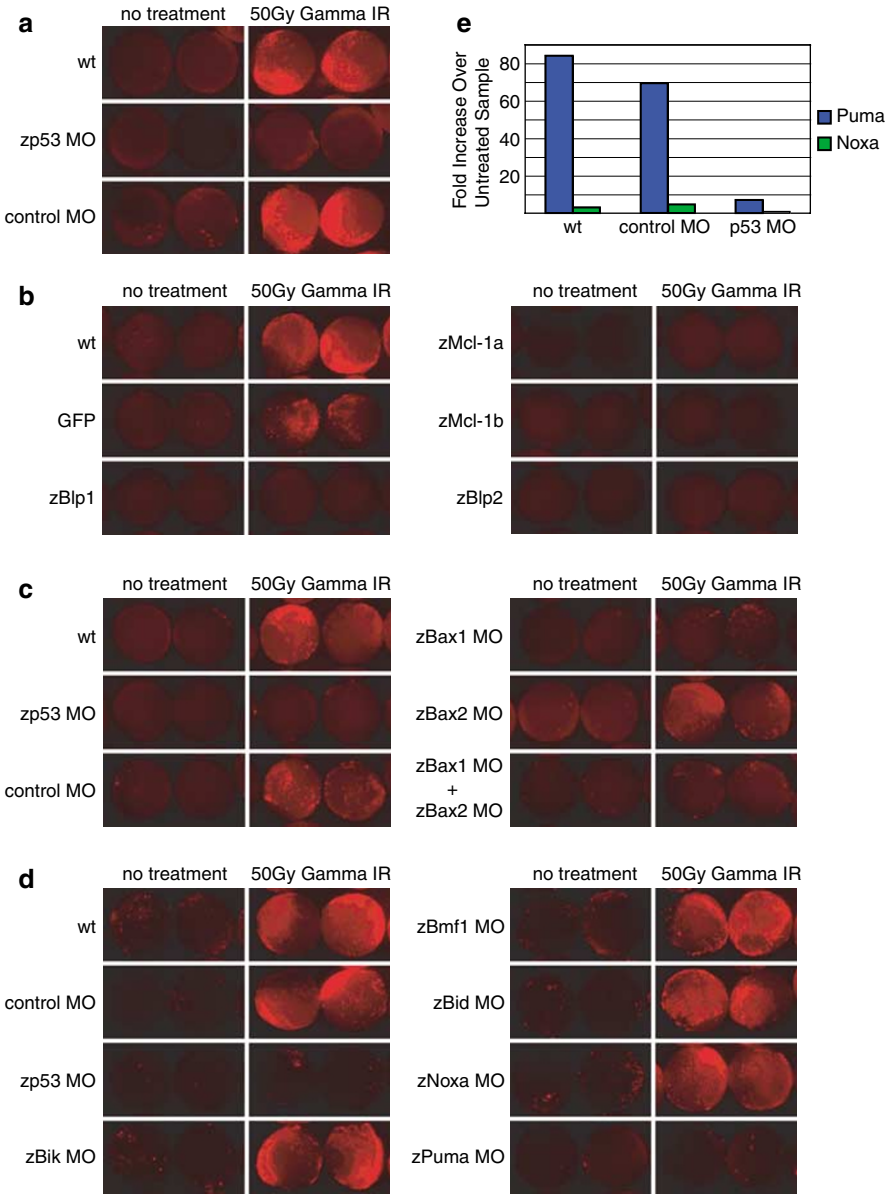


Figure 4 γ-Irradiation induces caspase-3 activation via the intrinsic apoptotic pathway in a *p53*-dependent manner. (**a**) Uninjected embryos, embryos injected with a *p53* MO, and embryos injected with a control MO were subjected to 50 Gy of γ-irradiation at 7 h.p.f. and stained for caspase-3 activity at 10 h.p.f. γ-Irradiation induction of caspase-3 activity is *p53*-dependent. Each panel is representative of at least two independent experiments. (**b**) Injection of 500 pg *zMcl-1a*, *zMcl-1b*, *zBlp1*, or *zBlp2* could efficiently inhibit caspase-3 activation in γ-irradiated embryos. Each panel is representative of at least two independent experiments. (**c**) Knockdown of *zBax1*, but not *zBax2*, could inhibit γ-irradiation-induced caspase-3 activation. Each panel is representative of four independent experiments. (**d**) Knockdown of *zPuma*, but not knockdown of any other BH3-only protein, inhibited caspase-3 activation in γ-irradiated zebrafish embryos. Each panel is representative of at least two independent experiments. (**e**) Taqman analysis reveals that *zPuma* is massively upregulated in γ-irradiated embryos in comparison to untreated (control) embryos. Expression of *zNoxa* was upregulated to a much lesser extent. γ-Irradiation-induced upregulation of both *zNoxa* and *zPuma* is dependent on *p53*

is an evolutionarily conserved proapoptotic DL of the extrinsic pathway that has several homologs in zebrafish, including *zDL1b* (Eimon *et al.*, accompanying manuscript). Apoptosis signaling by *zDL1b* can be blocked by overexpression of prosurvival *zBcl-2* family members (Eimon *et al.*, accompanying manuscript). However, it is unknown which endogenous prosurvival *Bcl-2* family members in zebrafish may function as inhibitors of extrinsic pathway augmentation. Therefore, we sought to determine whether knockdown of specific prosurvival *Bcl-2* genes could sensitize embryos to apoptosis induction by *zDL1b*. In wild-type embryos, ectopic expression of *zDL1b* had minimal effect on early embryonic viability. However, upon combined knockdown of *zMcl-1a* and *zMcl-1b*, overexpression of *zDL1b* caused rapid and massive apoptotic death. This effect was specific to *zMcl-1a* and *zMcl-1b*: combining either *zMcl-1a* or *zMcl-1b* MO with *zBlp2* MO showed only a minimal increase in sensitivity to *zDL1b*-induced apoptosis (Figure 5b). These data agree with the

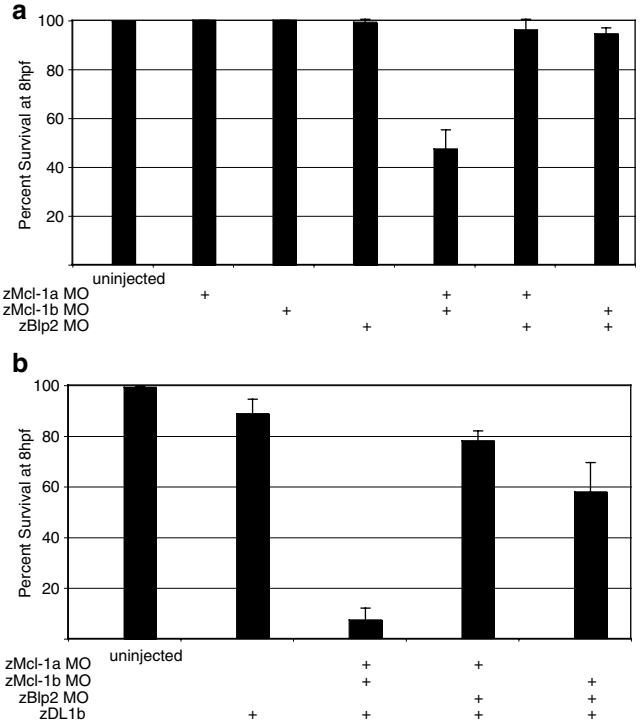


Figure 5 Simultaneous knockdown of *zMcl-1a* and *zMcl-1b* increases embryonic mortality, and sensitizes embryos to DL signaling. (**a**) Knockdown of *zMcl-1a*, *zMcl-1b*, or *zBlp2* alone has no effect on embryonic viability. Combined knockdown of *zMcl-1a* and *zMcl-1b* decreases embryonic viability by ~40%. Knockdown of *zBlp2* in combination with either *zMcl-1a* or *zMcl-1b* has no effect on embryonic viability. Error bars represent S.E.M. Data are compiled from a minimum of three independent experiments. (**b**) Knockdown of *zMcl-1a* and *zMcl-1b* sensitizes embryos to apoptosis induced by DL1b signaling. In contrast, knockdown of *zBlp2* in combination with *zMcl-1a* or *zMcl-1b* sensitizes embryos to DL1b-induced apoptosis only to a minor extent. Error bars represent S.E.M. Data are compiled from a minimum of three independent experiments

observation that in several mammalian cell lines, *Mcl-1* specifically modulates sensitivity to *Apo2L/TRAIL*-induced apoptosis.[30–32] Thus, *Mcl-1* may be a key modulator of DL-induced apoptosis in cells that require engagement of both the extrinsic and intrinsic pathways for commitment to apoptosis. Furthermore, these results indicate that endogenous *zMcl-1a* and *zMcl-1b* play an important role in curtailing apoptosis induction through the cell-intrinsic pathway in early zebrafish embryos.

Discussion

Our results show that the zebrafish genome contains full-length, functional homologs of virtually all the mammalian *Bcl-2* family members. Zebrafish offers a unique model to elucidate in greater detail the role of the various *Bcl-2* family members during embryogenesis *ex utero* in a genetically tractable vertebrate. Although the existence of some zebrafish *Bcl-2* family members has been reported previously,[14,15] several others were not known, and the functional relationship between zebrafish *Bcl-2* family members has not been systematically elucidated.

By amino-acid sequence comparison, we identified homologs of most mammalian *Bcl-2* family members, including BH3-only and multi BH domain proteins. The BH3-only family members are more divergent between zebrafish and mammals, but they share a high degree of homology within the critical BH3 domain. In both ectopic expression and MO knockdown experiments, the function of the majority of zebrafish *Bcl-2* family members as proapoptotic or prosurvival regulators was fully consistent with the established function of their corresponding mammalian homologs. These findings demonstrate a remarkable conservation of the structure–function relationship of each *Bcl-2* family member in vertebrate evolution.

zBax2 presented an intriguing exception to this stringent structure–function conservation. Even though it displays significantly greater sequence homology to mammalian Bax, zBax2 was functionally similar to human Bak,[24] in that it was unable to interact functionally with zBlp2, the homolog of mammalian *Bcl-2*. Thus, despite the lack of homology between zebrafish and human *Bak, zBax2* has evolved to display the same interactive selectivity as *hBak*.

In contrast to *zBlp1*, *zMcl-1a*, and *zMcl-1b*, *zBlp2* was only moderately successful in blocking apoptosis induced by ectopic expression of the BH3-only protein zBik. This finding suggests that zBik interacts weakly with zBlp2, providing further evidence for selectivity in the interactions of prosurvival and proapoptotic *Bcl-2* family members. Additional selectivity between Bcl-2 family proteins as reported previously[6,33] will be interesting to examine in zebrafish.

We used the zebrafish model to gain further insight into the relative importance of *Bcl-2* family members in response to DNA damage induced by γ-irradiation. Our results showed that in response to γ-irradiation, the apoptosis effector caspase-3 was activated in a *p53*-dependent manner. Overexpression of any of the zebrafish prosurvival *Bcl-2* members inhibited irradiation-induced caspase-3 activation. Knockdown of *zBax1* also ameliorated this response, confirming that the zebrafish *Bcl-2* family regulates the *p53*-dependent apoptotic response to γ-irradiation. Furthermore, γ-irradiation massively upregulated *zPuma* transcription through a *p53*-dependent mechanism, whereas only minimally inducing *zNoxa*. Knockdown of *zPuma*, but not of *zNoxa*, abrogated γ-irradiation-induced activation of caspase-3. Previous studies have demonstrated the relative importance of *Puma versus Noxa* in the apoptotic response to γ-irradiation of mammalian cells *in vitro* and within the adult mouse.[26] Our data support the conclusion that Puma is the primary initiator of γ-irradiation-induced apoptosis in intact, developing vertebrate embryos.

We examined the role of prosurvival *Bcl-2* family members during embryonic development. Knockdown of both *zMcl-1a* and *zMcl-1b* significantly decreased embryonic viability by 8 h.p.f. In contrast, knockdown of *zBlp2* alone or in combination with *zMcl-1a* or *zMcl-1b* had no effect on embryonic survival. These data suggest that *zMcl-1a* and *zMcl-1b* specifically act as essential gatekeepers during zebrafish embryonic development, protecting embryos from unchecked apoptotic death. Thus, *Mcl-1* plays a crucial antiapoptotic role during early embryonic development of the mouse[13] as well as the zebrafish, whereas *Bcl-2* is dispensable during early

embryogenesis in vertebrates. It remains to be determined whether, similar to mammalian *Bcl-2*, *zBlp2* plays a role later in embryogenesis.[12,34]

Our results show that *zMcl-1a* and *zMcl-1b* function also as gatekeepers against apoptosis induction by stimulation of the extrinsic pathway. In zebrafish embryos, knockdown of *zMcl-1a* and *zMcl-1b* substantially increased apoptosis triggered by ectopic expression of the *Apo2L/TRAIL* homolog *zDL1b*. Previous work suggests that in human hepatocellular carcinoma-derived cells,[32] human cholangiocarcinoma cells,[31] and NIH3T3 mouse fibroblasts,[30] *Mcl-1* is a critical modulator of sensitivity to apoptosis signaling by *Apo2L/TRAIL*. Hence, endogenous *Mcl-1* may be particularly important among prosurvival *Bcl-2* family members in controlling apoptosis induction by stimuli that engage both the extrinsic and intrinsic apoptosis pathways.

Several other components of the cell-intrinsic apoptosis pathway have yet to be elucidated in zebrafish. Zebrafish homologs to cytochrome *c* subunits have been identified.[35] Also, a homolog to mammalian *caspase-9* has been verified recently (Eimon *et al.*, accompanying manuscript), and potential homologs of *Apaf-1*, *XIAP*, and *SMAC/Diablo* (accession number XP_698996) have been reported[14] or predicted, although their functions have not been verified. Our data and previous reports demonstrate that the zebrafish has a functional *caspase-3* homolog.[36] Future studies will examine if the rest of the intrinsic apoptosis pathway is as functionally conserved as the *Bcl-2* gene family in zebrafish and mammals.

Materials and Methods

Zebrafish care

Adult Tubingen long-fin fish were obtained from the Zebrafish International Resource Center. Fish were maintained at 28.5°C according to the standard laboratory practices.[37] Embryos were collected ~30 min postfertilization and maintained in embryo media for further analysis.[37] All experiments on live animals were approved by the Genentech Inc. Institutional Animal Care and Use Committee.

Identification of zebrafish *Bcl-2* genes

Several zebrafish *Bcl-2* genes have been previously reported in the literature.[14,16,17,36,38,39] *zBmf1* and *zNoxa* homologs were identified by the Strasser group. Novel genes, with the exception of *zBik*, *zPuma*, and *zBax1*, were identified by BLASTing human and mouse protein sequences against the translated zebrafish genome and generating potential coding regions using GenomeScan.[40] *zBik*, *zPuma*, *zBmf2*, and *zBax2* were identified by Mukhyala *et al.* (manuscript in preparation). Coding regions were then amplified by RT-PCR with Qiagen OneStep RT-PCR kit according to the manufacturer's directions, and cloned by Topo TA cloning into pCRII (Invitrogen). Multiple RT-PCR products were sequenced to verify the correct coding sequence.

Synteny was established by comparing flanking genes to the zebrafish gene and human homolog via www.ensembl.org, version 39. Genes were designated syntenic if more than a single flanking gene was conserved between zebrafish and human.

Stage-specific and tissue-specific RT-PCR

RNA was isolated from dechorionated wild-type zebrafish embryos at the time points indicated using QiaShredder (Qiagen), followed by Qiagen RNeasy Mini-kit with DNaseI digestion according to the manufacturer's directions. Adult tissues were isolated from an adult female zebrafish. RNA was isolated similarly to embryonic tissue. RT-PCR (Qiagen OneStep) was performed on 50 ng of adult or 100 ng of total embryonic RNA for 30 cycles.

Synthetic mRNA synthesis and microinjection

Zebrafish cDNAs were cloned into the expression vector pCS108 (a gift from R Harland, University of California, Berkeley, CA, USA). Capped synthetic mRNA was generated by Ambion mMessage mMachine, and purified over NucAway spin columns (Ambion Inc.) according to the manufacturer's directions. mRNA was then diluted to the appropriate concentration in 1 × Danieu's solution[37] + 0.2% phenol red.

One- to four-cell stage embryos were injected with a total volume of 4.6 nl using a Nanoliter 2000 (World Precision Instruments) microinjector.

Antisense MO knockdown

MOs were designed around the translational start site of each transcript.[41] MOs were obtained from GeneTools LLC, then diluted to 1 mg/ml in 1 × Danieu's solution + 0.2% phenol red. A total of either 4.6 or 9.2 ng of MO was injected. In those experiments where a combination of two MOs was used, 4.6 ng of each MO was injected. An additional 4.6 ng of control MO was added to single MO injections when the experiment included comparison with two MO injections, bringing the total MO dose to 9.2 ng for each condition. One- to four-cell stage embryos were injected in a total volume of 4.6 nl via a Nanoliter 2000 (World Precision Instruments) microinjector. MO sequences are listed 5′–3′:

zMcl-1a MO:	5′-GCCTAAAATCCAAACTCAGAGCCAT-3′
zMcl-1b MO:	5′-TGTCGTTGTTTCTTCCAGCGAACAT-3′
zBlp1 MO:	5′-AGGTTGTTGCTCGTTCTCCGATGTC-3′
zBlp2 MO:	5′-GTCATAGCTAATTTCGTTAGCCATG-3′
zBax1 MO:	5′-TGAAAATAAGCGAACTGAAGAAGAC-3′
zBax2 MO:	5′-ATTTTTCGGCTAAAACGTGTATGGG-3′
zBid MO:	5′-GGTCAAAGTTCCTGTTGAAGTCCAT-3′
zBmf1 MO:	5′-ACACATCATCCTCGTCCTCATCCAT-3′
zNoxa MO:	5′-CTTTCTTCGCCATTTCAGCAAGTTT-3′
zPuma MO:	5′-TGCTTTCCATCTCTGGTCGGGCCAT-3′
zBik MO:	5′-CTACAAACAAGGACACAATGGTGGA-3′
p53 MO[42]:	5′-GCGCCATTGCTTTGCAAGAATTG-3′
Control MO:	5′-CCTCTTACCTCAGTTACAATTTATA-3′

The control MO was obtained from GeneTools LLC.

MOs to proapoptotic *Bcl-2* family members were selected based on their ability to inhibit apoptosis induced by ectopic expression of their cognate mRNA. As MO efficacy could not be verified for *zBok1*, *zBok2*, *zBmf2*, or *zBad*, we did not include these genes in knockdown analyses. MOs to prosurvival *Bcl-2* family members were selected based on their ability to abrogate cognate mRNA-mediated rescue from apoptosis triggered by ectopically expressed *zNoxa* (data not shown).

Antiactive caspase-3 staining

Active caspase-3 was detected as follows: embryos were fixed at 10 h postfertilization (h.p.f.) in 4% paraformaldehyde in phosphate-buffered

saline (PBS) for ~4 h at room temperature or overnight at 4°C, then dehydrated in methanol for a minimum of 2 h. After rehydration, embryos were washed with water, permeabilized in acetone for 7 min at −20°C, and washed again in water. After several washes with PBS + 0.5% Tween-20 (PBST), embryos were blocked for 2 h at room temperature in 5% fetal bovine serum, 2 mg/ml bovine serum albumin in PBST. Staining with rabbit antiactive caspase-3 antibodies (Pharmingen #559565) diluted 1 : 500 in block took place overnight at 4°C. Embryos were then washed several times in PBST before incubation with Cy3-conjugated goat anti-rabbit IgG antibodies (Jackson Immunology #111-166-003), also diluted 1 : 500 in block. Embryos were washed again with PBST before visualization using a Leica MZFL3 fluorescence microscope.

Fluorescence was quantified using ImageJ software (http://rsb.info.nih.gov/ij/). Briefly, the threshold was set to eliminate background fluorescence, and each embryo from a single clutch of embryos was analyzed to generate arbitrary fluorescence units.

γ-Irradiation

Embryos were γ-irradiated at approximately 7 h.p.f. in 1 ml of embryo media with 50 Gy, using a GammaCell 1000 Elite Cesium source (Nordion International Inc.). Embryos were subsequently moved to a tissue culture dish in a greater volume of embryo media and incubated for 3 h at 28.5°C until further analysis.

Quantitative real-time PCR

Taqman analyses were performed using Applied Biosystems 7500 Real-Time PCR System according to the manufacturer's directions. Primer and probe sequences are listed 5′–3′ below:

zGAPDH forward:	5′-TGC GTT CGT CTC TGT AGA TGT-3′
zGAPDH reverse:	5′-GCC TGT GGA GTG ACA CTG A-3′
zGAPDH probe:	5′-TGT GTG TGT GTG TTA GTT TCT TTT GAC AGT ATT TG-3′
zNoxa forward:	5′-CGA ACC TGT GAC AGA AAC TTG-3′
zNoxa reverse:	5′-CTG CGC GCA CTC TAC TAC A-3′
zNoxa probe:	5′-CGG TTT GCT CTT TCT TCG CCA TTT C-3′
zPuma forward:	5′-GAA CAC ACG GGT TAC AAA AGA C-3′
zPuma reverse:	5′-GAA AAT TCC CAG AGT CTG TAA GTG-3′
zPuma probe:	5′-ACG AGT GCA GGC GCT CTC CTT-3′

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