The Journal of Experimental Biology 211, 1874-1881
Published by The Company of Biologists 2008
doi:10.1242/jeb.012690

Extensive apoptosis and abnormal morphogenesis in pro-caspase-3 transgenic zebrafish during development

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Accepted 2 April 2008

SUMMARY

The pro-apoptotic caspase-3 gene has been shown to have key functions in the execution of apoptosis (programmed cell death) in vertebrate cells. However, the central role of caspase-3 in morphogenesis during development remains unclear. In this study, transgenic zebrafish that overexpress full-length pro-caspase-3 were generated to determine the effects of caspase genes on vertebrate morphogenesis and stress tolerance. The enhanced expression of the full-length pro-caspase-3 cDNA induced extremely high levels of caspase activity and extensive apoptosis in the transgenic embryos, and 33–46% of F2 embyos in the transgenic lines exhibited some form of morphological abnormality. Pro-caspase-3 transgenic zebrafish exhibited abnormal morphogenesis in the eyes, notochord, heart and yolk sac, suggesting that enhanced processing of pro-caspase-3 triggers significant apoptotic responses in the specific target tissues that are undergoing morphogenesis during development. The transgenic fish had reduced eye size and showed degeneration of the retina, including the photoreceptor cell layers, whereas pigmentation and lens formation were not affected. In addition, heart failure due to a weakened heartbeat and reduced circulation was noted in the pro-caspase-3 transgenic embryos. The transgenic embryos were markedly sensitive to stress conditions, such as UV irradiation at 2 or 5 mJ cm^{-2}. On the other hand, caspase-3 deficiency through injection of antisense morpholino oligo into embryos repressed apoptosis and enhanced stress tolerance after UV irradiation. Therefore, the caspase-3-mediated pro-apoptotic signalling pathway and its activation play critical roles in the induction of apoptosis and stress tolerance during zebrafish embryogenesis.

Key words: caspase-3, apoptosis, transgenic zebrafish, eye and heart formation, stress response, antisense morpholino oligo.

INTRODUCTION

Apoptosis (programmed cell death) is important in normal biological processes and in pathogenesis in fish (Lesser et al., 2001; Sardella et al., 2004; Sollid et al., 2003; Uchida et al., 2002) and higher vertebrates (Cohen et al., 1992; Ellis et al., 1991; Fernandes-Alnemri et al., 1994; Nicholson and Thornberry, 1997; Steller, 1995). The characterization of genes involved in apoptosis has been intensively pursued and has led to the identification of two major classes of genes, the *bcl2* family and the caspase family. Caspases are proteases that cleave target substrates with a specific peptide sequence (Chou et al., 2000; Enari et al., 1998). During apoptosis, the activation of caspases, which can be induced by nuclear, metabolic or externally activated stimuli, takes place in a cascade fashion and leads to nuclear engulfment and cell death (Chou et al., 2000; Thornberry and Lazebnik, 1998). The caspase-activated endonuclease (CAD)1, which cleaves and inactivates the inhibitor of CAD (ICAD), is responsible for DNA fragmentation at the linker regions between the nucleosomes (Enari et al., 1998; Sakahira et al., 1998). There is evidence that caspases contribute to the drastic morphological changes seen during apoptosis by proteolysing and disabling a number of key substrates, including Crk-associated substrate, focal adhesion kinase and rabaptin-5 (Cosulich et al., 1997; Kook et al., 2000; Liu et al., 1997; Wen et al., 1997). The most commonly activated caspase-3 mediates the limited proteolysis of these proteins.

Recent studies have highlighted some of the prominent features of apoptosis during development (Raff, 1996; Weil et al., 1997). In mammals, cardiomyocyte apoptosis results from cardiomyopathy and other cardiac disorders, and occurs in the myocardial tissue of patients with heart failure (Condorelli et al., 2001; Narula et al., 1996). Mice expressing caspase-3 in the muscle cells of the heart have increased rates of heart failure and apoptosis (Condorelli et al., 2001). Knockout mice lacking the caspase-3 gene showed skull defects with ectopic masses of supernumerary cells, reflecting defective programmed cell death during brain development and resulting in perinatal lethality (Kuida et al., 1996). Caspase-3-deficient mice showed an absence or delay of apoptosis-associated morphological changes, such as cytoplasmic blebbing and DNA fragmentation, in hepatocytes and thymocytes after Fas engagement (Zheng et al., 1998). Thus, caspase-3 is involved in the late stages of apoptosis in these cells (Wang and Lenardo, 2000). However, it is not clear whether apoptosis induced by overexpressed caspases represents the physiological functions of these caspases *in vivo*. In previous studies of zebrafish development, the fish embryo was demonstrated to be a useful model for stress-induced apoptosis under conditions such as heat shock and UV and γ-irradiation (Yabu et al., 2001a; Yabu et al., 2001b). Extensive apoptosis and caspase-3 activity were induced under these stress conditions. In addition, the transient overexpression of pro-caspase-3 in zebrafish embryos resulted in enhanced pro-apoptotic signals (caspase activation and ceramide generation) under normal developmental conditions *in vivo*. Therefore, the overexpression in transgenic embryos would be expected to enhance the pro-apotoptic signalling and abnormal morphogenesis regulated by a caspase-3-mediated mechanism under early embryogenesis.

In order to characterize the importance of caspase-3 in embryogenesis, we generated transgenic zebrafish that

overexpressed pro-caspase-3 in their embryos. These transgenic zebrafish were characterized under basal conditions at both the functional and molecular level. This study describes the effects of pro-caspase-3 overexpression and deficiency on the pro-apoptotic pathway regulating apoptosis in embryogenesis and stress tolerance during zebrafish development.

MATERIALS AND METHODS

Transgenic zebrafish

Zebrafish [*Danio rerio* (Hamilton 1822)] were maintained with commercial artificial feed (Tetrafin, Tetra Japan, Tokyo, Japan) at 28.5°C on a 14 h:10 h light:dark cycle (Westerfield, 1995). Eggs and embryos were collected and maintained at 28.5°C and were staged according to the time that had elapsed since fertilization, and based on morphological criteria (Westerfield, 1995). The zebrafish pro-caspase-3 cDNA, containing the complete 889 bp open reading frame (ORF), was amplified by PCR using the isolated full-length cDNA as a template with the sense primer 5′-TACTGTTT-AAAAGGGCTCGTTAAGCGG-3′ and an antisense primer corresponding to six histidine residues in front of a stop code, 5′-TTAGTGGTGGTGGTGGTGGTGAGGAGTGAAGTACATC-TCTTTGGT-3′, according to a previous paper (Yabu et al., 2001b). The reaction was carried out with *Ex*Taq polymerase (Takara, Kyoto, Japan) for 25 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 2 min. The amplified product was subcloned downstream from the human cytomegalovirus (CMV) promoter of the pTARGET mammalian expression vector (Promega, Madison, WI, USA), and the vector was named pCaspase-3. This plasmid construct was linked to the green fluorescent protein (GFP) gene (pEGFP-C1 vector; Clontech, Palo Alto, CA, USA) and was used for the generation of transgenic zebrafish (accession no. AB090853). Fertilized one-cell stage embryos were collected from wild-type zebrafish and microinjected with plasmid DNA, according to a previously described method (Yabu et al., 2001b). The injected zebrafish embryos, which were identified by green fluorescence from GFP expression, were cultured for 5 days at 28.5°C in sterilized tap water. Juvenile fish were cultivated to adulthood on a diet of paramecium, brine shrimp larvae and commercial feed. Because the transgenic embryos displayed bright green (GFP) fluorescence, the embryos that stably expressed the transgene were easily selected. Two independent transgenic lines, casp-1 and casp-2, were used for further studies. The F2 transgenic progeny from an F1 × wild-type cross were used for further analyses. Other transgenic lines overexpressing GFP, introduced by the pEGFP-C1 vector, were also used for this study as a negative control (Imamura et al., 2004).

Microinjection of morpholino oligonucleotide

The morpholino antisense oligonucleotide 5′-TTGCGTCCACACA-GTCTCCGTTCAT-3′ for zebrafish pro-caspase-3 (caspase-MO) was synthesized (Gene Tools, Boston, MA, USA) and used for knockdown experiments by microinjection at 1–10 ng into a one-cell stage zebrafish embryo. A morpholino oligonucleotide with five mispaired bases (5-mispaired control MO; 5′-TTGCCTCCAGAC-AGTGTCGGTTGAT-3′) was used for microinjection as a negative control.

Whole-mount terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) staining and immunostaining

Whole-mount TUNEL staining of zebrafish embryos was adapted from a tissue-sectioning method (Yabu et al., 2001a). Embryos were fixed overnight at 4°C in a 4% solution of formaldehyde in phosphate-buffered saline (PBS). The samples were washed twice in 100 mmol l^{-1} Tris-HCl (pH 7.5) buffer that contained 150 mmol l^{-1} NaCl and 0.1% Tween 20 (TBST) and then fixed in methanol at –20°C for at least 1 day. The samples were rehydrated following methanol fixation by washing three times for 15 min each in TBST at room temperature. The samples were transferred to TUNEL buffer (25 mmol l^{-1} Tris-HCl pH 6.6, 200 mmol l^{-1} sodium cacodylate, 5 mmol l^{-1} cobalt chloride and 0.25% bovine serum albumin) and washed for 30 min. Fluorescence labelling of fragmented DNA was carried out for 1 h at 37°C in TUNEL buffer that contained 1 mmol l^{-1} fluorescein-dUTP (Hoffmann-La Roche, Basel, Switzerland) and 50 units ml^{-1} terminal deoxynucleotidyl transferase (Hoffmann-La Roche). The reaction was stopped by washing the samples five times in TBST buffer for 5 min each at room temperature. The embryos were stained with alkaline phosphatase-labelled anti-fluorescein antibody with a chromogenic BM Purple AP substrate (Hoffmann-La Roche) according to the methods described by the manufacturer. The samples were observed after mounting in a TBST/glycerol solution (1:1). For histological observation, cross-sections of the TUNEL-stained embryos were prepared after paraffin embedding.

Whole-mount antibody staining with anti-caspase-3 (Yabu et al., 2001b) polyclonal antibody was performed as described previously (Westerfield, 1995). A fluorescent secondary antibody labelled with Cy3 (Amersham, Piscataway, NJ, USA) was used for fluorescence immunostaining. Photographs were taken using an Edge 3D microscope (Edge, Marina Del Rey, CA, USA).

For histological analysis, embryos were fixed in a 4% solution of formaldehyde in PBS, embedded in Historesin Plus (Leica, Wetzlar, Germany) and sectioned at 5 μm. Sections were stained with Methylene Blue–Azure II, and mounted with Permount (Fisher Scientific, Waltham, MA, USA).

Caspase assay

To detect the active form of caspase-3 in the zebrafish embryos, the hydrolysis of acetyl (Ac)-DEVD-α-methylcoumaryl-7-amide (MCA, Peptide Institute, Osaka, Japan) was assayed according to a method described previously (Yabu et al., 2001a). Each aliquot of five embryos was washed once with PBS and lysed in 50 μl of lysis buffer (20 mmol l^{-1} Hepes-KOH pH 7.5, 250 mmol l^{-1} sucrose, 50 mmol l^{-1} potassium chloride, 2.5 mmol l^{-1} magnesium chloride, 1 mmol l^{-1} dithiothreitol). The lysate was centrifuged at 10 000 ***g*** for 15 min at 4°C, and the supernatant was collected and used for assays of Ac-DEVD-MCA hydrolysis. The protein concentration was determined with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). One unit of enzyme activity was defined as the release of 1 nmol AMC per hour at 37°C.

Stress treatment of zebrafish embryos

Stress treatment of embryos was carried out according to a previously described method (Yabu et al., 2001a). Three groups, each containing 30 embryos, were incubated at 28.5°C in 2 ml of sterilized tap water in a six-well tissue culture dish (Corning, Corning, NY, USA). The dish was sealed with Parafilm (American National Can Co., Menasha, WI, USA) and kept in a water bath at the same temperature. The 12 h embryos were placed in a dish that contained 0.5 ml of sterilized tap water without a cover and were irradiated at 254 nm in a UV cross-linker (model FS-800, Funakoshi, Tokyo, Japan). The embryos were allowed to recover at 28.5°C, and the survival rates were measured daily. Statistical survival analysis was performed with Prism4 software (GraphPad Software, San Diego, CA, USA).

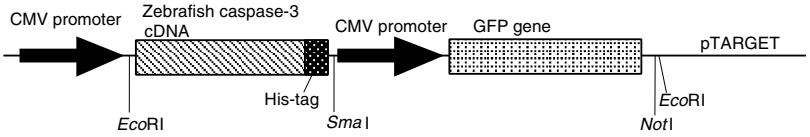

Fig. 1. The plasmid construct. GFP, green fluorescent protein.

RESULTS

Generation of transgenic lines and expression of the pro-capsase-3 transgene

To determine *in vivo* functions of pro-caspase-3, we generated germline-stable transgenic zebrafish expressing pro-caspase-3 under the control of the CMV promoter. The CMV-pro-caspase-3 cDNA connected to the CMV-GFP gene was introduced by microinjection into single-cell zebrafish embryos (Fig. 1), and two transgenic fish lines were obtained. The heterozygous F2 embryos of the transgenic casp-1 and casp-2 lines with GFP fluorescence from an F1 × wild-type cross were used for further apoptotic analysis. As a transgenic control, we used a transgenic line overexpressing GFP by the introduction of pEGFP-C1 vector (Imamura et al., 2005). During early development, the pro-caspase-3 transgenic embryos showed abnormal morphogenesis. Mortality was compared between the transgenic and wild-type fish groups on the 5th day post-fertilization. All of the wild-type and GFP control embryos were normal and alive, but 33% (N=160) of the transgenic casp-1 line and 46% (N=120) of the casp-2 line embryos showed abnormal phenotypes (Table 1).

The pro-caspase-3 transgenic embryos showed stable overexpression of the transgene product by immunofluorescent chemical detection (Fig. 2). We introduced the gene construct consisting of pro-caspase-3 cDNA with a His-tag at the C-terminal end to detect the transgene product in the transgenic zebrafish. His-tagged pro-caspase-3 was detected in the whole embryos of pro-caspase-3 transgenic fish at 24 h post-fertilization (h.p.f.), but not in the GFP control transgenic fish (Fig. 2). This finding indicates that the full-length pro-caspase-3 was ubiquitously expressed under the control of the CMV promoter in the transgenic zebrafish embryos.

Morphological effects of pro-caspase-3 overexpression in embryos

The tissue-selective processing and activation of overexpressed pro-caspase-3 leads, presumably, to extensive damage to the cells that express the transgene. Therefore, we conducted morphological analyses of the transgenic fish embryos.

The casp-1 and casp-2 transgenic lines clearly showed similar phenotypic disruptions in the formation of the eyes, heart, notochord and yolk sac (Fig. 3, Table 1). The hearts of the transgenic embryos developed abnormally (Fig. 4). The heart cavity of the transgenic embryos was enlarged, and the ventricle formed an elongated structure and was reduced in size. The contractility of the ventricle was weaker in transgenic embryos than in wild-type embryos, and blood circulation was reduced or ceased at 48 h.p.f. Heart rate measurements (beats min^{-1}) at 30 h.p.f. indicated that the heart rate of transgenic casp-1 embryos was approximately 82% that of wild-type embryos. The transgenic embryos were characterized by reduced eye size. Histological sections revealed the loss of retinal cells in the transgenic fish; this loss of retinal cells was not localized to any particular cell layer in the retina (Fig. 5). The photoreceptor cells displayed the most severe defects. However, pigmentation and lens formation in the eyes of transgenic fish were not affected. The pro-caspase-3 transgenic embryo showed abnormal (irregular) morphology of the notochord (Fig. 6). Therefore, overexpression of pro-caspase-3 in the transgenic embryos induced abnormal morphogenesis in the eyes, notochord, heart and yolk sac.

Extensive apoptosis in the pro-caspase-3 transgenic zebrafish embryos

The abnormal morphogenesis found in the pro-caspase-3 transgenic embryos was considered to be due to extensive apoptosis through enhanced pro-apoptotic signalling. This was confirmed by assaying caspase-3 activity, i.e. Ac-DEVD-MCA hydrolysing activity, at 3, 6, 9, 12 and 24 h.p.f. (Fig. 7). The caspase activity in the transgenic fish at 9 and 24 h.p.f. was, respectively, 4-fold and 9.5-fold the activity in the wild-type and GFP control fish lines, indicating that a caspase-dependent pro-apoptotic pathway was activated in the pro-caspase-3 transgenic embryos.

Whole-mount TUNEL staining showed the spatial distribution of apoptotic cells in the transgenic embryos. TUNEL-positive apoptotic cells were observed predominantly in the eyes, brain and spinal cord at 24 h.p.f. (Fig. 8). Before hatching at 36 h.p.f., the pro-caspase-3 transgenic embryos also showed extensive apoptosis in the eye (Fig. 9). In histological sections, many TUNEL-positive apoptotic retinal cells were detected in the pro-caspase-3 transgenic fish (Fig. 9), indicating that the tissue-selective proapoptotic mechanism occurred in the eye. This extensive tissue-specific apoptosis was considered to be the cause of abnormal morphogeneis in the pro-caspase-3 transgenic zebrafish embryos.

Responses of the pro-caspase-3 transgenic fish and the caspase-3-deficient embryos to stress

Previously, we have demonstrated that UV irradiation, as well as heat shock and γ-irradiation, induce extensive apoptosis through the caspase-3-mediated pro-apoptotic signalling pathway at the early stages of zebrafish development (Yabu et al., 2001a; Yabu et al., 2001b). Thus, UV irradiation can be used for bioassaying apoptosis during development to assess stress sensitivity and tolerance *in vivo*.

Table 1. Frequency of abnormal morphology of caspase-3 transgenic zebrafish embryos

			Abnormal phenotypes (%)					
Parental genotype	N	Normal (%)	Degenerated notochord	Curved body	Small eye	Enlarged heart cavity	Weakened heart beat	Degenerated entire embryo
Wild-type	120	100	0	0	0	0	0	0
Casp-1 (F1) × wild-type	160	67	28	13	19	16	9	3
Casp-2 (F1) × wild-type	120	54	19	34	28	22	8	2

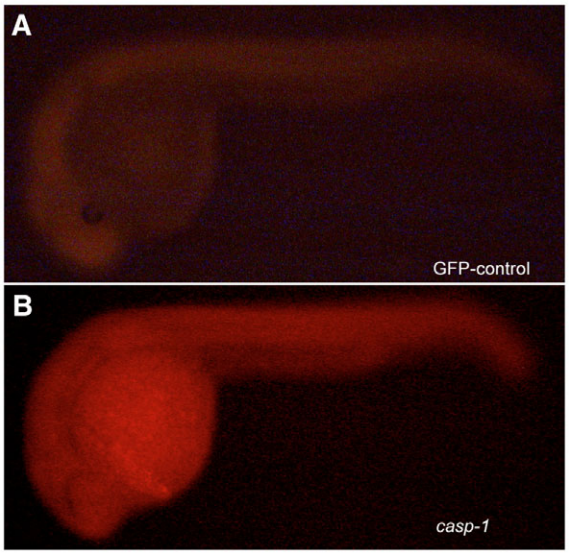

Fig. 2. Expression of the transgene product, His-tagged pro-caspase-3, in the transgenic embryos. The embryos were stained with anti-His-tag antibody and Cy3-fluoro-labelled anti-mouse IgG secondary antibody, and were observed by fluorescence microscopy. (A) GFP control. (B) Transgenic embryo expressing pro-caspase-3 (casp-1).

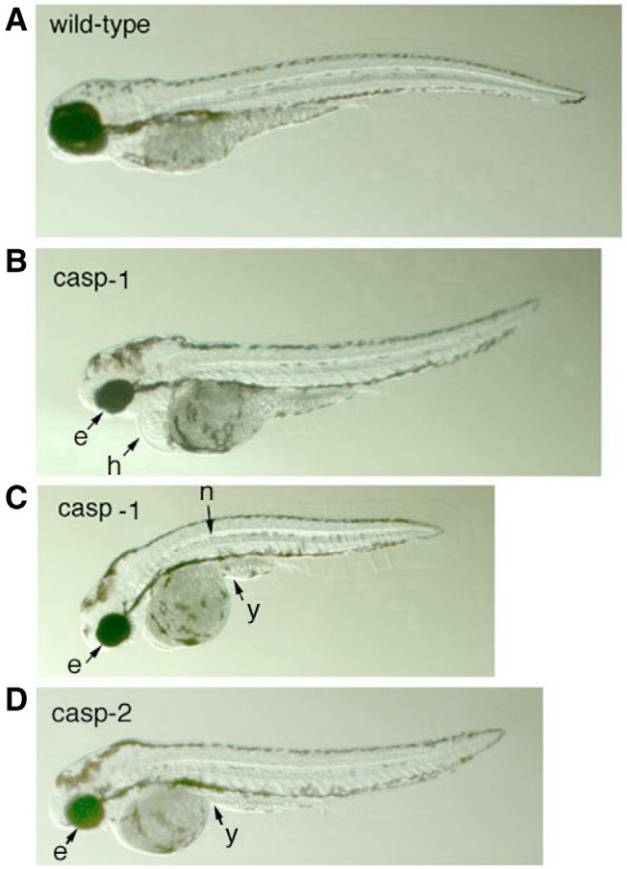

Fig. 3. Abnormal morphology of the pro-caspase-3 transgenic zebrafish. Arrows indicate abnormal morphology of the eye (e), heart (h), notochord (n) and yolk sac (y) of transgenic fish lines casp-1 (B,C) and casp-2 (D), at 48 h.p.f. (hours post-fertilization). An embryo expressing GFP was used as a control (A).

In order to understand the relationship between cellular pro-caspase-3 levels and stress tolerance *in vivo*, we examined the survival rates of transgenic zebrafish that overexpressed pro-caspase-3 under different stress conditions. When pro-caspase-3 transgenic fish embryos at 12 h.p.f. were exposed to UV irradiation at 0, 2 and 5 mJ cm^{-2}, the embryos displayed significantly lower survival rates than the GFP control embryos in a dose-dependent manner (Fig. 10). The survival rate of the pro-caspase-3 transgenic fish on the 4th day after UV irradiation at 5 mJ cm^{-2} was 17.6%, while that of the GFP control fish was 77.8%. These findings indicate that pro-caspase-3 expression levels in the embryos determine stress sensitivity.

To confirm that caspase-3-dependent apoptosis is essential for stress tolerance during zebrafish development, the specific effects of caspase-3 repression were observed after the microinjection of antisense morpholino oligonucleotide (MO) into zebrafish embryos. We designed a MO spanning the region +1 to + 25 (caspase-3-MO) of the zebrafish pro-caspase-3 mRNA, as predicted from its cDNA sequence, and injected caspase-3-MO into one-cell stage embryos. When a higher dose (10 ng) of caspase-3-MO was introduced into the caspase-3 transgenic and wild-type embryos, epiboly was arrested at 8–12 h.p.f., indicating that complete caspase deficiency caused the arrest of early embryogenesis (data not shown). When

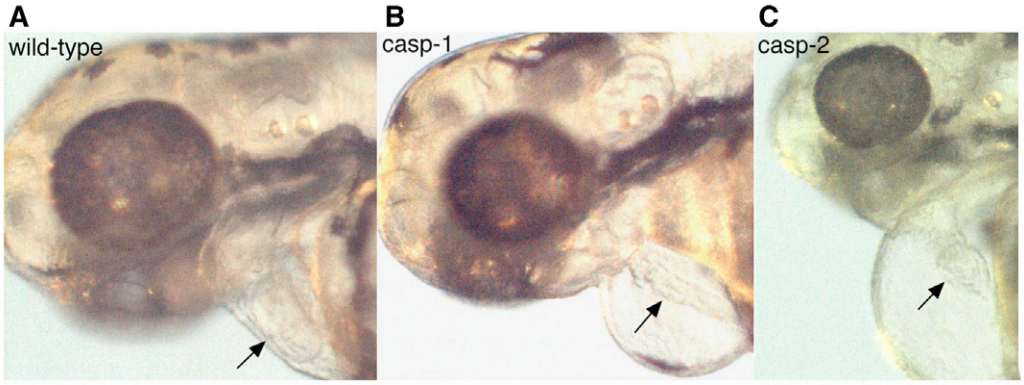

Fig. 4. Abnormal morphology of the heart of the pro-caspase-3 transgenic zebrafish. (A) wild-type embryo. (B,C) casp-1 and casp-2, transgenic embryos expressing pro-caspase-3. Arrows indicate the ventricles of embryos at 48 h.p.f. The heart cavity of the transgenic embryos was enlarged (casp-1 line). The ventricle formed an elongated structure and was reduced in size.

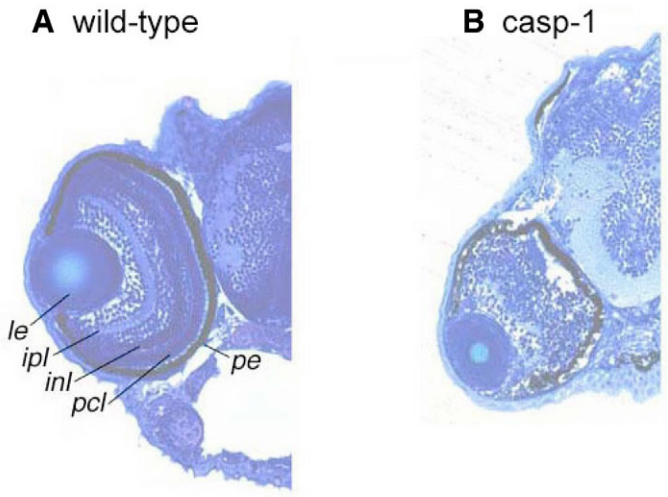

Fig. 5. Cross-sections of the eye of (A) wild-type and (B) pro-caspase-3 transgenic zebrafish (casp-1 line) at 48 h.p.f. le, lens; ipl, inner plexiform layer; inl, inner nuclear layer; pcl, photoreceptor cell layer; pe, pigmented epithelium.

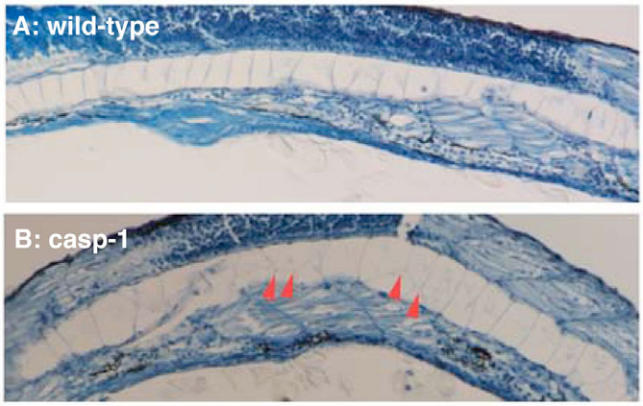

Fig. 6. Transverse sections of the notochord in (A) wild-type and (B) pro-caspase-3 transgenic zebrafish (casp-1 line) at 48 h.p.f. Arrowheads indicate abnormal (irregular) formation of the notochord in the transgenic embryo.

1 ng of caspase-3-MO was introduced into wild-type embryos, the embryos showed normal or slightly dorsalized phenotypes. The embryos in which a 5-mispaired control MO sequence had been introduced showed no apparent abnormal phenotypes. We examined the efficiency of the targeted knockdown by measuring Ac-DEVD-MCA hydrolysing activity and through the immunochemical detection of the active form of caspase-3 using anti-active caspase-3 antibody in the MO-injected embryos (Fig. 11). The MO-injected wild-type embryos had significantly lower activity than the control embryos. Thus, the caspase-3-MO was effective for the repression of caspase-3 expression in both wild-type embryos *in vivo*. We used embryos injected with 1 ng of caspase-3-MO for further experiments involving exposure to UV irradiation.

To examine the relationship between cellular pro-caspase-3 levels and stress tolerance *in vivo*, we exposed the caspase-3-deficient embryos (through MO injection) to UV irradiation at 5 mJ cm^{-2}. When embryos were exposed to UV irradiation at 12 h.p.f., the caspase-3-MO-injected embryos displayed a significantly higher survival rate than the control embryos (Fig. 12).

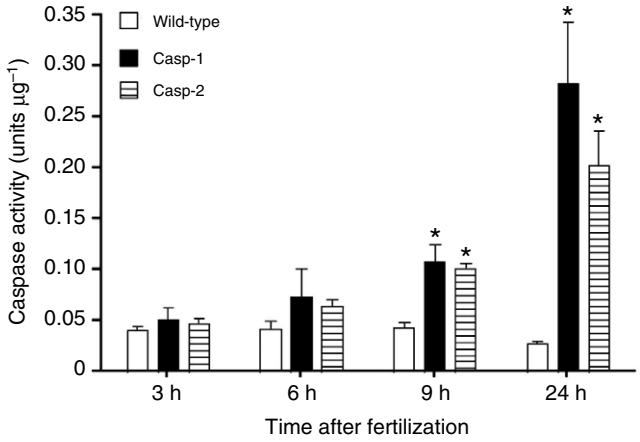

Fig. 7. Caspase activity in the transgenic zebrafish. Acetyl-DEVD-α-methylcoumaryl-7-amide (Ac-DEVD-MCA)-hydrolysing activity was measured in the embryos of casp-1 and casp-2 lines at 3, 6, 9 and 24 h.p.f. Pooled samples, each containing 10 embryos, were used for the assay. Each value is the mean ± s.d. of three independent experiments. Asterisks denote significant differences between wild-type and transgenic embryos (P<0.05, ANOVA).

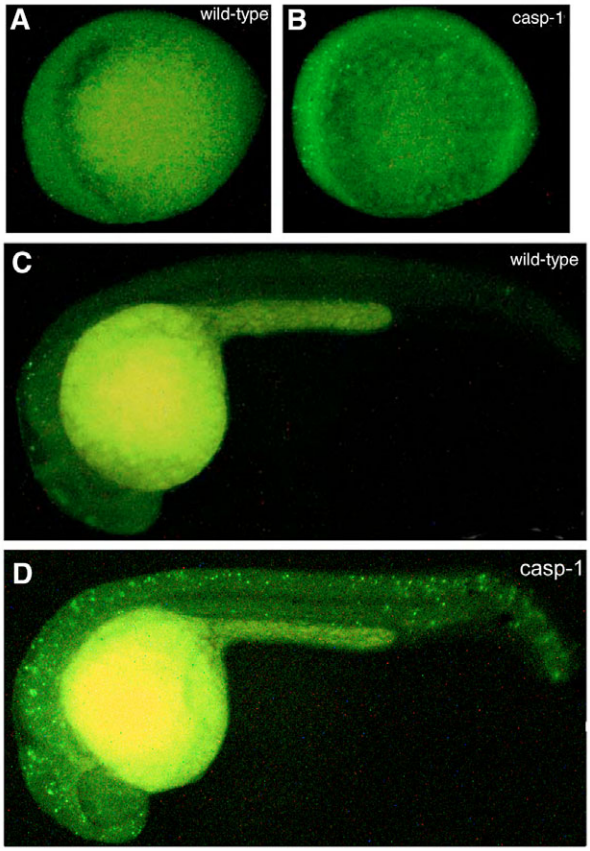

Fig. 8. Detection of apoptotic cells by fluorescent TUNEL staining of transgenic embryos. wild-type, GFP control embryos at 12 h.p.f. (A) and 24 h.p.f. (C). casp-1, transgenic embryos expressing pro-caspase-3 at 12 h.p.f. (B) and 24 h.p.f. (D).

Therefore, the present findings indicate that pro-caspase-3 expression levels in the embryos determine stress sensitivity and tolerance.

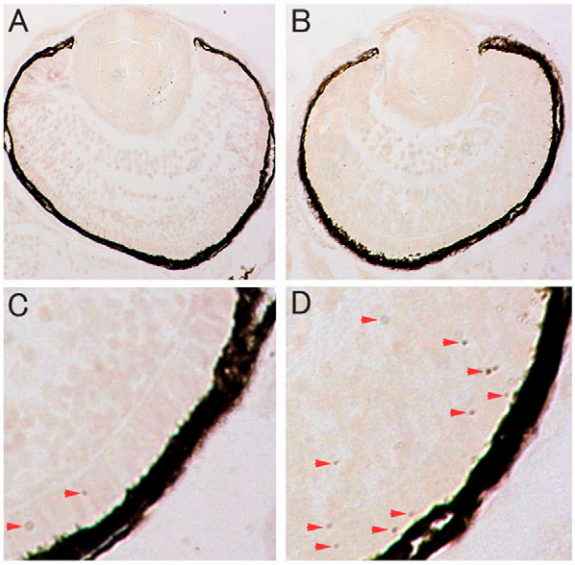

Fig. 9. Immunohistochemical detection of TUNEL-positive cells in the eye of a transgenic embryo expressing pro-caspase-3 (casp-1 line) at 36 h.p.f. Arrowheads indicate TUNEL-positive cells. (A,C) GFP-expressing embryo used as a control. (B,D) Transgenic embryo expressing pro-caspase-3.

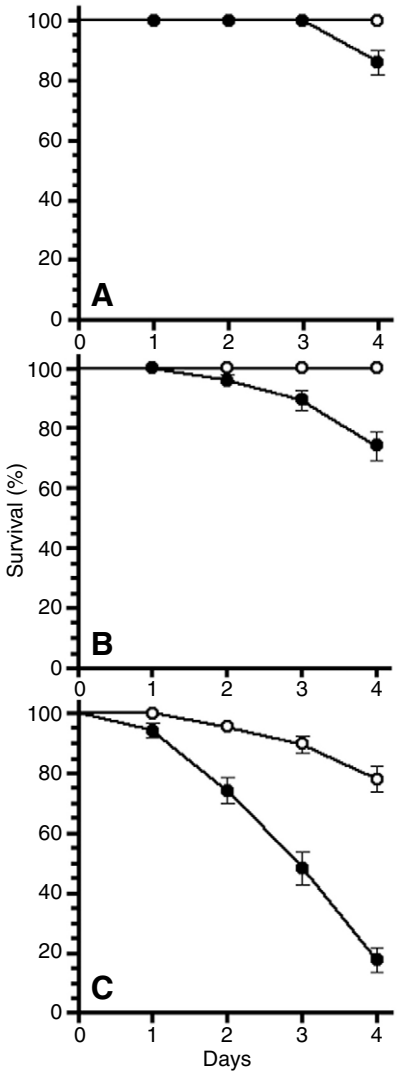

Fig. 10. Survival of transgenic embryos following UV irradiation. The casp-1 line (filled circles, N=90) and wild-type (open circles, N=90) at 12 h.p.f. were UV irradiated at 0 mJ cm^{-2} (A), 2 mJ cm^{-2} (B) or 5 mJ cm^{-2} (C), maintained at 28.5°C, and monitored daily for survival. The data were analysed using Kaplan–Meier survival analysis, and an asterisk indicates a statistically significant difference (P<0.05). Each value is the mean ± s.e.m.

DISCUSSION

Gain- and loss-of-function studies clearly demonstrate the effects of pro-caspase-3 on apoptosis and morphogenesis during development. Pro-caspase-3 is an important molecule in the cellular suicide cascade that can be activated in cellular membranes or mitochondria by environmental stimuli (Chou et al., 2000; Thornberry and Lazebnik, 1998). Caspase-3 activity levels are markedly increased in zebrafish embryos that have been exposed to heat shock, UV or γ-irradiation (Yabu et al., 2001a), and it has been suggested that caspase-3 is responsible for early development through ceramide signalling (Yabu et al., 2001b). Apoptosis has been characterized in zebrafish embryos under both normal developmental and severe stress conditions (Yabu et al., 2001b). Therefore, it was of interest to consider whether developmental functions could be modified by pro-caspase-3 overexpression and deficiency. Our findings showed that pro-caspase-3 overexpression induced extensive apoptosis and abnormal formation of the eye, notochord and cardiac system. These findings suggest critical roles for pro-caspase-3 in regulating multiple processes during early embryogenesis. Interestingly, tissue-specific expression patterns of apoptotic cells and abnormal morphology were observed in the pro-caspase-3 transgenic zebrafish.

In this study, we introduced pro-caspase-3 with a His-tag at the C-terminal end to allow detection of the protein in the transgenic zebrafish. Previously, we characterized the biochemical properties of recombinant His-tagged caspase-3 expressed in *Escherichia coli* (Yabu et al., 2001b). The bacterially produced recombinant caspase-3 showed specific activity against substrate Ac-DEVD-MCA, similar to mammalian caspase-3 (Yabu et al., 2001b). In addition, the transiently expressed pro-caspase-3 with a His-tag sequence at the C-terminus in cultured fish cells was activated and induced apoptosis and ceramide generation by limited proteolytic processing under stress conditions (Yabu et al., 2001b). Thus, the gene construct is well designed for examining caspase function and stress-induced apoptosis *in vivo*. In this study, the full-length pro-caspase-3 cDNA was stably overexpressed during zebrafish development under the control of the CMV promoter, which exhibits ubiquitous expression in zebrafish embryos (Fig. 2). From these previous studies and the present findings, the His-tagged zebrafish pro-caspase-3 used in this study was considered to have the ability to trigger endogenous pro-apoptotic signalling activation *in vivo*. Therefore, the differential effects of apoptosis and abnormal morphogenesis in the eyes and heart could be the result of a tissue-specific enhanced processing of pro-caspase-3 in these organs, rather than increased expression.

The activation of pro-caspase-3 is strictly repressed *in vitro* and *in vivo* under normal physiological conditions (Nicholson and Thornberry, 1997). These findings suggest a differential regulation of the increased sensitivity to stress stimuli (Yabu et al., 2001a), the increased expression of Bcl2 (Chou et al., 2000), and/or the decreased expression of Bax (Nicholson and Thornberry, 1997) in the tissues of developing transgenic embryos. Therefore, the zebrafish model overexpressing pro-caspase-3 is important and

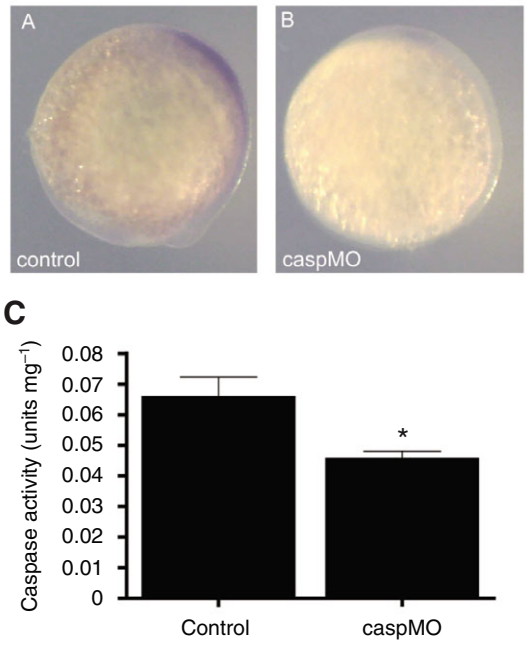

Fig. 11. Repression of caspase-3 expression in the caspase-3-morpholino antisense oligonucleotide (MO)-injected embryos. The active form of caspase-3 was detected by immunohistochemistry with anti-active caspase-3 antibody. (A) control, embryos injected with MO (1 ng) at 9 h.p.f. (B) caspMO, embryos injected with caspase-3-MO (1 ng) at 9 h.p.f. (C) Caspase-3 activity against Ac-DEVD-MCA was compared between the control MO-injected and caspase-3-MO-injected embryos at 9 h.p.f. Each value indicates the mean ± s.d. of three independent experiments. An asterisk denotes a statistically significant difference between the control MO-injected and the caspase-MO-injected embryos (P<0.05, Student's t-test). The activity of the caspase-3-MO-injected embryos was significantly lower than that of the controls.

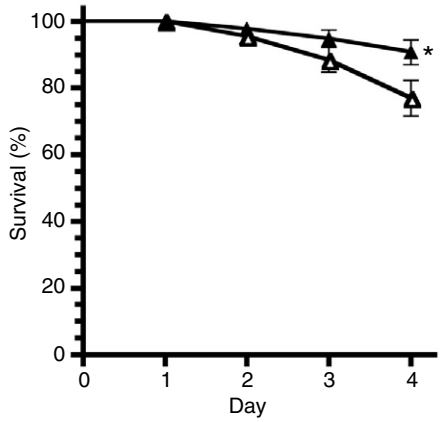

Fig. 12. Enhanced stress tolerance in the caspase-3-MO-injected embryos following UV irradiation. Survival ratio was compared between the control MO-injected and the caspase-MO-injected embryos. MO was introduced by microinjection into one-cell stage embryos. Open triangles, control embryo injected with control MO (N=90); filled triangles, wild-type embryo injected with caspase-3-MO (N=90). The data were analysed using the Kaplan–Meier survival analysis, and an asterisk indicates a statistically significant difference (P<0.05). Each value indicates the mean ± s.e.m.

useful for delineating structural changes mediated by differential regulation of apoptosis in zebrafish embryos.

A phenotype found to be associated with pro-caspase-3 overexpression was the defective formation of the eye and notochord. Several mutations that affected the development of the retina, brain and heart were noted in previous screens for genetic defects in zebrafish embryogenesis (Chen et al., 1996; Malicki et al., 1996; Stainier et al., 1996). The retinal mutations were classified into six phenotypic categories: neuronal patterning defect, cyclopia, defect of the outer retina, growth retardation, non-specific retinal degradation, and retinal degradation associated with defective pigmentation. In this study, the pro-caspase-3 transgenic fish had reduced eye size and degeneration of the retina and photoreceptor cell layers, but had normal pigmentation and lens formation, suggesting that the specific generation of retinal cells was induced by excessive caspsase-3 activation. These defects in the transgenic fish were similar to those of previously reported mutant phenotypes, such as *turbulent* and *ziemniok* (Malicki et al., 1996). Therefore, caspase-3-mediated pro-apoptotic signalling may regulate retinal differentiation and development, and retinal degeneration may result from enhanced apoptotic signalling in response to different environmental stimuli, such as stress and treatment with apoptosis-inducing reagents.

Another phenotype observed in the pro-caspase-3 transgenic embryos was abnormal cardiac formation and function. We observed heart failure due to a weakened heart and reduced circulation, which has also been seen in fish with known mutations, such as *pipe heart* (Stainier et al., 1996) and *pipe line* (Chen et al., 1996). Heart-targeted overexpression of caspase-3 in mice increased infarct size and depressed cardiac contractility after ischaemia–reperfusion injury (Condorelli et al., 2001). Therefore, the caspase-3-mediated pro-apoptotic pathway may play an important role in the development of the heart, eye and notochord. Although many zebrafish mutants with neural degeneration and developmental defects have been generated (Chen et al., 1996; Malicki et al., 1996; Stainier et al., 1996), the genes associated with these mutations have rarely been identified. There is the possibility that these morphological defects may be a secondary consequence of general retardation caused by extensive apoptosis during early development.

In transgenic mice, overexpression of the human caspase-3 gene did not induce morphogenetic defects during normal development but leads to greater apoptosis under stress conditions (Kerr et al., 2004). The transgenic mice showed significantly larger lesions when they were subjected to focal cerebral ischaemia–reperfusion injury. These results indicate that mice overexpressing human caspase-3 are essentially normal; however, they have increased susceptibility to degenerative insults. On the other hand, the present findings provide evidence that pro-caspase-3 overexpression, even in a partly activated state, is lethal under normal developmental conditions. Although in the case of the transgenic embryos with extremely high caspase activity almost all of the embryos survived under normal conditions, abnormal morphogenesis was found especially in the eyes, heart and spinal chord. In addition, approximately 60% of the F2 fluorescent progeny did not exhibit developmental abnormalities. Thus, caspase-3 activation and apoptosis may occur in only a limited number of specific tissue and cell types in the embryos.

In the human breast cancer cell line MCF-7, pro-caspase-3 overexpression showed a 3.7-fold higher specific enzyme activity but was not toxic and did not affect background apoptosis (Friedrich et al., 2001). Interestingly, the pro-caspase-3-transfected cells were more sensitive to cytotoxic drugs compared with control cells. Thus, overexpression of caspase-3 in the transgenic zebrafish embryos may affect only a limited number of apoptotic cells under normal growing

conditions and may enhance stress sensitivity especially in situations where activation of pro-apoptotic signalling is disturbed.

Apoptosis may be regulated for the purpose of eliminating unnecessary cells after cell differentiation during embryogenesis, as was shown in the nematode *ced-3* mutant, which has many extra cells due to the prevention of almost all programmed cell death (Avery and Horvitz, 1987). Therefore, the proper temporal and spatial regulation of apoptosis may be maintained by the tissue-specific expression of intercellular morphogenetic signalling factors, such as BMP4 and other TGF-β superfamily members, as pointed out in the case of digit formation in mouse and chicken (Monsoro-Burq, 1996; Yokouchi et al., 1996).

Furthermore, the overexpression of pro-caspase-3 in transgenic embryos under stress conditions of low-dose UV irradiation enhanced mortality (Fig. 10), while caspase-3 deficiency decreased mortality (Fig. 12). Environmental stresses have been shown to be critical mediators of apoptosis during zebrafish embryogenesis (Yabu et al., 2001a). The pro-caspase-3 transgenic fish were very sensitive to low doses (2 and 5 mJ cm^{-2}) of UV irradiation (Fig. 10). This suggests that the activation of caspase-3 in apoptosis can be affected by UV irradiation and that UV irradiation and pro-caspase-3 may act as mediators of the same pathway. Therefore, pro-caspase-3 may regulate the progression of pro-apoptotic signalling and play a critical role in the survival of fish embryos under normal and stress conditions *in vivo*.

In conclusion, the stable overexpression of pro-caspase-3 induced extensive apoptosis and abnormal morphogenesis in the eyes, notochord, heart and yolk sac during zebrafish development. The enhanced processing of pro-caspase-3 appears to trigger significant apoptotic responses in these specific target tissues. High pro-caspase-3 levels may enhance stress sensitivity and death of the organism *in vivo*. As a model for the characterization and analysis of the pro-apoptotic pathway, the transgenic zebrafish offers a high-quality, high-throughput bioassay tool for determining the biological effects of chemical compounds as well as for dissecting biological pathways.

This work was supported, in part, by grants from the Japan Society for the Promotion of Science, the Japan Science and Technology Corporation, Fisheries Research Agency, and the Ministry of Agriculture, Forestry and Fisheries of Japan.

REFERENCES

Avery, L. and Horvitz, H. R. (1987). A cell that dies during wild-type *C. elegans* development can function as a neuron in a ced-3 mutant. *Cell* **51**, 1071-1078.

Chen, J. N., Haffter, P., Odenthal, J., Vogelsang, E., Brand, M., van Eeden, F. J., Furutani-Seiki, M., Granato, M., Hammerschmidt, M., Heisenberg, C. P. et al. (1996). Mutations affecting the cardiovascular system and other internal organs in zebrafish. *Development* **123**, 293-302.

Chou, K. C., Tomasselli, A. G. and Heinrikson, R. L. (2000). Prediction of the tertiary structure of a caspase-9/inhibitor complex. *FEBS Lett.* **470**, 249-256.

Cohen, J. J., Duke, R. C., Fadok, V. A. and Sellins, K. S. (1992). Apoptosis and programmed cell death in immunity. *Annu. Rev. Immunol.* **10**, 267-293.

Condorelli, G., Roncarati, R., Ross, J., Jr, Pisani, A., Stassi, G., Todaro, M., Trocha, S., Drusco, A., Gu, Y., Russo, M. A. et al. (2001). Heart-targeted overexpression of caspase-3 in mice increases infarct size and depresses cardiac function. *Proc. Natl. Acad. Sci. USA* **98**, 9977-9982.

Cosulich, S. C., Horiuchi, H., Zerial, M., Clarke, P. R. and Woodman, P. G. (1997). Cleavage of rabaptin-5 blocks endosome fusion during apoptosis. *EMBO J.* **16**, 6182-6191.

Ellis, R. E., Yuan, J. and Horvitz, H. R. (1991). Mechanisms and functions of cell death. *Annu. Rev. Cell Biol.* **7**, 663-698.

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A. and Nagata, S. (1998). A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* **391**, 43-50.

Fernandes-Alnemri, T., Litwack, G. and Alnemri, E. S. (1994). CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1ß-converting enzyme. *J. Biol. Chem.* **269**, 30761-30764.

Friedrich, K., Wieder, T., von Haefen, C., Radetzki, S., Janicke, R., Schulze-Osthoff, K., Dorken, B. and Daniel, P. T. (2001). Overexpression of caspase-3 restores sensitivity for drug-induced apoptosis in breast cancer cell lines with acquired drug resistance. *Oncogene* **20**, 2749-2760.

Imamura, S., Ojima, N. and Yamashita, M. (2004). *In vivo* regulation of the CDC48 gene promoter in zebrafish embryos. *Mar. Biotechnol.* **6**, S1-S7.

Kerr, L. E., McGregor, A. L., Amet, A. L. E., Asada, T., Spratt, C., Allsopp, T. E., Harmar, A. J., Shen, S., Carlson, G., Logan, N. et al. (2004). Mice overexpressing human caspase 3 appear phenotypically normal but exhibit increased apoptosis and larger lesion volumes in response to transient focal cerebral ischaemia. *Cell Death Differ.* **11**, 1102-1111.

Kook, S., Shim, S. R., Choi, S. J., Ahnn, J., Kim, J. I., Eom, S. H., Jung, Y. K., Paik, S. G. and Song, W. K. (2000). Caspase-mediated cleavage of p130cas in etoposide-induced apoptotic Rat-1 cells. *Mol. Biol. Cell* **11**, 929-939.

Kuida, K., Zheng, T. S., Na, S., Kuan, C., Yang, D., Karasuyama, H., Rakic, P. and Flavell, R. A. (1996). Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature* **384**, 368-372.

Lesser, M. P., Farrell, J. H. and Walker, C. W. (2001). Oxidative stress, DNA damage and p53 expression in the larvae of atlantic cod (*Gadus morhua*) exposed to ultraviolet (290–400 nm) radiation. *J. Exp. Biol.* **204**, 157-164.

Liu, X., Zou, H., Slaughter, C. and Wang, X. (1997). DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell* **89**, 175-184.

Malicki, J., Neuhauss, S. C., Schier, A. F., Solnica-Krezel, L., Stemple, D. L., Stainier, D. Y., Abdelilah, S., Zwartkruis, F., Rangini, Z. and Driever, W. (1996). Mutations affecting development of the zebrafish retina. *Development* **123**, 263-273.

Monsoro-Burq, A. H., Duprez, D., Watanabe, Y., Bontoux, M., Vincent, C., Brickell, P. and Le Douarin, N. (1996). The role of bone morphogenetic proteins in vertebral development. *Development* **122**, 3607-3616.

Narula, J., Haider, N., Virmani, R., DiSalvo, T. G., Kolodgie, F. D., Hajjar, R. J., Schmidt, U., Semigran, M. J., Dec, G. W. and Khaw, B. A. (1996). Apoptosis in myocytes in end-stage heart failure. *N. Engl. J. Med.* **335**, 1182-1189.

Nicholson, D. W. and Thornberry, N. A. (1997). Caspases: killer proteases. *Trends Biochem. Sci.* **22**, 299-306.

Raff, M. C. (1996). Size control: the regulation of cell numbers in animal development. *Cell* **86**, 173-175.

Sakahira, H., Enari, M. and Nagata, S. (1998). Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* **391**, 96-99.

Sardella, B. A., Matey, V., Cooper, J., Gonzalez, R. J. and Brauner, C. J. (2004). Physiological, biochemical and morphological indicators of osmoregulatory stress in 'California' Mozambique tilapia (*Oreochromis mossambicus* x *O. urolepis hornorum*) exposed to hypersaline water. *J. Exp. Biol.* **207**, 1399-1413.

Sollid, J., De Angelis, P., Gundersen, K. and Nilsson, G. E. (2003). Hypoxia induces adaptive and reversible gross morphological changes in crucian carp gills. *J. Exp. Biol.* **206**, 3667-3673.

Stainier, D. Y., Fouquet, B., Chen, J. N., Warren, K. S., Weinstein, B. M., Meiler, S. E., Mohideen, M. A., Neuhauss, S. C., Solnica-Krezel, L., Schier, A. F. et al. (1996). Mutations affecting the formation and function of the cardiovascular system in the zebrafish embryo. *Development* **123**, 285-292.

Steller, H. (1995). Mechanisms and genes of cellular suicide. *Science* **267**, 1445-1449.

Thornberry, N. A. and Lazebnik, Y. (1998). Caspases: enemies within. *Science* **281**, 1312-1316.

Uchida, D., Yamashita, M., Kitano, T. and Iguchi, T. (2002). Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish. *J. Exp. Biol.* **205**, 711-718.

Wang, J. and Lenardo, M. J. (2000). Roles of caspases in apoptosis, development, and cytokine maturation revealed by homozygous gene deficiencies. *J. Cell Sci.* **113**, 753-757.

Weil, M., Jacobson, M. D. and Raff, M. C. (1997). Is programmed cell death required for neural tube closure? *Curr. Biol.* **7**, 281-284.

Wen, L.-P., Fahrni, J. A., Troie, S., Guan, J.-L., Orth, K. and Rosen, G. D. (1997). Cleavage of focal adhesion kinase by caspases during apoptosis. *J. Biol. Chem.* **272**, 26056-26061.

Westerfield, M. (1995). *The Zebrafish Book.* Oregon: University of Oregon Press.

Yabu, T., Todoriki, S. and Yamashita, M. (2001a). Stress-induced apoptosis by heat shock, UV and g-ray irradiation in zebrafish embryos detected by increased caspase activity and whole mount TUNEL staining. *Fish Sci.* **67**, 333-340.

Yabu, T., Kishi, S., Okazaki, T. and Yamashita, M. (2001b). Characterization of zebrafish caspase-3 and induction of apoptosis through ceramide generation in fish fathead minnow tailbud cells and zebrafish embryo. *Biochem. J.* **360**, 39-47.

Yokouchi, Y., Sakiyama, J., Kameda, T., Iba, H., Suzuki, A., Ueno, N. and Kuroiwa, A. (1996). BMP-2/-4 mediate programmed cell death in chicken limb buds. *Development* **122**, 3725-3734.

Zheng, T. S., Schlosser, S. F., Dao, T., Hingorani, R., Crispe, I. N., Boyer, J. L. and Flavell, R. A. (1998). Caspase-3 controls both cytoplasmic and nuclear events associated with Fas-mediated apoptosis *in vivo*. *Proc. Natl. Acad. Sci. USA* **95**, 13618-13623.