EXPRESSION AND FUNCTION OF THE SMALL HEAT SHOCK PROTEIN HSP27

DURING EMBRYOGENESIS OF ZEBRAFISH *DANIO RERIO*

By

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Abstract

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The expression of the small heat shock protein 27 (Hsp27) is increased in response to diverse stress conditions. During early embryogenesis of mammals, including humans, Hsp27 is also constitutively expressed in skeletal and cardiac muscles. Results of previous *in vitro* studies conducted using cultured mammalian myoblasts have suggested that Hsp27 plays a role in regulating cell differentiation or preventing apoptotic cell death during myogenesis. However, *in vivo* studies testing this conclusion have not been conducted. Recently, we identified a homolog of Hsp27 in zebrafish, *Danio rerio,* and characterized the expression of Hsp27 in skeletal and cardiac muscles in zebrafish embryos. Here, we examined the role of Hsp27 in embryogenesis using specific morpholino oligonucleotides to knockdown expression of Hsp27 in the whole zebrafish embryo. Surprisingly, development of zebrafish embryos lacking Hsp27 was normal in the absence of stress. However, Hsp27 knockdown embryos showed decreased motility and heart rate beatings compared to control embryos after sublethal heat shock. Analysis of skeletal muscle birefringence in living embryos before and immediately after heat shock revealed that the Hsp27 knockdown is correlated with short term loss of muscle integrity after injury. Defects in skeletal muscle tissues were also persistent in Hsp27 knockdown

embryos 18 hours after injury. Finally, knockdown of Hsp27 resulted in dramatic disruption of motor neuron networks in heat stressed, but not control embryos. These findings demonstrate that Hsp27 is required for injury resistance of muscle tissues and nerves, but not morphogenesis, in zebrafish embryos.

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Chapter 1

INTRODUCTION

1.1 Background

Heat shock proteins (HSPs) play essential roles in all stages of an organism's life. Well known functions of HSPs include folding of nascent proteins and refolding of proteins damaged as a consequence of thermal and other stresses [1-3]. Less well known, but nonetheless vital functions include participation as cofactors in receptor mediated signaling, scaffolding proteins for signaling elements, maintenance of cellular redox states and regulation of cytoskeletal structures [4-8]. Failure of these activities underlies numerous and important human diseases [9]. Moreover, the functions of HSPs are conserved in all living organisms from bacteria to humans [10].

HSPs represent a family of non-related proteins that have been grouped together based on one common feature – they are induced as a result of stress. An entire family has been identified and classified on the basis of molecular weight [11]. The high molecular weight proteins (ranging in size from approximately 60 to 110 kDa) posses the ATP-binding domain and they are linked with the cytoprotective properties and chaperoning activity in the cell. In contrast, the small molecular weight proteins (sHsp) are a very diverse group of proteins ranging in monomeric masses from 12 to 43 kDa. The sHsp all possess an evolutionarily conserved C-terminal region called the crystallin domain, but lack the ATP-binding domain. Despite the diversity among the members in sequence and size, they all form large oligomers and have a dynamic quaternary structure [12]. In addition, sHsp are found

in a range of cellular compartments and bind a variety of substrates, therefore, they attracted much attention as they are implicated in many different stresses [12-14]. One of the most widely studied sHsp has a molecular mass of 27 kDa (Hsp27). Among the ten members of sHsps in humans, Hsp27 (or its homolog Hsp25 in mice) is considered a true heat shock protein characterized by an enhanced expression in response to stressful conditions [15, 16], whereas the expression of other sHsp members family is induced by stress.

1.2 Physiological role of Hsp27

Hsp27 is constitutively expressed in some cell types, including all types of muscle cells [17, 18]. *In vitro* experiments have proposed that Hsp27 acts as a phosphorylation-state dependent molecular chaperone that can assist the refolding of proteins from terminal aggregation [15]. Therefore, binding of Hsp27 to proteins in their non-native conformations could be part of the chaperone network. Another role of Hsp27 is linked to its ability to inhibit actin polymerization by behaving as an actin-capping protein [19]. It is not yet clear whether such inhibition is due to its direct interaction with actin itself or with another actin-binding protein. However, it was demonstrated that cells with increased phosphorylation of Hsp27 showed increased recovery from treatments with cytoskeletal disruptors, such as cytochalasin D or severe heat shock [20, 21]. Thus, Hsp27 in its phosphorylated form could play a role in reorganization and stabilization of F-actin architecture.

Phosphorylation regulates oligomeric structure of Hsp27 as well as its distribution in the cell. In response to stresses Hsp27 is rapidly phosphorylated at three serines: 15, 78 and 82 by the p38 mitogen-activated protein kinase protein-activated kinase 2

(MAPKAPK2) [20, 22, 23]. Under normal conditions Hsp27 is present in high-molecular weight oligomers with unclear quaternary structure [24]; however, upon stress-induced phosphorylation, Hsp27 is dissociated into smaller oligomers, including dimers that could associate with cytoskeletal structures, such as microfilaments (F-actin) and cellular junctions [7, 25]. It was also demonstrated that dimers of Hsp27 do not exhibit protective functions, but larger oligomers form aggregates that possess such activity [26]. However, further studies contradict this hypothesis that phosphorylation of Hsp27 is indeed necessary for cell protection [27, 28]. Also, phosphorylation mediates nuclear translocation of Hsp27 and its association with intra-nuclear components in a specific manner [29]. However, the role of nuclear translocation of Hsp27 is not fully understood.

In mammals, Hsp27 has a broad tissue distribution, and it is stress-induced in most cell types [15, 17]. Interestingly, Hsp27 is also constitutively expressed in all types of muscle cells [30-32]. However, its role in muscles is not completely understood at this time. In addition to muscle tissues, Hsp27 was also detected in embryonal neurons of the spinal cord and the purkinje cells [33]. Current findings suggest that expression of Hsp27 is elevated in damaged or developing axonal cells suggesting its protective function during neuritogenesis and further neurite growth [34]. Missense mutations in the human *hsp27* gene have also been found to lead to neuropathies including Charcot-Marie-Tooth disease (CMT), distal hereditary neuropathy (HMN), and Alexander-disease [35, 36].

Although the exact mechanism of Hsp27 function in the cell is not yet understood, its overexpression is linked to increased resistance to death during oxidative stress [7,

37], heat shock [38], hypoxia [39], toxic metals [40], and other injuries (reviews: [16, 41, 42]. It was also shown that Hsp27 negatively regulates apoptosis [43-45]. These later findings may explain the correlation between elevated levels of Hsp27 and poor prognosis in patients with gastric, liver, prostate carcinoma, and osteosarcomas [46]. A number of hypotheses have been proposed in which Hsp27 confers increased cell protection against injury. One of these hypotheses is based upon the chaperone-like activity of Hsp27 preventing aggregation of denatured proteins. Another hypothesis attributes Hsp27 as an anti-apoptotic agent that influences the cytochrome C mediated pathway [43, 45]. Exactly which of these mechanisms occurs *in vivo* is yet to be determined. Studies of Hsp27 overexpression in *Drosophila* showed increased lifespan within motor neurons by more than 30% as well as increased resistance to oxidative injuries induced by paraquat and temperature [47].

1.3 Hsp27 expression during embryogenesis

Studies in flies, fish, frogs and mice have concluded that Hsp27 is upregulated early during development [33, 48-50]. Developmental expression of Hsp27 is especially evident in myoblasts and developing muscle tissues *in vivo*, and this expression pattern is recapitulated in part in cultured mammalian myoblasts undergoing differentiation *in vitro* [51]. These results suggest that Hsp27 expression is evolutionarily conserved and may be an important feature of myogenesis in chordate animals. Additional *in vitro* data have provided support for this hypothesis. For example, the expression of antisense *hsp27* mRNA has been shown to inhibit differentiation of cultured murine myoblasts and result in enhanced cell death of

cultured embryonic stem cells [52]. Therefore, it was proposed that Hsp27 acts as a switch between differentiation and apoptosis in murine stem cells.

Another study by Ito and colleagues addressed the role of Hsp27 and its phosphorylation during C2C12 myoblast differentiation that was induced by serum withdrawal [53]. Their primary finding suggested that phosphorylated Hsp27 negatively affects myogenic differentiation. However, these studies involved inhibitors of p38 MAP kinase that is not only linked to Hsp27 phosphorylation, but also stimulates myogenic transcription factor MEF2 which is crucial for differentiation of myoblasts *in vivo* [54]. Therefore, the role that Hsp27 plays during of myoblasts is not clear. As a result, there is a gap in understanding the purpose of upregulation of Hsp27 expression during development. This deficit of understanding can be resolved by studies on Hsp27 knockouts. However, preliminary experiments in mammals are complicated by the fact that a Hsp27 gene knockout is embryonic lethal, apparently due to problems with embryo implantation. Several studies reported a sharp increase of Hsp27 in rat and human endometrium after ovulation [34, 55, 56]. In view of known functions of Hsp27, it is likely that it is involved in protection of the endometrial proteins against factors that may lead to protein denaturation and embryo abortion. However, recent publication by the Moskophidis group has developed a mouse model with targeted *hsp27* gene disrupted expression [57]. It is early to draw definitive conclusions about the role of Hsp27 *in vivo* but the availability of *hsp27*-deficient mice grants with opportunity to not only to further develop understanding of Hsp27 cytoprotective functions but also discover its roles during embryo development.

Chapter 2

HSP27 IS REQUIRED FOR STRESS TOLERANCE BUT NOT FOR

MORPHOGENESIS OF ZEBRAFISH *DANIO RERIO*

2.1 Introduction

Heat shock proteins play essential roles in all stages of an organism's life. Well

known function of heat shock proteins include folding of nascent proteins and

refolding of proteins damaged as a consequence of thermal and other stresses.

However, less well known but nonetheless vital functions include participation as

cofactors in receptor mediated signaling, scaffolding proteins for signaling elements,

maintenance of cellular redox states and stabilization of cytoskeletal arrays (recent

reviews [16, 42, 58, 59]). High molecular weight heat shock proteins use the energy

of ATP to modify or stabilize the conformation of other proteins. In contrast, small

heat shock proteins (sHsp) lack ATPase activity, but can undergo phosphorylation

dependent transition between high molecular weight oligomers and smaller subunits

to acquire its cytoprotective functions.

Hsp27 is one of the most widely expressed and distributed member of the sHsp

family. In addition to stress-induced expression, Hsp27 is constitutively expressed in

some cell types, including muscle cells [60]. The role of Hsp27 in muscle is not

completely understood. There is some evidence that hsp27 is required for

differentiation of muscle cells. Foe example, Hsp27 expression begins early during

differentiation of fibroblast-like myoblasts into multinucleate muscle cells. This

pattern of expression has been observed both in myoblast cell cultures as well as

murine embryos [33]. Recently, our laboratory identified an Hsp27 homolog in

zebrafish *Danio rerio* [61]. We demonstrated that zebrafish Hsp27 is expressed in developing skeletal and heart muscle tissues, lens, and head region. Moreover, we have generated a transgenic fish line that expressed cyan fluorescent protein (CFP) under the control of Hsp27 promoter. As a result, the reporter was detected predominately in cardiac and skeletal muscle under normal conditions (Appendix A).

Studies in zebrafish showed that *hsp27* mRNA levels were transiently up-regulated and heat-inducible during embryogenesis, supporting a conserved role for Hsp27 in developing vertebrate embryos [33, 49, 50]. The primary advantage of the zebrafish system lies in its ease for genetic manipulation. We designed antisense Hsp27 specific morpholino oligonucleotides to prevent transcription of *hsp27* mRNA. Moreover, fish embryogenesis takes place without maternal involvement, and hence, the requirement for implantation is alleviated, which provides an opportunity to study gene knockdown in the developing embryos. We tested our primary hypothesis that Hsp27 knockdown would disrupt normal development of zebrafish embryos through negative regulation of myoblast differentiation. Surprisingly, we found that expression of Hsp27 is not required for normal organogenesis in zebrafish embryos. These results are similar to those observed in the developing knockout mice lacking related small heat shock proteins, α-B crystallin and HSPB2 [62]. However, our studies indicate that Hsp27 is needed for stress tolerance during development. Specifically, embryos lacking Hsp27 were more susceptible to the motor neuron damage during heat shock compared to the embryos with intact Hsp27 levels. These data reveal that Hsp27 plays a unique and essential role in generating stress tolerance in intact embryos and provide new insights into the functional significance of Hsp27 *in vivo*.

2.2 Materials and Methods

Embryos and fish maintenance and heat shock

Adult zebrafish, *Danio rerio*, were reared and maintained as described previously [61]. Embryos were collected from the wild-type crosses and maintained at 26°C incubator in "Embryo medium" (10% Hank's buffer with full strength calcium and magnesium) [63]. Heat shock treatment for all experiments consisted of placing embryos in a circulating water bath (Fischer Scientific, IsoTemp 2150) in sealed 6-well plates for 30 min at 39°C. This treatment resulted in 95% survival for all groups of embryos (data not shown).

Production of antibody

The cDNA sequence coding for Hsp27 in zebrafish was isolated from RNA of zebrafish embryos with gene specific primers using reverse transcriptase polymerase chain reaction (RT-PCR) [61]. Expression vectors were generated in the pGEX6p-Hsp27 and transformed into *E.coli* strain BL21 and protein production induced by addition of isopropyl-ß-D-thiogalactopyranoside (IPTG). The expressed glutathione *S*-transferase (GST) fusion protein was purified using the Talon system (Biosciences-Clontech of Palo Alto, CA). Purified antigens were injected into New Zealand white rabbits using TiterMax Gold adjuvant during the first immunization step. Two months later the immunization with the incomplete Freund's adjuvant was repeated to boost the immune response. Final bleed was performed two weeks after the boosting injections. The antiserum was tested against purified recombinant Hsp27 and whole protein extracts from zebrafish embryos expressing Hsp27 and lacking Hsp27 as a consequence of morpholino knockdown.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting

Whole zebrafish embryos were collected in pools of 20 embryos/sample and homogenized in SDS-PAGE sample buffer (100 mM Tris/HCl (pH8), 50 mM EDTA, 1% SDS, 50 mM dithiothreitol, 0.01% bromphenol blue, 10% glycerol). Samples were sonicated on ice and cleared by centrifugation at 10,000g for 10 min at 4°C. The concentration of total protein in resulting samples was measured using Protein Assay Kit (Bio-Rad). Except when otherwise indicated, 30 μg of total protein was loaded into each lane of a gel composed of 4% acrylamide stacking gels and separated in 12% gels at 150V [64]. Resolved proteins were transferred onto nitrocellulose membranes (GE Water & Process Techologies, NitroBind, 0.22 μm) and membranes processed for Western blot detection of proteins using the polyclonal antisera described above or anti-α-sarcomeric actin that recognizes zebrafish actin (Sigma, mouse ascites fluid, #A2172, clone 5C5) and horse radish peroxidase labeled secondary antibodies. Western blots were developed and proteins analyzed as described previously [61]. Experiments were repeated with three independent protein preparations. For quantitative comparisons of expression levels, measured integrated optical density (IOD) values of bands detected on Westerns for Hsp27 were normalized to values measured from samples collected at 0 hours post fertilization (hpf).

Morpholino oligonucleotide microinjections

Antisense morpholino (MO) oligonucleotides were obtained from GeneTools, LLC (Philomath, OR). MO oligonucleotide sequences were: Hsp27 antisense (α27), 5′– GTTTTG AAGAGTTGTT TTTCGGCTC–3' and scrambled control (scr), 5′– GGAGCTTAGTGATGTTCTGTTCTTT–3'. The antisense Hsp27 MO used in our

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studies was complimentary to the 5' non-transcribed region of Hsp27 mRNA at position −10 corresponding to the translation start. The scrambled morpholino has the identical nucleotide composition arranged in random order and was used as a negative control for all morpholino experiments. MO were diluted in sterile water to 0.15 mM and frozen at -80°C. Zebrafish embryos were injected at one- to four-cell stage with either MO solution or water alone as a control, using a Narishige (MMN-1) coarse micromanipulator and Applied Scientific Instrumentation (MPPI-2) microinjector. Needles were pulled from the borosilicate filaments (Sutter Instruments Co, Novato, CA; 1.00mm x 0.78mm x 10 cm) using a pipette puller (Sutter Instruments Co, Novato, CA; Model P-87) with the following parameters: heat – 384, pull – 150, velocity – 80, time – 200, and pressure – 200. Injections were carried out as described previously [65] and injected embryos were cultured in "Embryo medium" at 26°C.

Fluorescent labeling of embryos

To image neurons, 50 hpf embryos were dechorionated, fixed for two hours in 4% paraformaldehyde in PBS (pH 6.4) at room temperature and stored overnight in PBST (PBS, 0.05% Tween-20, Sigma) at 4°C. Embryos were then permeabilized for 10 min in 0.5% Trypsin (Trypsin-EDTA, 1X, Gibco), 1% Triton X-100 (Sigma) in PBS with gentle shaking. Embryos were washed four times with 1% Triton in PBST for 10 min each and blocked for 1 hr at room temperature with 10% normal goat serum in PBST. Embryos were then incubated overnight at 4°C with primary antibodies against acetylated tubulin (Sigma, mouse ascites fluid, #T-6793, clone 6-22B-1) diluted to 1:300 in 10% goat serum and 1% BSA in PBST with gentle shaking. Embryos were washed for 3 hours at room temperature in three changes of PBST.

Embryos were then incubated with fluorescein isothiocyanate (FITC) labeled secondary antibodies (Jackson Immunochemicals, West Grove, PA, #115-095-071) and rinsed in the same manner. To image myofibrils, permeabilized embryos were stained for F-actin with rhodamine labeled with phalloidin (0.1 μg/mL) for 1 hr at room temperature, and then rinsed twice for 1 hour in PBST. Fluorescently labeled embryos were mounted on slides with Mowiol containing an antifade agent (DABCO, Sigma) and imaged using Zeiss LSM 510M confocal microscope (Carl Zeiss Inc., Thornwood, NY) and a 20X 1.2 NA glycerol objective. For imaging of neurons, a through-focus series of images in the Z dimension were collected for each embryo at 3μm intervals midway between the end of the developing gut and the tip of the tail. For analysis of myofibrils, the 12th myotomes, positioned at the caudal tip of the developing gut, were imaged.

Swimming assay and heart beat measurements

Embryos injected with water, scr-MO or antisense Hsp27 MO were raised to 36 hpf and divided into two groups. One group was heat shocked at 39°C for 30 min, while the other was untreated (control). Both groups were incubated overnight at 26-28°C. Manually dechorionated 48-50 hpf embryos were placed in a 10 cm petri dish in "Embryo medium" in a temperature controlled chamber at 26°C. Swimming was initiated by touching the embryo on the side. Swimming behavior was recorded for 10 sec with a CCD camera (Panasonic, Secaucus, NJ; WV-BP314) at a frame rate of 23 frames/sec. Images were captured and stored using a Macintosh station equipped with a Scion LG3 image capture board. The XY coordinates of each embryo in each frame of videos was determined using NIH Image (NIH, Bethesda, MD) and exported to Microsoft Excel which was used to calculate the total distance

between embryo positions in sequential frames. Total distance and rate of

swimming for each embryo were averaged from two swimming trials. The number of

embryos examined in three independent experiments was: water injected: control

(38), heat shock (35); scr-MO injected: control (37), heat shock (30); antisense

Hsp27 MO injected: control (36), heat shock (33).

Heart rates were measured by counting heart beats in 15 sec intervals for 40-42 hpf

embryos injected with water, scr-MO or antisense Hsp27 MO. Similarly to the

swimming assay, embryos in each category were subdivided in two groups and left

untreated (control) or heat shocked as described above. The beats were counted by

eye using a dissecting microscope Nikon (SMZ-2T). Data were obtained from a total

of 16 embryos in each test group in three independent trials.

Analysis of myofibril integrity *in vivo*

Embryos were injected with water, scrambled MO and antisense Hsp27 MO at one-

to four-cell stage and incubated at 26°C. 30 hpf embryos were manually

dechorionated in "Embryo medium" containing 160µg/ml Tricane (Sigma). Myofibril

birefringence, an indicator of actin filament content, was imaged using a Zeiss

AxioVert 200M equipped for polarized light microscopy and a 20X 0.45 NA objective

lens. Embryos were oriented at 45° relative to the polarizer analyzer. Camera

settings and exposure parameters were kept constant throughout experiments. For

each individual embryo, myotomes directly above (dorsal to the tip of the developing

gut) were imaged before and immediately after heat shock (39°C for 30 min), and the

birefringence intensity of myomeres measured using NIH Image (NIH, Bethesda,

MD). Ratios of values before and after heat shock were measured and average ratios calculated for each group of embryos.

Analysis of motor neuron integrity

Confocal Z-stacks of images were combined using an algorithm that placed the brightest pixel in each image into a final projected image. All images were obtained at the same magnification and covered about 6 muscle segments, or myotomes. Projected images were then analyzed in three ways. First, three investigators assessed overall neuron integrity on a scale of zero to four. A score of four was assigned to images showing uniform staining of all motor neuron pairs innervating all visible myotomes. A score of zero was assigned to images that lacked any intact motor neuron pairs. The number of neuronal pairs in each field of view were also evaluated. A neuron pair was counted if both branches were distinct and visible. Finally, the number of cell bodies in the area of the spinal chord stained positively with anti-acetylated-tubulin antibodies was counted. All images were analyzed by three independent investigators in a blinded manner.

Myotome area measurements

Overall integrity of muscle segments was assessed by measuring the area of the 12th myotome for each embryo, using tools in ImageJ 1.37v (NIH, Bethesda, MD). Values were obtained in three experiments with the following total number of embryos: water injected: control (22), heat shock (20); scr MO injected: control (23), heat shock (22) antisense Hsp27 MO injected: control (22), heat shock (20).

Statistical analysis

Average values measured for treated embryos were compared to values measured from untreated, water injected control embryos using a Student's T-test. Calculated "p" values of less than or equal to 0.05 were considered an indication of statistically significant differences in average values.

2.3 Results

Hsp27 expression during zebrafish embryogenesis

We have previously demonstrated upregulation of zebrafish *hsp27* mRNA levels in zebrafish embryos [61]. In the present study, we developed a polyclonal antibody against full length zebrafish Hsp27 protein. Figure 1A shows a Western blot of equal amounts of total protein isolated from embryos at varying hours post fertilization (hpf) probed with our antisera. A single protein band of approximately 22 kDa (the predicted MW of zebrafish Hsp27) is detected, consistent with our previous analysis of *hsp27* mRNA expression kinetics. Hsp27 expression is detected as early as 8 hpf, and IOD values increase approximately 7 fold between 9 and 24 hpf. Unlike *hsp27* mRNA expression levels that declined at 36 hpf as noted in our previous study [61], Hsp27 protein levels remained constant between 24 hpf and hatching at 72 hpf, although whole-embryo content of Hsp27, calculated as a fraction of total protein, did decline at 1 week post fertilization. It is notable that only a single protein band was detected on these blots, indicating that zebrafish embryos do not upregulate expression of other small heat shock proteins in response to Hsp27 knockdown.

Morpholino knockdown of Hsp27

To investigate the functional role of Hsp27 in zebrafish embryos, we performed gene knockdown of Hsp27 using oligonucleotide morpholinos. The 5' untranslated region of zebrafish *hsp27* gene sequence, the complementary sequence, the sequence of our anti-Hsp27 morpholino oligonucleotide (MO) sequence, and the sequence of the scrambled control MO are shown in Figure 2A. Figure 2B shows results of injecting antisense (α27) and scrambled (scr) morpholinos at varying initial concentrations into the yolks of one- to four-cell stage embryos on Hsp27 expression level, as evaluated by Western blotting. Total harvested protein on blots was also detected using Ponceau S staining. Injection of the antisense, Hsp27 specific MO totally prevented expression of Hsp27 at all concentrations shown and at concentrations as low as 0.1 mM (not shown). In contrast, injection of 1 mM solutions of the scrambled MO had no effect on Hsp27 expression. Also evident is that no immunologically related proteins are upregulated in embryos after Hsp27 knockdown.

We next examined the effectiveness of MO knockdown as a function of stress and time. Figure 2C shows Hsp27 expression changes in embryos injected with either antisense Hsp27 or scrambled MO at 0.15 mM, followed by heat shock (39°C for 30 min at 30 hpf) and subsequent recovery at 26°C for 13 hours prior to protein harvest. Anti-sarcomeric actin Western blotting is shown as a loading control. Injection of scrambled MO did not prevent upregulation of Hsp27 expression in response to heat shock. However, Hsp27 was not be detected in either control or heat shocked embryos injected with antisense Hsp27 MO. Finally, the efficacy of gene knockdown was examined as a function of time post injection (Fig. 2D). One- to four-cell stage embryos were injected with either water, scrambled MO, or antisense Hsp27 MO, and harvested at indicated times and Western blots probed for Hsp27 and

sarcomeric actin, as a loading control. Additionally, Figure 2D indicates that

antisense Hsp27 MO injections, performed in the yolk at the one- to four-cell stage

were effective in reducing Hsp27 protein expression to undetectable levels over a

period of up to one week post-fertilization. Finally, the total amount of expressed

Hsp27, as a proportion of total protein, declines dramatically between embryos

harvested early during development and larvae collected 1 week post-ferilization.

Zebrafish embryonic development and muscle function is normal in the

absence of Hsp27

Previous studies have indicated that Hsp27 could play a role in regulating

differentiation or survival of mammalian embryonic cell types [52, 66]. However,

comparison of the overall morphology of embryos injected with water, scrambled MO

and antisense Hsp27 MO and developed until 36 hpf did not reveal overt changes in

the appearance of resulting embryos (Fig. 3A). To provide a quantitative

assessment of the morphology of these embryos, we measured the area of the yolk

sack (Fig. 3B), the length of the tail (Fig. 3C) and the diameter of the lens (Fig. 3D),

for each embryo. None of these parameters showed statistically significant

differences in average value among tested groups. Average measurements ($\pm$ SD)

of yolk were 0.22 mm^2 $\pm$ 0.01 (n=23) for water injected, 0.20 mm^2 $\pm$ 0.02 (n=24) for

scrambled MO, and 0.21 mm^2 $\pm$ 0.02 (n=22) for antisense Hsp27 MO (Fig. 3B).

Average tail lengths for water injected embryos were 1.99 cm $\pm$ 0.12 (n=30), for

scrambled MO 2.01 cm $\pm$ 0.06 (n=30), and 1.98 cm $\pm$ 0.09 (n=29) for antisense

Hsp27 MO (Fig. 3C). Finally, lens diameter was 67.2 µm $\pm$ 7.6 (n=22) for water

injected, 65.6 µm $\pm$ 7.3 (n=23) for scrambled MO, and 64.3 µm $\pm$ 6.1 (n=21) for

antisense Hsp27 injected embryos (Fig. 3D). In addition, results described below

indicate that morphological development of nerves and muscle segments (myotomes), as well as motility of embryos, are unaltered in unstressed embryos. Taken together, these data indicate that Hsp27 is dispensable for normal morphogenesis in zebrafish under normal conditions.

Motility and heart function are compromised in heat shocked embryos lacking Hsp27

Hsp27 has been shown to protect actin filament arrays from ischemic and temperature induced disassembly in cultured muscle and non-muscle cells [38, 67, 68]. We therefore examined whether Hsp27 expression was required for muscle function in zebrafish embryos after heat shock. First, the effect of Hsp27 knockdown on the function of skeletal muscle was analyzed in swimming behavior assay. Figure 4A shows representative swimming paths of 50 hpf embryos from each experimental group tested without stress or after 18-hour recovery from a heat shock applied at 30 hpf. Note that the delay in analysis in this experiment was necessary, in part, because embryos younger than 50 hpf exhibited little swimming activity. We also quantified parameters of swimming behavior in these embryos (Fig. 4B and 4C). Under control conditions, water injected embryos traveled (± standard error of the mean (SEM)) 4.69 cm ± 0.28 (n=36) at 2.52 cm/s ± 0.07 (n=32), scrambled MO injected embryos showed similar results 4.70 cm ± 0.42 (n=37) at 2.77 cm/s ± 0.10 (n=40) and antisense Hsp27 MO embryos swam 3.8 cm ± 0.28 (n=36) at 2.51 cm/s ± 0.07 (n=41). In embryos recovering from heat shock, water injected embryos traveled (±SE) 4.14 ± 0.37 cm at 2.59 ± 0.07 cm/s (n=35), scrambled MO injected embryos traveled 4.27 ± 0.32 cm at 2.44 ± 0.06 cm/s (n=40) and antisense Hsp27 MO injected embryos traveled 1.27 ± 0.16 cm at 1.22 ± 0.04 cm/s (n=33). No

statistically significant differences in measured values for motility were found in studies of embryos under control conditions. These results, similar to the morphological data presented above, illustrate that functional development of muscle tissues is normal in zebrafish embryos in the absence of Hsp27. In contrast, Hsp27 knockdown animals, but not embryos injected with water or scrambled MO, displayed a statistically significant (p<0.05) reduction in average swimming rate (approximately 50%) and 75% reduction in swimming distance, compared to water injected or scrambled MO-injected control animals (Fig. 4B and 4C, ⊠ shaded bars).

Because we previously detected *hsp27* gene expression in hearts of zebrafish embryos [61], we also assessed whether the loss of Hsp27 expression could affect heart functioning in this experimental system (Fig. 5). Under normal conditions, embryos that were injected with water, scrambled MO and antisense Hsp27 MO had heart rates of 116 beats/min ± 8 (n=16), 117 beats/min ± 7 (n=16) and 107 beats/min ± 7 (n=16), respectively (Fig. 5, ◼ solid bars). In embryos recovering from heat shock, measured values for heart rate in the same groups were 102 ± 7 (n=16), 98 ± 5 (n=16), and 81 ± 5 (n=16) (Fig. 5, ⊠ shaded bars). There was no statistically significant difference in these average values. Heat shock of embryos injected with water or scrambled MO resulted in a small decrease in average heart rates, relative to control non-stressed animals, but this decrease was not statistically significant. However, in contrast, heat shock of animals lacking Hsp27 resulted in an approximately 20% decrease in the heart rate, relative to non-stressed antisense Hsp27 MO injected animals of the same stage (Fig. 5, ⊠ shaded bars).

Morpholino knockdown of Hsp27 results in immediate and persistent defects in muscle architecture after heat shock

The defects in swimming behavior reported above could have resulted because of damage to muscle tissues, motor neurons, or both. We first examined the integrity of muscle tissues both immediately after heat shock and after an overnight recovery at times when swimming assays were described and performed. To assess integrity of muscle tissues in individual embryos before and after heat shock, we imaged muscle tissues in living, anesthetized embryos using polarized light microscopy and measured the intensity of muscle fiber birefringence of the same embryo before and after heat stress. Figure 6A shows representative images and Figure 6B and 6C show results of quantitative analysis for a total of eight embryos in each experimental group. Average values ($\pm$ standard error of the mean (SEM)) for birefringence of muscle fibers were not significantly different between experimental groups before heat shock (Fig. 6B). Additionally, birefringence of muscle tissues in water and scrambled MO injected embryos was not significantly altered by heat shock (Fig. 6C), compared to values measured in the same animal before heat shock. However, embryos lacking Hsp27 expression as a consequence of antisense Hsp27 MO injections showed a significant loss, which was approximately 50% of measured muscle birefringence, after heat shock.

Muscle integrity of embryos was also assessed immediately after performing swimming assays by fluorescence imaging of fixed, phalloidin stained embryos. Figure 7A shows representative images of control and heat shocked embryos for each experimental group. At this time point, no significant differences were observed in the phalloidin staining of muscle tissues among the embryos examined

(data not shown), suggesting that repair of actin filaments occurred during the interval between heat shock and at times when swimming assays were performed. We also failed to detect obvious lesions in muscle structure between groups of embryos (Fig. 7A) or changes in number or integrity of muscle cell nuclei (not shown). However, when we measured the total area of the 12th myotome in each embryo, a small but statistically significant (*= $p < 0.05$) decrease in myotome area was detected after heat shock in animals lacking Hsp27 (⊠ shaded bars), but not in those injected with water or scrambled MO (Fig. 7B).

Morpholino knockdown of Hsp27 results in loss of motor neuron integrity in heat shocked embryos

Although experiments presented here demonstrate that the loss of Hsp27 function results in defects in muscle structure, these defects did not appear sufficient to account for the dramatic loss of mobility observed in the swimming assays. Consequently, we examined the integrity of motor neurons in embryos fixed immediately after swimming assays at 50 hpf (Fig. 8). Water injected embryos incubated under control conditions and probed with anti-acetylated-tubulin antibodies displayed brightly and uniformly stained pairs of motor neuron processes innervating each muscle segment (white arrows, Fig. 8A). Embryos injected with scrambled MO showed a similar staining pattern under control conditions. However, neuronal processes in Hsp27 knockdown animals displayed less uniform staining when fixed under control conditions and the same age (Fig. 8A). These differences were much more dramatic in heat shocked animals. Water and scrambled MO injected embryos contained clearly detectable pairs of motor neurons at each muscle segment (white arrows, Fig. 8A). Conversely, Hsp27 knockdown animals displayed fewer stained

motor neurons. In addition, heat shocked Hsp27 knockdown animals displayed a large number of acetylated-tubulin positive cell bodies in the area of the spinal cord (gray arrows, Fig. 8A). Such accumulation of acetylated-tubulin cell bodies could have resulted from the collapse of the tubulin in axonal parts of neuron cells.

To assess these changes in a population of embryos, images of acetylated-tubulin staining were scored blindly by three independent investigators for overall integrity of motor neurons (Fig. 8B), number of intact motor neuron pairs (Fig. 8C) and number of acteylated-tubulin positive cell bodies (Fig. 8D), as described in Materials and Methods. Examination of these data suggest that the loss of Hsp27 resulted in a reduction of the integrity of motor neurons and uniformity of anti-acetylated-tubulin staining, even under control conditions (■ solid bars), and also that heat shock caused some disruption of motor neurons in water and scrambled MO injected animals (▨ shaded bars). On the other hand, these differences were not statistically significant with a p value of ≥ 0.05. In contrast, Hsp27 knockdown animals showed statistically significant changes in all measured parameters of neuronal integrity (p≤0.05).

2.4 Discussion

Hsp27 expression is upregulated early during development of mammals and other vertebrate embryos including frogs and fish [33, 49, 50]. Developmental expression of Hsp27 is especially evident in myoblasts and developing muscle tissues in vivo, and this expression pattern is recapitulated in cultured mammalian myoblasts undergoing differentiation *in vitro* [15, 66]. These observations have supported the view that Hsp27 plays a role in embryogenesis or myogenesis. *In vitro* studies of

mammalian myoblast differentiation have appeared to confirm this hypothesis. For

example, reduction of Hsp27 expression in stable transformed cell lines expressing

full-length antisense Hsp27 mRNA inhibits differentiation of mammalian myoblasts

and promoted apoptosis of embryonic stem cells [66]. In contrast, a transgenic

mouse line lacking Hsp27 expression has been produced that lacks obvious

developmental defects [57]. However, such stable lines of animals could have

arisen by a process that selects embryos that can compensate for the loss of Hsp27.

In previous studies, we have demonstrated that zebrafish Hsp27 is homologous to

mammalian Hsp27 at the sequence level and provides thermoprotection to

mammalian cells in a manner that is functionally indistinguishable from human

Hsp27 [61]. In addition, we showed that the pattern of Hsp27 expression in

zebrafish embryos closely mimics that observed in mammalian embryos [49]. In the

present work we report results of the first transient gene knockdown studies of

Hsp27 in a vertebrate animal. Similar to results recently reported for stable

transgenic mice lacking Hsp27 [57], we find that morphogenesis of zebrafish

embryos is normal in the absence of Hsp27 (Fig. 3). It remains possible that loss of

Hsp27 expression in our experiments was compensated for by expression of a

related protein. This possibility has not been completely eliminated by studies

conducted here or in the previous published studies of Hsp27 knockout mice.

However, we and others have detected a major form of α-B crystallin in zebrafish,

but have been unable to detect the expression of this protein in muscle tissues under

any circumstances [49, 69]. Haung *et al* also observed no upregulation of

mammalian -B crystallin in mice lacking Hsp27. Other members of the crystallin

family are expressed in zebrafish and mRNA for at least one can be detected in

muscle tissues [70, 71]. However, Western blotting studies using a polyclonal

antisera against zebrafish Hsp27 did not detect expression of closely related proteins in antisense Hsp27 MO injected animals, even after heat shock (Fig. 2). In addition, dual gene deletion mice have been created that lack genes for the related small heat shock proteins -B crystallin and HSPB2. Although these animals develop muscle and skeletal defects as a function of age, they successfully complete embryogenesis in an apparently normal manner [72]. Taken together, the present and previous results support the hypothesis that sHsp expression is dispensable for normal vertebrate embryogenesis, but plays a role in stress tolerance and maintenance of muscle tissues.

Despite the apparent lack of a requirement for Hsp27 during development, studies in a variety of vertebrates make clear that Hsp27 is expressed early and transiently during myogenesis. For example, we previously showed that Hsp27 mRNA expression in zebrafish 24 hpf embryos was greater than that of control adult muscle tissues [49]. A similar transient elevation in sHsp expression has been reported in tissues of embryonic and perinatel mammals [57, 73]. Here, we also demonstrate that Hsp27 protein expression one day post fertilization is greater than that observed at one week of development (Fig. 2). It remains possible that a subtle developmental requirement for Hsp27 during myogenesis can be revealed by further experiments, or that the assays of muscle function in the present study failed to detect subtle changes resulting from the absence of Hsp27. For example, image analysis of acetylated tubulin staining in control embryos reveal a reduction in the integrity of motor neurons in animals lacking Hsp27 under control conditions (Fig. 8B and 8C). This reduction did not achieve statistical significance in the absence of stress in our studies, but may have if a greater number of embryos had been

analyzed. It is possible that Hsp27 specific morpholinos used in our studies produced a slightly greater toxic effect on injected embryos than did the scrambled morpholino, producing this effect. However, some *in vitro* studies have shown that Hsp27 can modulate outgrowth of neurons [34, 74]. The discrepancies between results of studies examining the role of Hsp27 during myogenesis *in vitro* [15, 66] and *in vivo* [57]) indicate these results should be interpreted with caution, but our results do not rule out a functional role for Hsp27 during development of motor neurons in zebrafish embryos. Further studies may resolve this issue. An alternative possibility is the transient expression of Hsp27 in developing heart and muscle tissues is not necessary for development *per se* but is none the less advantageous for an embryo that cannot change its location in response to environmental changes, such those of fish and amphibians. Such a scenario might predict that Hsp27 expression would be elevated in metabolically active tissues especially vulnerable to a combination of metabolic and environmental stresses. Once larvae emerge from their chorions, the animals would be free to move away from areas of environmental stress, and high levels of constitutive Hsp27 expression seen in young embryos might not be necessary. Similarly, David *et al*, have proposed that heat shock proteins are elevated in mammalian embryos to protect them from stress and potential hypoxic injury during birth [73].

Hsp27 is required for stress tolerance of muscle and nerve tissues in zebrafish embryos.

Previous studies have demonstrated that overexpression of Hsp27 can enhance recovery of muscle and nerve function in animal models after acute injury [75, 76]. However, these studies have not directly addressed whether endogenous Hsp27

plays a unique role in providing such protection, or if upregulation of other chaperones could substitute for Hsp27 during the response to stress. Additionally, previous studies examining protective effects of Hsp27 overexpression in intact animals have employed transgenic mice that ubiquitously express Hsp27 under the direction of -actin promoters [75, 76]. Thus, these previous studies could not resolve whether the protection of muscle function by Hsp27 results from activities of endogenous Hsp27 in muscle cells, or in associated tissues such as vascular and nerve tissues. Finally, previous studies addressing protective effects of Hsp27 on muscle function have also not included analysis of cell injury and subcellular reorganization. Our study addresses some of these issues. First, heat shock has been shown to ubiquitously upregulate expression of other heat shock proteins in zebrafish, including members of the Hsp70 and Hsp90 families and small heat shock protein Hsp47 [77]. Although the expression of these proteins was not directly examined in this study, our results make clear that mechanisms underlying the stress-dependent regulation and function of the other heat shock proteins cannot compensate for the loss of Hsp27 in zebrafish embryos. Our results demonstrate, directly and for the first time, that Hsp27 is uniquely required for the protection muscle function in an intact vertebrate embryo. We have also shown that Hsp27 is expressed under control conditions in a limited number of tissues in the zebrafish embryo and that constitutive expression is most strongly detected in skeletal and cardiac muscle tissues [49]. An unresolved question was whether the level of expression observed under control conditions was sufficient to provide stress resistance or might serve some other purpose. Our analysis of myofibril integrity immediately after heat shock showed dramatic loss of myofibril birefringence in animals lacking constitutively expressed Hsp27 (Fig. 6). Because the time course of

this experiment (30 min between the start of heat shock and image analysis) was too short to allow significant upregulation of Hsp27 in either muscle cells or associated cell types, our results strongly support the view that constitutive, muscle cell specific expression of Hsp27 is sufficient to promote myofibril integrity in injured muscle cells. Additionally, previous studies from several laboratories, including our own studies in zebrafish, have shown association of Hsp27 with myofibrils in injured muscle cells. It is noteworthy that translocation of Hsp27 from the cytosolic to nuclear cell compartment observed in non-muscle cells is not commonly reported for differentiated muscle cells [75, 78, 79]. Proposed nuclear functions of Hsp27 include regulation of mRNA splicing in injured cells [80] and interaction with nuclear apoptotic signaling elements such as DAXX [81]. We have previously shown that zebrafish Hsp27 enters the nucleus of heat stressed fibroblasts in culture and that an EGFP-zebrafish Hsp27 fusion protein enters nuclei of epidermal but not muscle cells in heat shocked zebrafish embryos [61]. Here, however, we detected only minor defects in muscle tissues over time scales appropriate for apoptosis and protein synthesis (Fig. 7). Based on data, it appears that the primary function of Hsp27 in injured muscle cells is to stabilize myofibrils during acute injury.

The loss of Hsp27 expression has a dramatic effect on the integrity of motor neurons in intact embryos subject to sublethal injury (Fig. 8). Based on the observation that a significant expression of Hsp27 in peripheral nerve tissue of zebrafish embryos was not noted under control conditions by *in situ* hybridization [49] or using immunostaining procedures in preliminary studies (not shown). It appears that Hsp27 expression is upregulated post-injury in order to promote stress tolerance of peripheral nerve processes. Moreover, such upregulation occurs over a time frame

that is consistent with the demonstrated role of Hsp27 in modulating apoptotic

signaling processes in neuronal cells [74, 82]. However, it is interesting that in our

studies loss of acetylated tubulin staining of nerve processes in Hsp27 knockdown

animals correlated with an increase in acetylated tubulin staining of cell bodies in the

region of the spinal cord (Fig. 8). To our knowledge, this is the first indication that

redistribution of markers for stable microtubules accompanies nerve cell injury in

vivo. Because these cell bodies are present 14-16 hours post injury, it is possible

that these cells survived initial injury and that Hsp27 is therefore dispensable for their

survival. However, the depletion of acetylated tubulin staining in nerve processes

and its appearance in cell bodies within the spinal cord region may be interpreted as

resulting from the collapse of the cytoskeleton in nerve processes or defects in

anterograde transport of stable microtubules. Our results support a novel role for

Hsp27 in the maintenance of neuronal cell structure in injured motor neurons.

Interestingly, mutation in Hsp27 has been shown to be a cause of the inherited

peripheral neuropathy Charcot Marie Tooth Syndrome [36]. Our results indicate that

the zebrafish embryo may be useful new model for studying the role of Hsp27 in

peripheral neuropathies and other nerve cell processes.

Finally, evidence is presented that indicates a high degree of conservation in the

expression and function among vertebrate Hsp27 orthologues. Many recent insights

into the molecular function of sHsps have been derived from studies of the yeast

crystallin family member Hsp26 [13]. However, the high diversification of sHsps

during vertebrate evolution [83] argues that these proteins may have adopted unique

roles in vertebrate tissues that may be difficult to elucidate from studies of simple

organisms. Consistent with this latter view, results of the present study demonstrate

that Hsp27 performs unique functions is a restricted subset of embryonic tissues in

the vertebrate embryo.

Chapter 3

CONCLUSIONS AND FUTURE DIRECTIONS

Role of Hsp27 during embryogenesis

Hsp27 expression is upregulated early during development of mammals [33] and other vertebrate embryos including frogs and fish [49, 50]. Developmental expression of Hsp27 is especially evident in myoblasts and developing muscle tissues *in vivo*, and this expression pattern is recapitulated, in part, in cultured mammalian myoblasts undergoing differentiation *in vitro* [51]. These observations have supported the view that Hsp27 plays a role in embryogenesis or myogenesis. *In vitro* studies of mammalian myoblast differentiation have appeared to confirm this hypothesis. For example, reduction of Hsp27 expression in stable transformed cell lines expressing full-length antisense Hsp27 mRNA inhibits differentiation of mammalian myoblasts and promoted apoptosis of embryonic stem cells [52]. Hsp27 knockout mice have not been produced, further supporting the view that Hsp27 plays a critical role in development. The present work represents the first gene knockdown studies of Hsp27 in a vertebrate animal. Surprisingly, although the expression of Hsp27 in developing muscle tissues is conserved between fish and mammals, morphogenesis of zebrafish embryos is normal in the absence of Hsp27. It is possible that embryogenesis at temperatures greater than 28°C (the rearing temperature of zebrafish embryos) requires Hsp27 expression, or that the developmental requirement for Hsp27 in muscle tissues evolved subsequent to the diversion of fish and mammals. However, we previously showed considerable conservation of primary sequence and cellular functioning between zebrafish and human Hsp27 [61]. Therefore, these scenarios seem unlikely.

It is also possible that the loss of Hsp27 expression in our experiments was compensated for by the expression of an alternate, related protein. For example, another member of the small heat shock protein family has been identified in zebrafish and it is expressed in adult muscle tissues, albeit at low levels [49]. This possibility was not addressed in the present study. On the other hand, we note that our polyclonal antisera did not detect expression of closely related proteins in antisense Hsp27 MO injected animals, even after heat shock. Our data support the view that Hsp27 expression is dispensable for normal vertebrate embryogenesis. Partial support for this hypothesis comes from studies of transgenic gene knockout mice lacking the expression of heat shock transcription factors, that display normal development but deficits in stress tolerance [84]. The current lack of Hsp27 knockout mice may be explained by a requirement for Hsp27 in reproductive tissues in mammals. For example, Hsp27 expression is upregulated in endometrial tissues of rat and humans during pregnancy [55, 56]. Because fish embryogenesis takes place without maternal involvement, this requirement would not affect embryogenesis in fish.

Despite the apparent lack of a requirement for Hsp27 during development, studies in a variety of vertebrates including frogs, mammals and our own studies in zebrafish, make it clear that Hsp27 is expressed early and transiently during myogenesis. It remains possible that a subtle developmental requirement for Hsp27 during myogenesis can be revealed by further experiments, or that the assays of overall larval motility in the present study failed to detect subtle changes in muscle architecture or function in the absence of Hsp27. An alternative possibility is that

transient expression of Hsp27 during development is advantageous to an embryo that cannot change its location in response to environmental changes. Such a scenario might predict that Hsp27 expression would be elevated in metabolically active tissues especially vulnerable to a combination of metabolic and environmental stresses. Once larvae emerge from their chorions, the animal would be free to move away from areas of environmental stress, and high levels of constitutive Hsp27 expression seen in young embryos might be less necessary. Presently, it is not clear whether functional expression levels of Hsp27 are higher in non-placental embryos than mammalian embryos, as might be predicted by this hypothesis.

Role of Hsp27 in stress tolerance during development of zebrafish

Currently, the role of *in vivo* Hsp27 in stress tolerance was investigated by overexpressing of the wild-type or non-phopshorylatable mutant of Hsp27 [75]. These findings suggest that Hsp27 overexpression in transgenic mice exhibits significant cytoprotective properties in the heart and brain by reducing caspace-3 mediated cell death in the hippocampus and reducing infract size in cardiac ischemia. Another study of Hsp27 *in vivo* overexpression found that it provides a substantial rescue of motor neurons 5-6 months following nerve injury [76]. Both of these studies complement our findings of Hsp27 role for protecting the integrity of muscle and nerve tissues. At this point, it is obvious that embryos lacking Hsp27 were more prone to heat shock injury. As a result, Hsp27 knockdowns exhibited dramatic defects in their swimming behavior, which is intimately linked to the function of muscle and motor neurons. Perhaps, Hsp27 in muscle tissues plays a role of a rapid response agent preventing irreversible denaturation and aggregation of the cytoskeletal proteins and subsequent collapse of the whole contractile apparatus.

This view is supported by a number of *in vitro* experiments where increased cytoskeleton protection is concomitant with translocation of Hsp27 from the cytosolic fraction to the pellet suggesting that Hsp27 is essential for protecting actin filaments [21]. Moreover, experiments with cytoskeleton disruptors (such as cytochalasin D) showed stabilization of actin fibrils in Hsp27 overexpressing cells. Our results suggest that Hsp27 knockdown embryos exhibit not only short, but long term defects in skeletal muscle fibers after exposure to heat stress. Muscle birefringence data show that immediately after heat shock the structural integrity of muscle fibers in Hsp27 knockdown embryos was significantly lower in comparison to control embryos. Such effect has not been shown before partly because most studies examined the function of Hsp27 by overexpression. Thus, it was not clear whether the outcomes of these experiments were physiologically relevant. However, in this study, we demonstrate that endogenous protein contributes to preserving the structural integrity of skeletal muscle in zebrafish embryos. These findings provide additional support for the hypothesis that Hsp27 in muscle cells may act as a rapid response agent during stressful conditions. Further experiments are currently underway that will determine the exact mechanism by which Hsp27 contributes to the stability of muscle tissues.

Despite the fact that less is understood about the expression of Hsp27 in neurons compared to muscle tissues, it is clear that Hsp27 is implicated as a survival promoting factor. *In vitro* data suggest that Hsp27 may play a role in neuritogenesis and subsequent neurite extension, and potentially in the patterning of this growth [85]. Moreover, overexpression of Hsp27 in transgenic mice contributes to a substantial rescue of motor neurons after stress [76], and, here we report that Hsp27

knockdown embryos results in loss of motor neuron integrity in heat shocked

embryos. Specifically, Hsp27 knockdown resulted in some reduction of the integrity

of motor neurons and uniformity of their pattern, even under control conditions, and

also that heat shock caused some disruption of motor neurons in water and

scrambled MO injected animals. These observations complement the findings that

were done in Hsp27 overexpression mice [75, 76]. As Sharp *et al* report, in Hsp27

overexpressing mice, the surviving motor neurons were able to regenerate and

resulted in a 90% improvement of motor neuron number [76]. The increase in motor

neuron number in Hsp27 overexpressing mice was associated with improved muscle

force and contractile speeds. Even though we measured different parameters than

the Sharp group, we found that Hsp27 knockdown embryos after heat shock

performed poorly on the swim tests. Therefore, it is likely that such performance of

Hsp27 knockdown embryos in swimming assays was associated with neuronal

damage that had occurred during the heat shock.

An unfortunate limitation of our morpholino oligonucleotide technique is in the

transient nature of a knockdown. Morpholino-induced knockdown efficacy

decreases though dilution as the total cytosolic volume of embryos increases by

growth. In addition, some fraction of morpholino oligonucleotides is constantly in

tight binding with target mRNA and cannot bind another mRNA until the bound

complex is degraded [86]. At this point, we do not have any evidence that Hsp27

deficient embryos develop normally into adulthood and produce viable and fertile

progeny. Therefore, we can not make any conclusion with regard to the long term

effects of Hsp27 knockdown which would be otherwise possible with the classical

genetic Hsp27 knockout. Nonetheless, this is the first study of *in vivo* Hsp27

knockdown in the whole animal that shows no significant effects on organogenesis of developing embryos. Based on our findings we conclude Hsp27 is required for injury resistance of muscle tissues and nerves, but not morphogenesis, in zebrafish embryos.

Future directions

The present study examines the role of Hsp27 in embryogenesis using specific morpholino oligonucleotides to knockdown expression of Hsp27 in the whole zebrafish embryo. As a result, we have discovered that Hsp27 is required for injury resistance of muscle tissues and nerves, but not morphogenesis, in zebrafish embryos. Further investigation into the molecular aspects is required in order to deduce the definitive mechanism of Hsp27 protective functions in muscle and neurons. Initial steps may include identification of potential targets of Hsp27 interactions as well as determination of the phosphorylation state of Hsp27 during control and heat shock conditions. The identification of interaction targets will lead to determination of exact pathway by which Hsp27 expressing cells acquire resistance to injury. It is likely that Hsp27 functions are phosphorylation dependent, and therefore identification of vital kinases will provide further integration of Hsp27 into the protective mechanisms. Besides analysis of the interaction targets of Hsp27 and its phosphorylation state, it will also be crucial to carefully examine whether muscle and neurons in Hsp27 knockdown are more prone to apoptosis compared to control embryos. There is large body of *in vitro* studies that examined anti-apoptotic properties of Hsp27, but no *in vivo* evidence is provided to date.

It would be also important to test different types of stresses. One of the incomplete sides of this study lies in the assessment of cardiac muscles. We used heart rates to measure cardiac performances; instead it would be crucial to perform analysis of cardiac output by, perhaps, measuring pressure and blood flow rates in Hsp27 knockdown embryos. In addition, it would be important to subject hearts to ischemic injury followed by reperfusion and assess cardiac functionality in the absence of Hsp27.

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Figure 1. Expression of Hsp27 during zebrafish development.

Panel **A**: Western blot of equal amounts of total protein from embryos of different ages through the whole course of embryonic development. Stages are indicated as hours post fertilization (hpf). Panel **B***:* Quantification of integrated optical density of bands on Hsp27 Western blots. X axis: hours post fertilization (hpf). Y axis: Hsp27 expression fold increase during developmental course. Values are calculated as means $\pm$ standard deviation of three independent experiments.

Figure 1
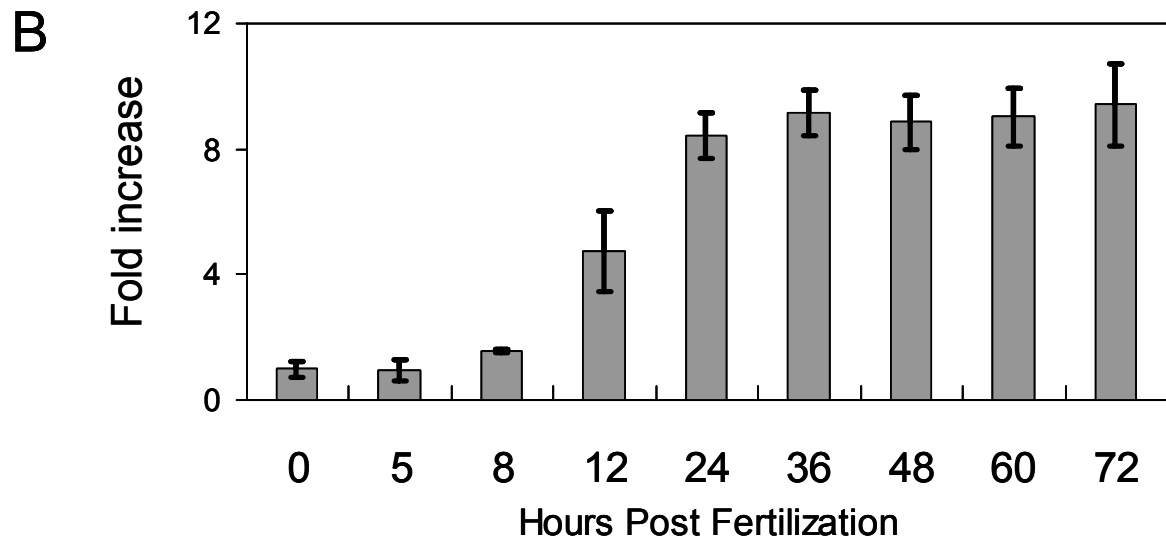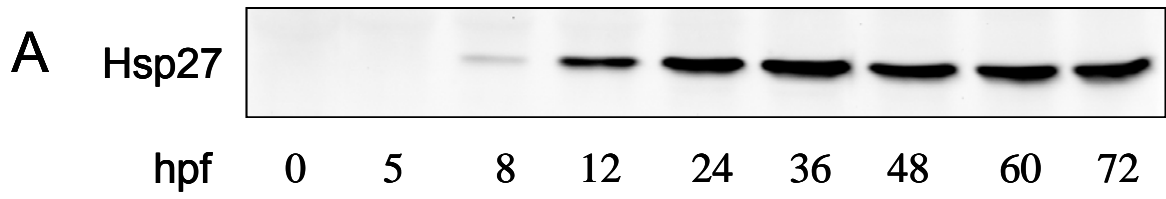
Figure 2. Morpholino-mediated knockdown of Hsp27 expression in zebrafish embryos.

Panel **A**: Alignment of the 5' untranslated region of the zebrafish Hsp27 gene with the antisense Hsp27 specific morpholino sequence (zfHsp27 MO) and the scrambled control morpholino sequence (Scr-MO). Identical nucleotides are highlighted with gray. Translation start is indicated by bent arrow and the start codon is in bold font. Panel **B**: Western blot analysis of Hsp27 expression in 30 hpf embryos injected at the one- to four-cell stage embryos with antisense Hsp27 MO (α27) and scrambled-MO (scr). Total protein was visualized using Ponceau S staining. Numbers are MO concentrations injected (mM). 'M' indicates a MW marker lane, with MW values of markers shown in kDa. Predicted position of zfHsp27 protein on gel is marked by arrow. Panel **C**: Knockdown of Hsp27 is effective in heat shocked zebrafish embryos. Zebrafish embryos were injected with 0.15 mM antisense Hsp27 MO (α27 lanes) and scrambled MO (scr lanes) at the one- to four-cell stage and developed for 30 hours (CN), then heat shocked for 30 min at 39°C and recovered for 18 hrs (HS). Total protein was assessed for expression of Hsp27at 50 hpf and actin staining used as a loading control. Panel **D**: Hsp27 knockdown is effective for up to seven days. Zebrafish embryos were injected with water (water lanes), scrambled MO (scr lanes) or antisense Hsp27 MO (α27 lanes) at one- to four-cell stage and developed for 24, 48, 72 hours, as well as 1 week (24h, 32h, 72h, and 1wk, respectively). At each time point embryos were collected and equal amounts of total protein assessed for expression of Hsp27 and actin by Western blotting. Note that Hsp27 is missing in antisense Hsp27 MO injected embryos even at age of 1 week.
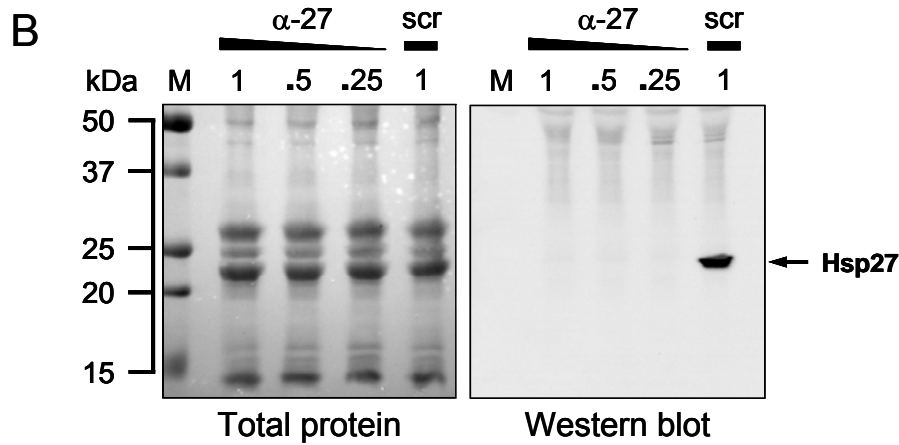
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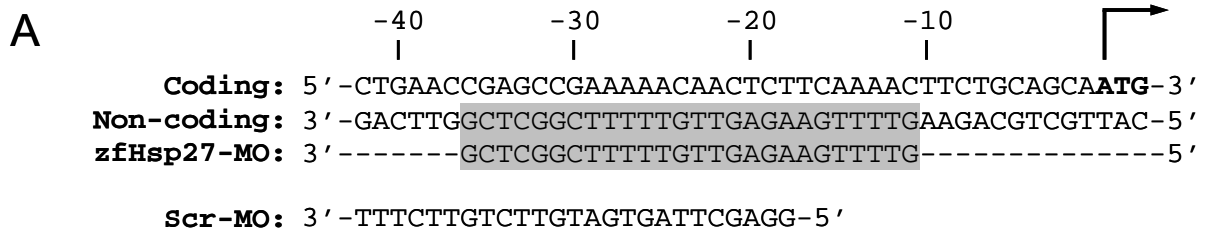**Figure 2**

A

```
                  -40           -30           -20           -10
                   |             |             |             |
Coding:      5'-CTGAACCGAGCCGAAAAACAACTCTTCAAAACTTCTGCAGCAATG-3'
Non-coding:  3'-GACTTGGCTCGGCTTTTTGTTGAGAAGTTTTGAAGACGTCGTTAC-5'
zfHsp27-MO:  3'-------GCTCGGCTTTTTGTTGAGAAGTTTTG--------------5'

Scr-MO:      3'-TTTCTTGTCTTGTAGTGATTCGAGG-5'
```

B

Total protein   Western blot

C

D
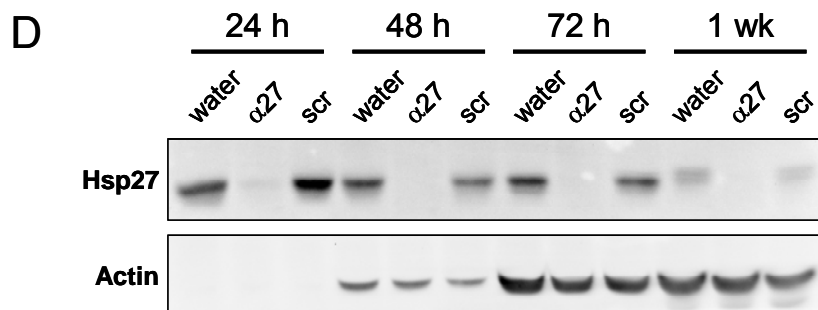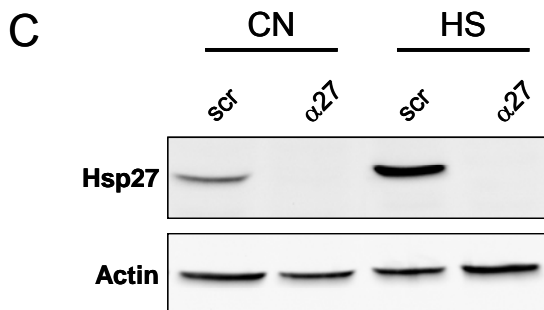
Figure 3. Morphogenesis is not altered in Hsp27 knockdown embryos.

Panel **A**: Overall morphology of water, scrambled MO (scr) and antisense Hsp27 MO (α27) injected embryos. Hsp27 knockdown embryos showed normal organogenesis compared to water and scrambled MO injected animals. Scale bar = 1mm. Arrows point to the beginning and the end of the measured tail length. Panel **B**: Comparable analysis of the yolk area, a measure of nutrient utilization. Bars represent mean values (μm^2) of 22 water, 23 scrambled MO and 21 antisense Hsp27 MO injected embryos $\pm$ standard error of the mean (SEM). Panel **C**: Analysis of the tail lengths. Embryo tails were measured from the tip of the tail (dark arrow) to the yolk mass (white arrow, Fig. 3A). Bars represent mean values (cm) of 30 water and scrambled MO, and 29 antisense Hsp27 MO embryos, $\pm$ SEM. Panel **D**: Comparable analysis of the eye lens diameter. Bars represent mean values (μm) of 22 water, 24 scrambled MO, and 22 antisense Hsp27 MO injected embryos $\pm$ SEM. Embryos analyzed for B–D were collected in a total of three independent trials.

Figure 3
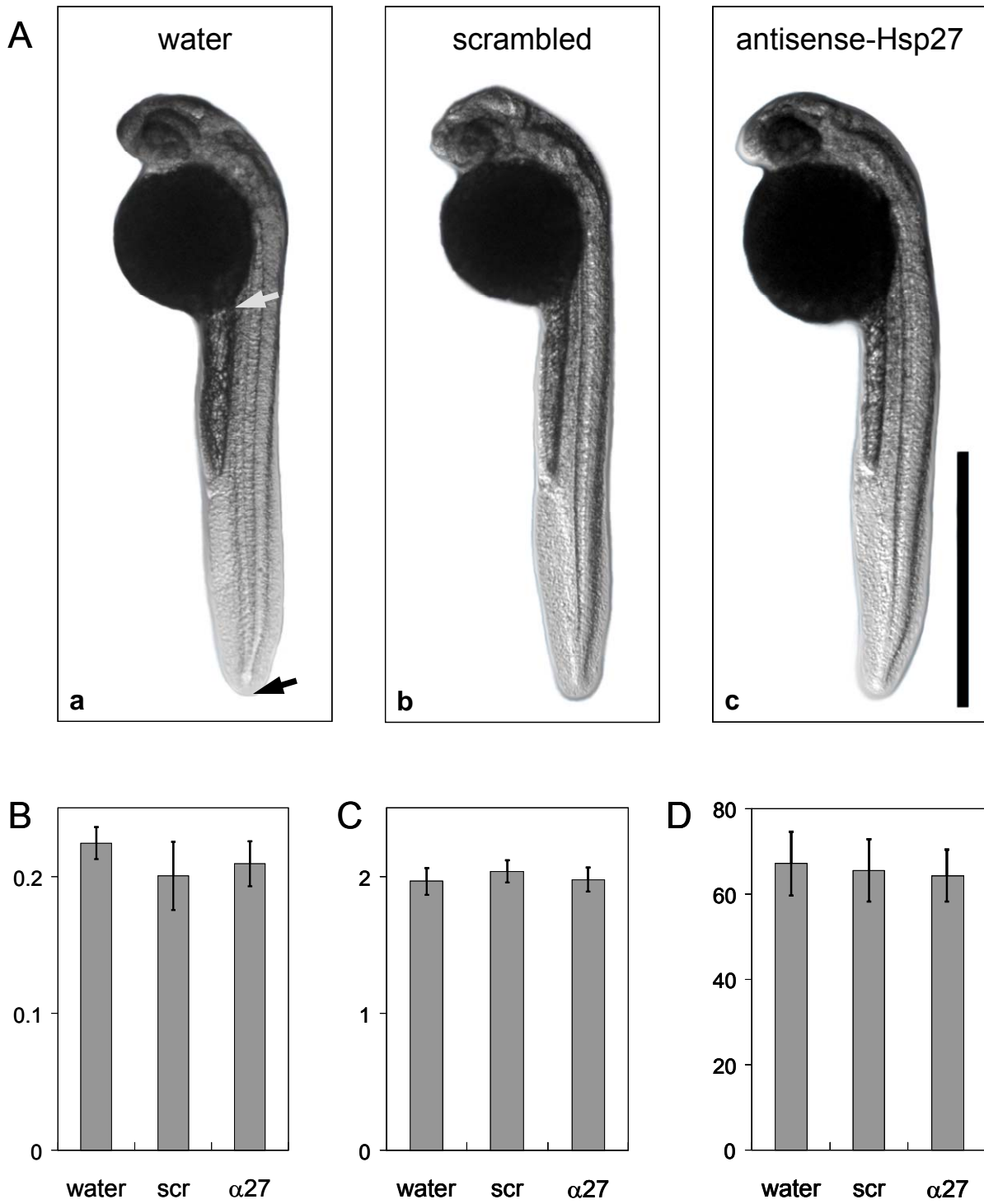
Figure 4. Comparable analysis of swimming behavior of morpholino injected and control embryos.

Panel **A**: Representative swimming paths of water and MO injected embryos. All injected embryos showed similar swimming under control conditions (control). After heat shock, antisense Hsp27 MO injected embryos showed reduced swimming ability. Details of heat shock and recovery are described in Materials and Methods. Scale bar = 1 cm. Panel **B**: Measured mean swimming rates $\pm$ standard error of the mean (SEM). Panel **C**: Measured mean swimming distance $\pm$ SEM. There was no statistically significant difference in swimming rates (**B**) or distances (**C**) among water and MO (scr, α27) injected embryos under control conditions (□ solid bars). However, Hsp27 knockdown (α27) correlated with statistically significant (*=p<0.05) loss in swimming rates (**B**) and distances (**C**) 14 hours post injury (▨ shaded bars), while scrambled MO injected embryos did show these differences. The means of a total of water injected: control (38), heat shock (35); scrambled MO injected: control (37), heat shock (30); antisense Hsp27 MO injected: control (36), heat shock (33) in three independent trials.

Figure 4
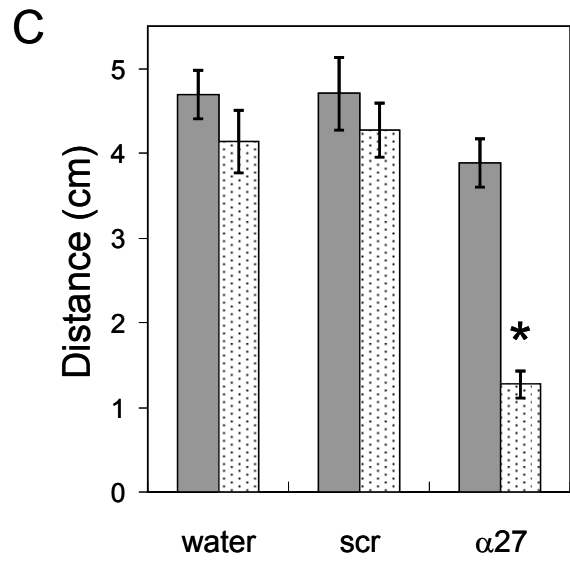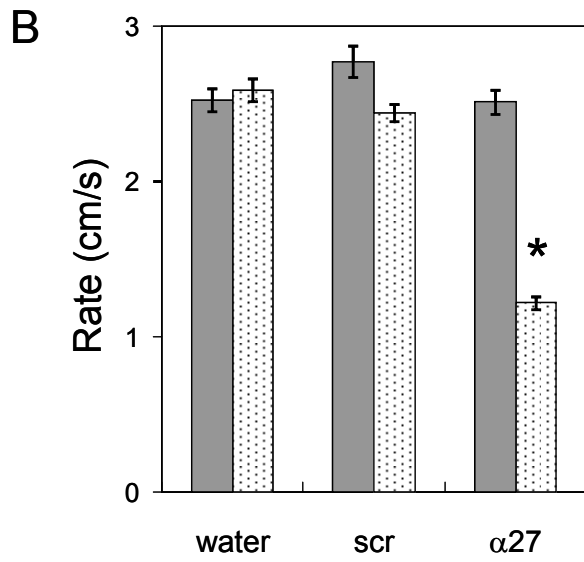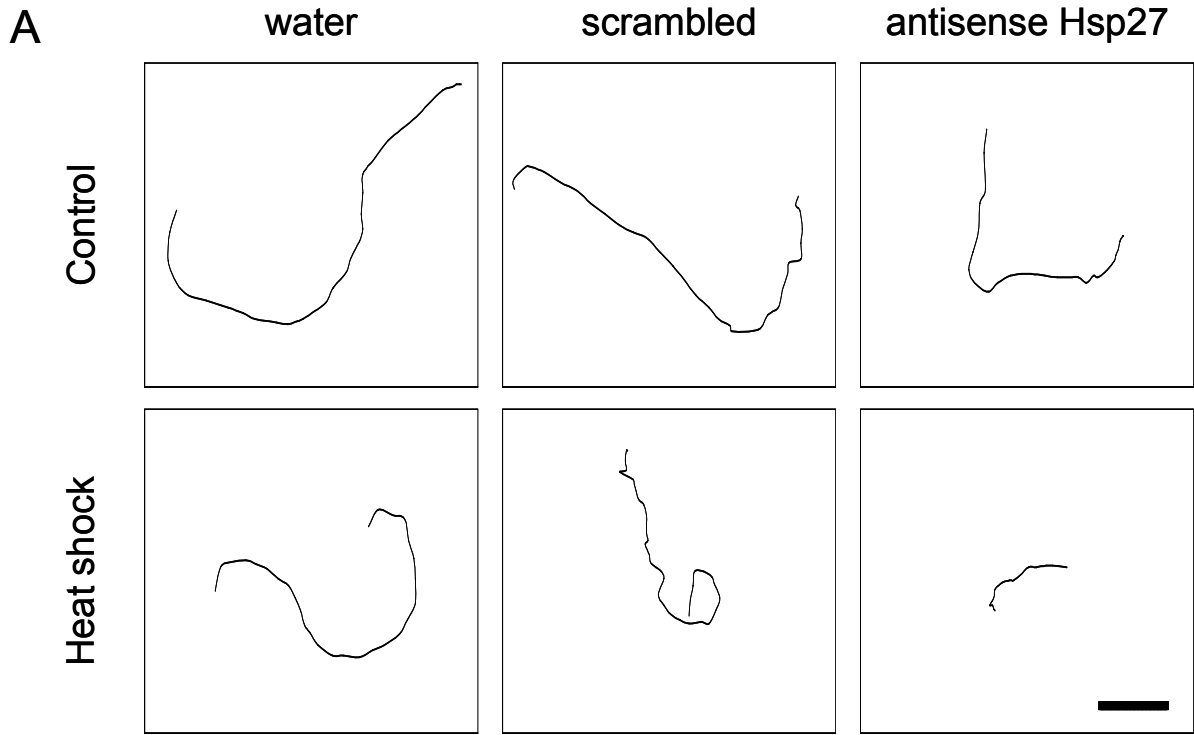
Figure 5. Alteration of heart beat frequency in Hsp27 knockdown embryos.

Heart rates under control conditions (▣ solid bars) were similar for embryos injected with water, scrambled MO (scr) or antisense Hsp27 MO (α27). Heart rates measured after heat shock at 30 hpf and subsequent 18 hours of recovery (▨ shaded bars) were reduced, but antisense knockdown correlated with a greater and statistically significant decrease than for other groups of embryos. Bars represent means and standard error of the mean measured for heart beats in 16 embryos in each experimental group over three independent trials.

Figure 5
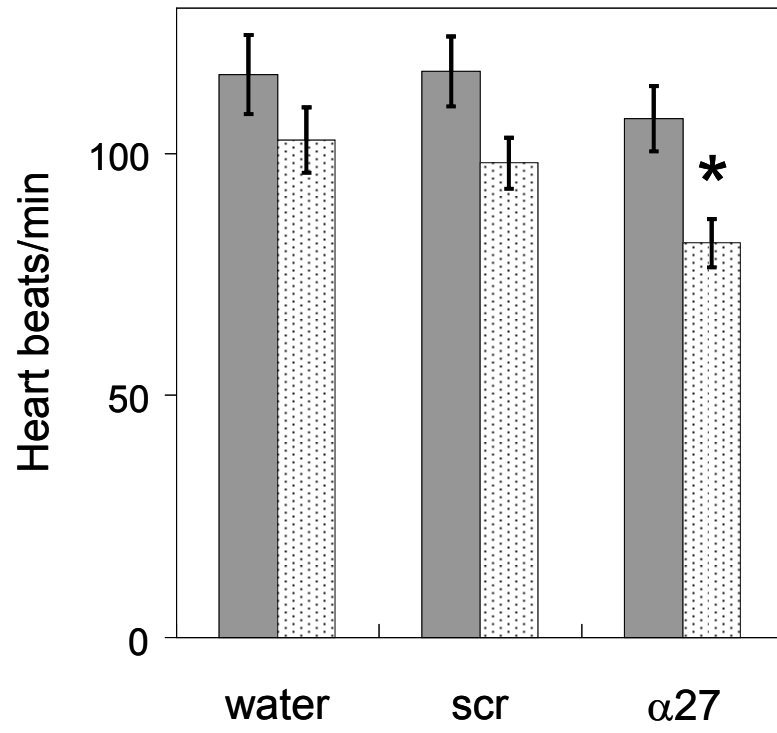
Figure 6. Loss of myofibril integrity after heat shock in Hsp27 knockdown embryos.

Polarized light birefringence of muscle fibers was imaged for water, scrambled MO (scr) and antisense Hsp27 MO (α27) injected embryos. Panel **A***:* Representative images of myofibril birefringence in 30 hpf embryos before (Control) and immediately after heat shock at 39°C for 30 min (Heat shock). Panel **B**: Mean myofibril birefringence among groups of injected embryos showed no differences under control conditions. Panel **C**: Myofibril birefringence measured after heat shock, normalized to that measured in the same animal under control conditions. The injection of antisense Hsp27 MO (α27) correlated with statistically significant (*=p$\leq$0.05) loss of muscle fiber birefringence measured immediately after heat shock compared to water and scrambled MO injected controls. Bars show the mean and standard error of the mean of values measured for 8 embryos in each experimental group.
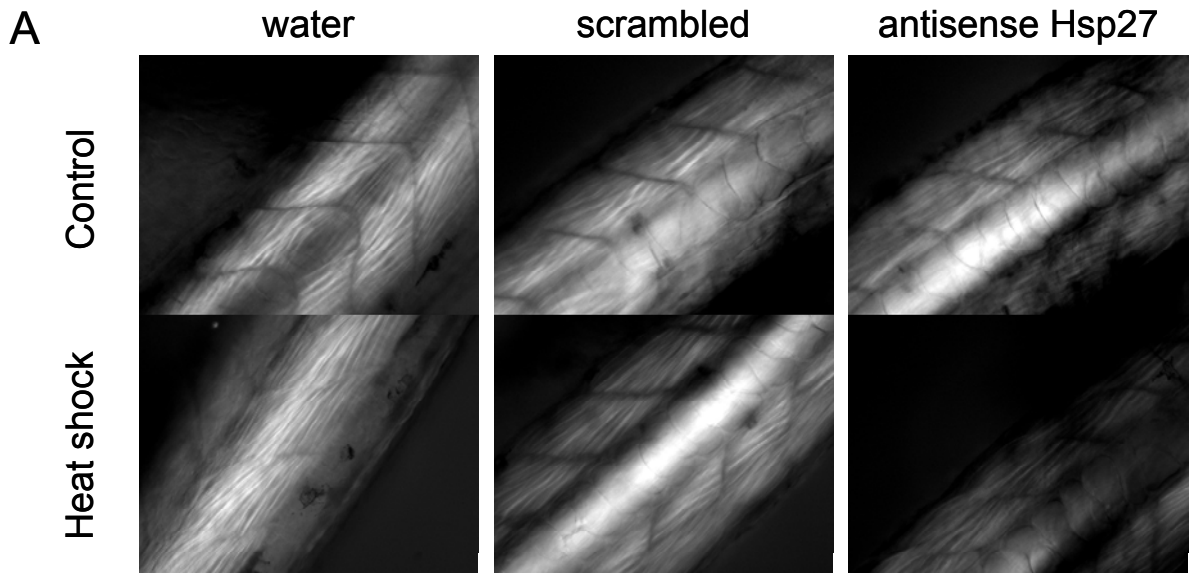
Figure 6
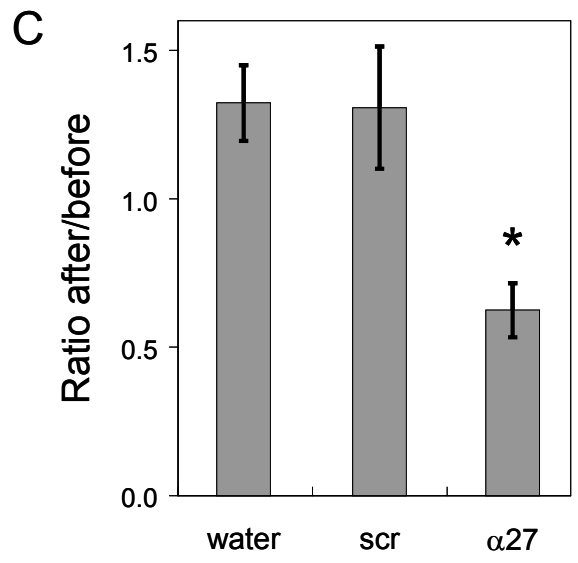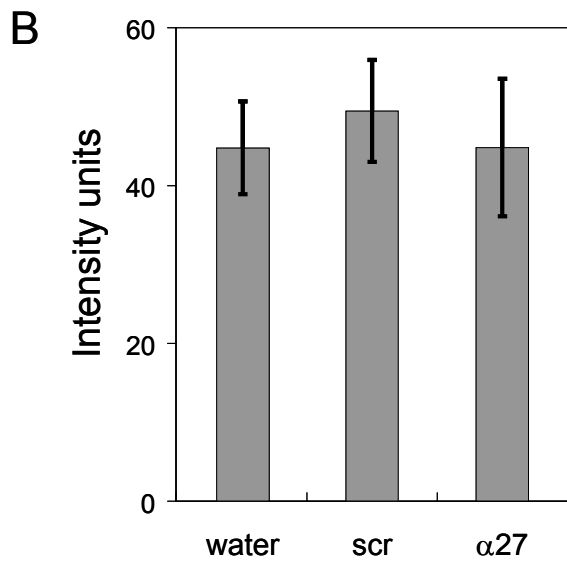
Figure 7. Myotome area in injected embryos.

Panel **A**: Confocal microscopy images of the 12th myotome of 50 hpf embryos stained for F-actin with rhodamine-phalloidin. Arrow indicates 12th myotome. Scale bar = 50μm. Note, there is no change in the intensity of actin arrays between experimental groups before and after heat shock. Panel **B**: Total area measurements of the 12th myotome in water, scrambled MO, and antisense Hsp27 MO injected embryos at 50 hpf. There is no difference ($p > 0.05$) in the mean myotome area among experimental groups under control conditions (■ solid bars). However, antisense Hsp27 MO injected embryos (α27) demonstrate statistically significant (*=$p \leq 0.05$) reduction in the myotome area after heat shock (⊠ shaded bars). Bars show the means of calculated areas $\pm$ SEM for 21 embryos in each experimental group measured over three independent trials.
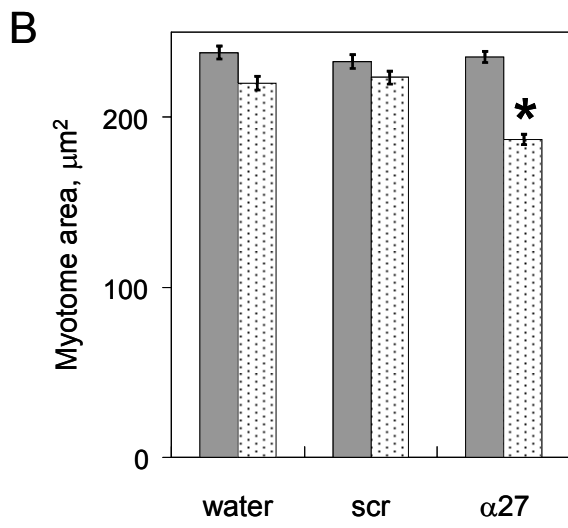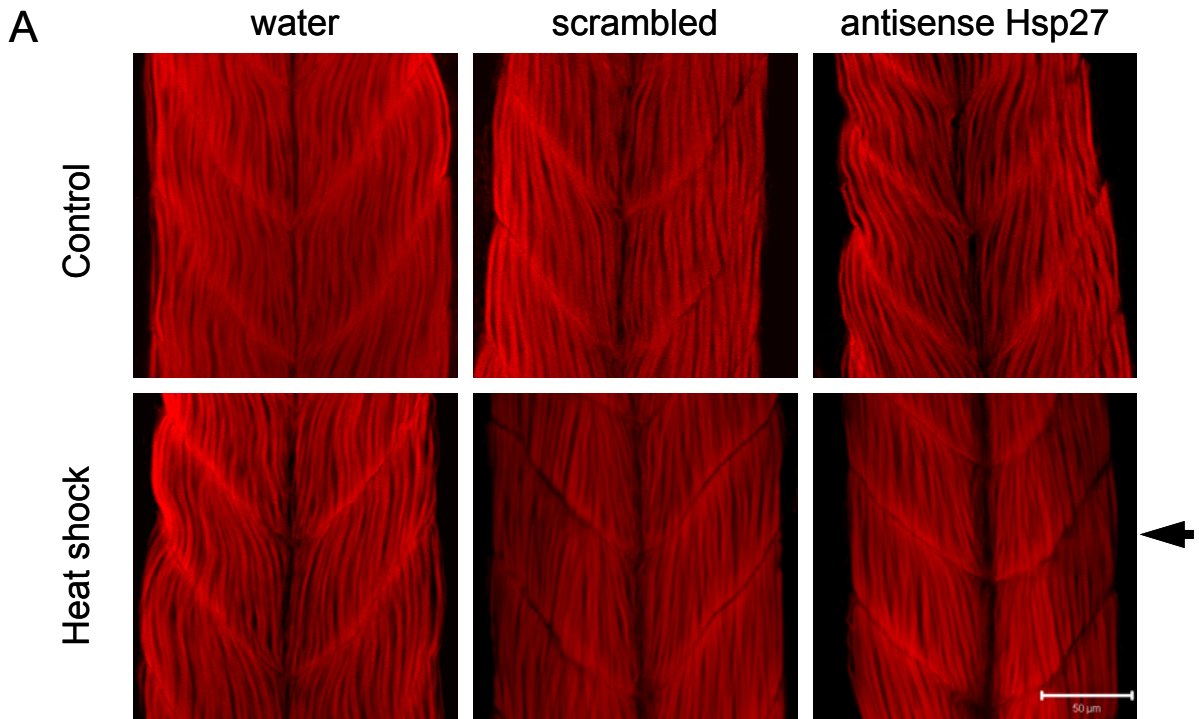
Figure 7

Figure 8. Morpholino-mediated knockdown of Hsp27 produces motor neuron

defects in heat shocked embryos.

Panel **A**: Anti-acetylated tubulin immunofluorescent staining for motor neurons of

control and heat shocked embryos after swimming analysis. Processes of intact

motor neurons in water injected embryos display bright and repetitively uniform

staining (white arrows, upper left panel) in water and scrambled MO injected

embryos, whereas antisense Hsp27 MO injected embryos show somewhat less

uniform staining patterns. Heat shocked embryos display less intense staining of

motor neurons whereas cell bodies that are stained with anti-acetylated tubulin

antibodies is more pronounced (gray arrow heads, lower right panel). Scale bar =

100 μm. Panel **B**: Scores for overall motor neuron integrity. The overall integrity of

neurons was significantly decreased after heat shock in Hsp27 knockdown animals.

Panel **C**: Number of intact motor neuron pairs in injected embryos. The number of

intact neuron pairs is significantly reduced in Hsp27 knockdown embryos after heat

shock compared to water and scrambled MO injected embryos. Panel **D**: Average

number of acetylated-tubulin positive cell bodies along the spinal cord of the embryo.

Hsp27 knockdown embryos had significantly greater number of acetylated-tubulin

cells after heat shock. All image scores were processed blindly by three

independent observers. Bars represent mean values obtained from 10 water, 9

scrambled MO, and 11 antisense Hsp27 MO injected embryos under control

conditions (■ solid bars); and 10 embryos in each group under heat shock (⊠

shaded bars). Asterisks denote statistical significance where p$\leq$0.05.

Figure 8
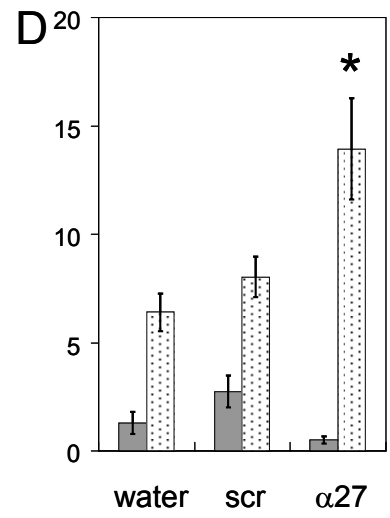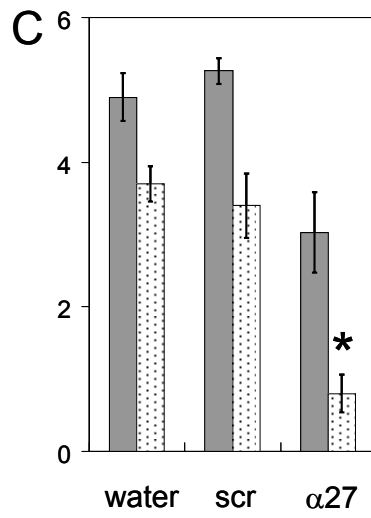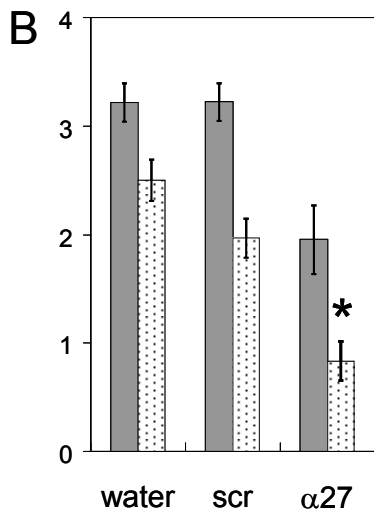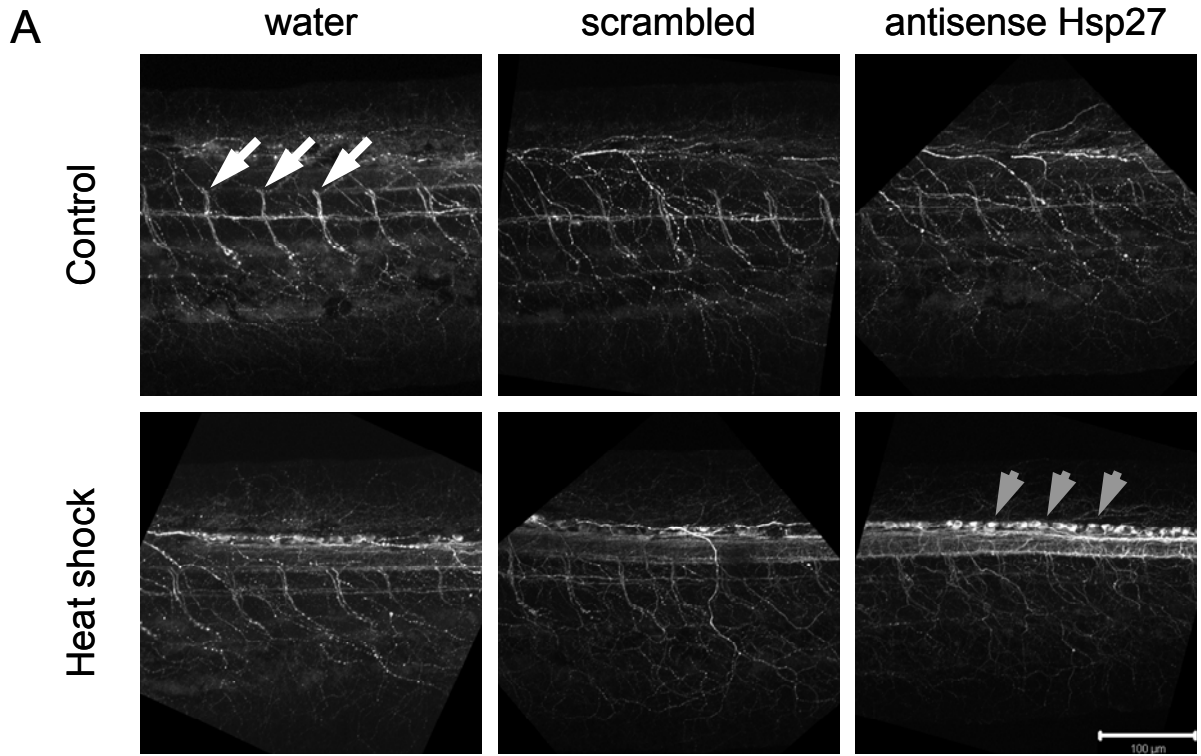
APPENDIX A

TRANSGENIC ZEBRAFISH LINE EXPRESSING CYAN FLUORESCENT PROTEIN REPORTER UNDER THE CONTROL OF HSP27 PROMOTER

We have generated a transgenic fish line that expresses cyan fluorescent protein (CFP) reporter under the control of Hsp27 promoter. The 300 bp proximal promoter region was isolated from the genomic DNA and sequenced. On the figure below, Panel **A** shows 330 bp of Hsp27 zebrafish promoter region. Start codon is labeled in bold (ATG). Subsequently, 330 bp PCR fragment was subcloned into the CFP expressing vector in the orientation as diagrammed in Panel **B**. We have generated a PCR product that contained the 300 bp of Hsp27 promoter and the sequence coding to CFP. This product was then injected in to the one-cell stage embryos. The mosaic animals were pre-selected by fluorescence and raised to adulthood. Selfing on these mosaic animals resulted in the first generation (F_1) that was subsequently selected for CFP fluorescence and raised to adulthood to produce the second generation of (F_2), which was backcrossed with the parents to result in a 100% fluorescing progeny (F_3). Subsequent matings generated 100% CFP fluorescing progeny. Panel **C** shows 52 hpf embryos under control and heat shock conditions. Side **"a"** illustrates the overall morphology of Hsp27-CFP embryos. Note that CFP signal is detected predominantly in the heart and skeletal muscles. Enlarged images of control (side **b**) and heat shock (side **c**) embryos show that after heat stress the CFP expression is detected in the head epithelial cells and the lens.

Figure Appendix A

A

```
1      AAGAAAGATC AAGCTCCCAA AATGCAATTC AAAATTCACA AAATACAAAA
51     AACTTTAATT CACCAACATA TTGTTCACAG TCCAGTATTA AATACACACT
101    ATCAGCCTTT GCCTCAGATT CCTTCCTCAT GCGAGGGCCG TCCCTGAATA
151    GACTTTCTAG TCACTTCTAT AATTATCCGG ACGGGACTGG GCTGTTGCAG
201    AAGTGTACTG AAGTGGGTGT GTCCTCCAGA AAGCCTTCTT ATAAAAGTCC
251    CAGCCCCGTG CAAACAATCA GAACACACAC ACACTCTGAA CCGAGCCGAA
301    AAACAACTCT TCAAAACTTC TGCAGCA**ATG**
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B | 300bp – Hsp27 promoter |—| **Cyan Fluorescent Protein** |

C
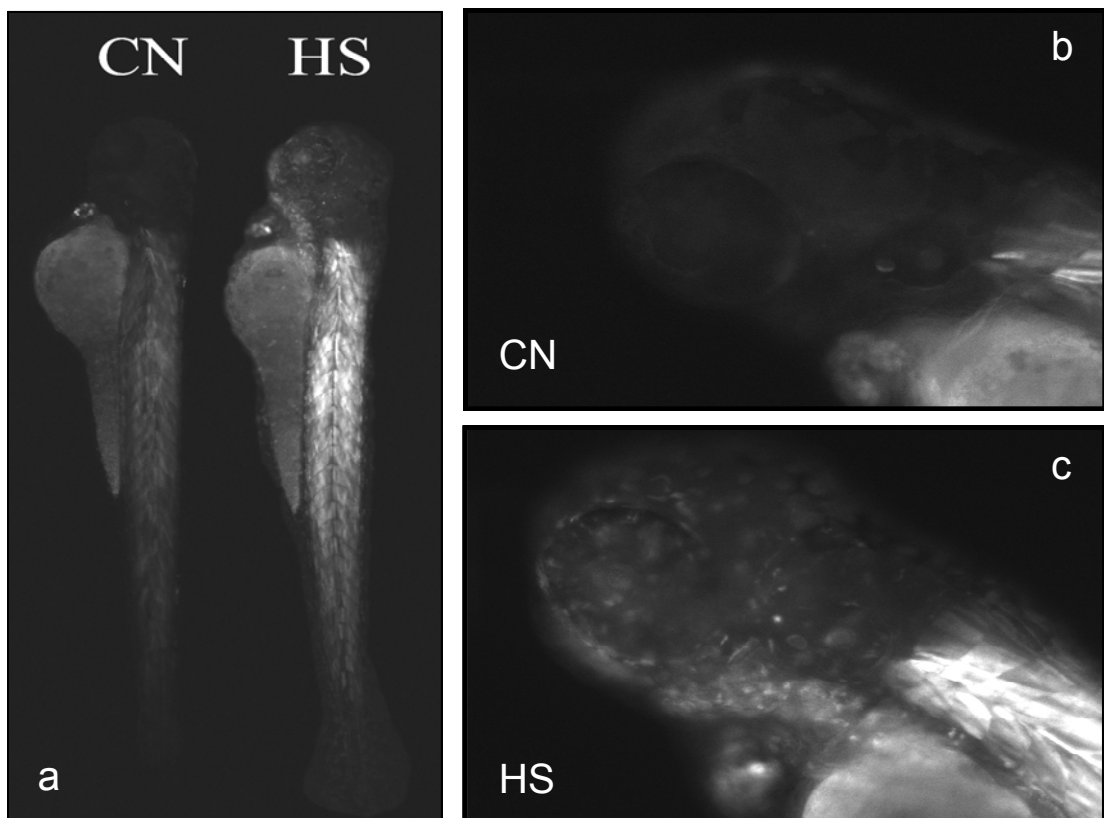
LIST OF ABBREVIATIONS

$\alpha 27$ – antisense Hsp27 morpholinos

CFP – cyan fluorescent protein

CN – control conditions

E.coli – *Escherichia coli*

IOD – integrated optical density

hpf – hours post fertilization

HS – heat shock conditions

Hsp – heat shock proteins

Hsp27 – heat shock protein with molecular weight of 27 kDa

PCR – polymerase chain reaction

Scr – scrambled morpholinos

SD – standard deviation

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM – standard error of the mean

sHsp – small heat shock proteins