Chapter 2

Materials and Methods

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2.1 Embryo collection

Male and female zebrafish (*Brachydanio rerio*) pairs were set up in breeding boxes the evening before embryos were required. The following morning, upon light cycle activation (at 8.30am), the female is stimulated to release her eggs, which are subsequently fertilized by the male. Both the male and female zebrafish are separated from the embryos by a mesh divider to prevent them from eating their offspring. To obtain precise staging of the embryos, the male and female can be separated overnight within the same breeding box (by the mesh divider), and then the following morning put together. Embryos were collected and raised in either Egg Water (0.18g/l sea salt, 2mg/l methylene blue) or 0.3 X Danieaus (17.4mM NaCl, 0.21mM KCl, 0.12mM $MgSO_4$, 0.18mM $Ca(NO_3)_2$, 1.5mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)) at 28^{o}C. Embryos were staged according to their morphology as outlined by(Kimmel et al., 1995).

2.2 Morpholino oligonucleotide design and injection

Antisense morpholino oligonucleotides (MOs) were used to block translation at the start or splice site of a given gene. All MOs used were 24-25 oligomers of approximately 50% GC content and were generated and designed by Gene Tools LLC, see Table 2.1 for MO sequences. All ordered MOs were checked for by performing a BLAST (Basic Local Alignment Search Tool) search of the Ensembl zebrafish genome to ensure that each MO specifically matched the target sequence.

Table 2.1 Sequence of MOs used in this thesis.

MO	Sequence 5′-3′	N° of bases identical to capZα1	N° of bases identical to capZα2
CapZa1 ATG	GCTCGTGACCATAATAGGGCAAAAC		0/25
CapZa1 splice 1	AATGCTTTATTTTTACCTTCTCCTC		16/25
CapZa1 splice 2	GCTTGTAGCTCTTACCTGATAATCG		13/25
CapZa2 ATG	AAGCTGTTCCTCAAAGTCCGCCATC	21/25	
CapZa2 splice	AGATTACTCACCTGATCTTCACTTC	13/25	
CapZb ATG	CCAACTGCTGCTCATTCATGTCTGC		
CapZb splice	GGGCTGGCTACTCACAGGTCTCTG		
Desmin ATG	TTGGGTTTGTACGCTGTGTGAATGC		
Desmin splice	ATAAAGTACATACAGCTCTGAAAGC		
SK Tmod4 ATG	TCTCTGGGATCACTCTTAGACATAC		

All MOs received were diluted in 100µl of Milli-Q (MQ) H_2O. The concentration of each MO in mg/ml was subsequently determined by measuring the MO (diluted 1 in 800 in 0.1M HCL) at 265nm with a spectrophotometer (Amersham Biosciences Ultrospec 1100 *pro*), and then using this reading in the following formula:

$$\text{Absorbance at 265nm} \times \text{Dilution factor} \times \frac{\text{Molecular Weight}}{\text{Molar Absorbance}}$$

The molecular weight and molar absorbance were stated in the Gene Tools LLC data sheet accompanying each requested MO. The MO was subsequently diluted in morpholino buffer (5mg/ml phenol red, 4mM HEPES (pH7.2), 160mM KCl) or MQ H_2O to produce final concentrations ranging between 3-10ng/1.4nl. Needles for injecting MO into embryos were made by a needle puller (David Kopf Instruments, Model 720) using F-type capillaries (Kwik-Fil TM borosilicate glass capillaries with filament). 1.5µl of MO was loaded per needle, once in the holder (World Precision Instruments), which is controlled by a three axis micromanipulator (Narishige), the tip of the needle was cut open with a blade. The amount of MO ejected from the needle was controlled by a compressed air flow control panel (World Precision Instruments

Pneumatic Pico Pump PV820), and measured using a graticule (0.05mm striations). Each needle was calibrated by producing a droplet with a diameter of 0.3mm (14nl) from ten pulses, therefore each single pulse ejected 1.4nl. The MO was then injected into the yolk of 1-2 cell stage embryos aligned along the edge of a slide placed in a petri dish.

2.3 General molecular biology techniques

2.3.1 Purification of DNA using phenol chloroform

The volume (vol) of each DNA sample was firstly made up to 100µl with MQ H_2O and then phenol:chloroform:isoamyl alcohol (25:24:1) was added. The solution was vortexed for 30 s and centrifuged (14645 xg) for 5 min at 4^{o}C. The aqueous top layer was placed in a new tube and vortexed for 30 s with an additional 100µl of chloroform:isoamyl alcohol (24:1). The solution was then centrifuged again for 5 min at 4^{o}C. The top layer was pipetted into a new tube and 0.1 vol of 3M sodium acetate and 3 vols of 100% ethanol were added. The solution was left at -20^{o}C for 1 hr then centrifuged (16168 xg) for 30 min. The pellet was subsequently washed in 70% ethanol and resuspended in 20µl of MQ H_2O.

2.3.2 DNA extraction from agarose gels

DNA bands were observed under UV light (365 nm) and cut out of a 2% agarose gel. A Qiagen gel extraction kit was then used to extract the DNA from the gel according to the manufacturer's protocol. Three vols of buffer QB was added to each gel piece and the gel dissolved by incubation at 55^{o}C for 10 min. The solution was then transferred to a column and centrifuged for 1 min (16168 xg), the column was then washed with PE buffer and the DNA was eluted in 30µl of MQ H_2O.

2.3.3 Restriction digestions

All digests were performed according to enzyme manufacturer's instructions (Promega or New England Biosciences (NEB), using appropriate buffers at the recommended temperature (temp), for 2 hrs in a total vol between 20-40µl.

2.3.4 Ligation

The NEB Quick Ligation™ kit was used ligate digested PCR products into the appropriate vector according to the manufacturer's protocol. Linearized vector (50ng) was mixed with a three fold molar excess of insert in a final vol of 10µl. Then 10µl of 2 X Ligation buffer and 1µl of T4 DNA ligase was added to each sample and incubated for 5 min at room temp. The ligated vector was then transformed into chemically competent cells by heat shock as described in the following section.

2.3.5 Ligation using TOPO TA® and TOPO Blunt II® vectors

PCR product (2-4µl) was mixed with 0.5µl of TOPO® vector (0.5ng), 1µl of NaCl (1.2 µmol) and made up to a vol of 6µl with MQ H_2O. The solution was incubated for 30 min at room temp. The ligated plasmids were then transformed into Invitrogen TOP10 chemically competent cells by incubating 2µl of ligation mix with 25µl of competent cells for 30 min on ice. Cells were heat shocked for 1 min at 42^{o}C and then recovered in ice for 5 min. SOC (2% Tryptone, 0.5% yeast extract, 8.55mM NaCl, 20mM $MgSO_4$ $7H_20$, 20mM dextrose monohydrate) (250µl) was then added to the cells and the sample was shaken at 200 rpm at 37^{o}C for 1 hr, before being plated onto Kanamycin (50µg/ml) (TOPO® Blunt II vector) or Ampicillin LB agar plates (100µg/ml) (TOPO® TA vector). If the cloning was successful single colonies formed on the plates after they had been incubated at 37^{o}C overnight.

2.3.6 Colony PCR

Each single colony was mixed with 0.2mM dNTPs, 0.4µM of forward and reverse primer, 1 X REDTaq PCR reaction buffer and REDTaq® polymerase (0.06U/µl). PCR conditions were as follows: 94 ^{o}C for 2 min followed by 35 cycles of 94 ^{o}C for 30 s, 60^{o}C for 1 min, 72 ^{o}C for 2 min, and finally 72 ^{o}C for 3 min.

2.3.7 Extraction and purification of plasmids

Individual colonies were picked and grown in 2ml of 2 x YT (1.6% tryptone, 1% yeast extract, 85.5mM NaCl) and the appropriate antibiotic (Kanamycin 50µg/ml and Ampicillin 100µg/ml), shaking at 200 rpm overnight at 37^{o}C. Cultures were spun down and plasmids were extracted using the QIAprep® miniprep kit. Cells were resuspended in 250µl of solution 1 containing RNase A (1mg/ml), then 250µl of solution 2 was added, samples mixed gently and finally 350µl of solution 3 was added, the samples were mixed again and then centrifuged (16168 xg) for 10 min. The supernatant was then transferred to a QIAprep spin column and centrifuged for 1 min (16168 xg). The column was washed with PE buffer (1 min at 16168 xg) and then the plasmid DNA was eluted with 50µl of MQ H_2O. Plasmid concentrations were quantified on a Nano Drop®-1000 spectrophotometer at 260nm. An OD of 1 at 260nm equates to 50µg/ml of double stranded DNA.

2.4 DNA sequencing of PCR products and plasmids

DNA sequencing was performed by the Sanger Centre Small Sequencing Facility using the ABI PRISM big dye terminator cycle sequencing ready reaction kit according to the manufacturer's instructions, in an ABI 3730x1 automatic sequencer. 100ng of plasmid or 15-25ng of PCR product and 0.4pmols of primer were provided for each sequencing reaction.

2.5 Bioinformatics

DNA sequence was analyzed and manipulated using the Lasergene software package. Determining the genotype of adult fish and embryos by sequencing was performed by aligning sequences in GAP4 and identifying nucleotide changes. Orthologous alignments and phylogenetic trees were made in MegAlign using the Clustal W method. The Ensembl search engine was used to find zebrafish sequence, and NCBI and Ensembl were used to find orthologous sequences.

2.6 RNA extraction and RNA *in vitro* synthesis

2.6.1 RNA extraction from zebrafish embryos

Total RNA was isolated at various stages by homogenizing 30-50 dechorionated embryos in 500µl of TRIzol® reagent (Invitrogen). The homogenized tissue was then incubated for 5 min at 30^{o}C. Chloroform (0.1ml) was subsequently added to each sample and shaken vigorously for 15 s, then incubated for a further 3 min at 30^{o}C. The solution was centrifuged for 15 min (13362 xg) at 4^{o}C and the upper aqueous phase was removed to a fresh tube before the RNA precipitated by adding 0.25ml of isopropanol. Samples were then incubated for 10 min at 30^{o}C, centrifuged for 10 min (16168 xg) at 4^{o}C, the supernatant was removed and pellet washed once in 75% ethanol and centrifuged for 5 min (5220 xg). The pellet was then air dried for 5-10 min at room temp and the RNA finally dissolved in RNase free water by incubation for 10 min at 55^{o}C.

2.6.2 Capped RNA synthesis

DNA (2µg) was incubated with 1X transcription buffer (Promega), 9.26mM DTT (dithiothreitol), 0.926mM CAP structure, 60U of RNA Polymerase, 100U of RNAse inhibitor 0.926mM ATP, UTP, CTP and 92.6µM GTP, for 20 min at 37^{o}C. More GTP was then added to give a final GTP concentration of 0.74mM and the solution was incubated for a further hour. Roche DNAse I (30U) was added and the solution incubated for 45 min at 37^{o}C, then the RNA was purified in a BD Biosciences CHROMA SPIN™ column. The column was first prepared by centrifuging it for 5 min at 1258 xg, flow through was discarded and the column re-centrifuged again under the same conditions, prior to the addition of the RNA sample to the column. The RNA was eluted from the column by centrifugation at 1811 xg for 5 min. The RNA concentration was subsequently determined by measuring the absorbance at 260nm on the Nano Drop®-1000 spectrophotometer. An OD of 1 at 260nm equates to 40µg/ml of RNA.

2.6.3 Anti-sense RNA probe synthesis for whole-mount *in situ* hybridizations

Each RNA probe was synthesized from 0.4µg of linearized plasmid, 1 X Transcription buffer, 0.01M DTT, 1 X DIG (Digoxigenin) RNA labeling Mix (Roche), 52.5U of RNase Inhibitor and 20U of RNA Polymerase in a total vol of 20µl. The solution was incubated at 37^{o}C for 2 hrs, and then the volume was increased to 50µl with MQ H_2O. DNase I (20U) was also added to digest the plasmid template. The sample was incubated for a further 30 min at 37^{o}C before the RNA was recovered in a CHROMA SPIN™ column as outlined in the previous section. After measuring the RNA concentration on the Nano Drop®-1000 spectrophotometer the RNA was diluted to 10 X working concentration (10µg/ml) in RNA *in situ* hybridization (ISH) buffer. The probes generated and the plasmids they were derived from are shown in Table 2.2.

Table 2.2 *In situ* probes generated to detect the expression of the zebrafish *capz* subunits. The primers used to generate each insert are shown in the appendix, Table 3.

Probe	size	Vector	Enzyme used to linearize plasmid	Polymerase used to transcribe RNA
capzα1	599 bp	pCR®-blunt II-TOPO®	Spe I	T7
capzα2	480 bp	pCR®-blunt II-TOPO®	BamHI	T7
capzβ	596 bp	pCR®-blunt II-TOPO®	XhoI	Sp6

2.7 Whole-mount mRNA *in situ* hybridizations

Whole-mount mRNA ISHs were performed as described in (Thisse et al., 1993). Embryos at various stages were fixed in 4% PFA in PBS overnight at 4°C before being stored in 100% methanol at -20°C prior to use in whole-mount ISHs. Embryos stored in methanol were initially rehydrated by washing for 5 min in decreasing concentrations of methanol (75%, 50%, 25%) diluted in PBT (1 X PBS / 0.1% Tween 20). They were then washed four times in PBT for 5 min each. Embryos over 24 hpf were permeabilized with Proteinase K (10µg/ml) for 15 min. All embryos were re-fixed in 4% PFA for 20 min then washed in PBT five times for 5 min each. Embryos were subsequently pre-hybridized in 800µl of hybridization buffer (50% formamide, 5 X SSC, 0.1% Tween 20, 50µg/ml Heparin, 500 µg/ml Torula RNA) for 2 hrs at 68°C prior to hybridization with 200µl of RNA anti-sense probe diluted in hybridization buffer (final concentration 1µg/ml) overnight at 68°C. The next day the embryos were washed briefly in hybridization wash buffer (does not contain Heparin and Torula RNA) followed by 75% hybridization wash buffer (HWB)/2XSSC at 68°C for 15 min, 50% HWB /2XSSC at 68°C for 15 min, 25% HWB/2XSSC at 68°C for 15 min, 2XSSC at 68°C for 15 min, two washes in 0.2XSSC/50% formamide for 30 min each at 68°C. All subsequent washes were done at room temp starting with 75% 0.2XSSC/PBT for 10 min, 50% 0.2XSSC/PBT for 10 min, 25% 0.2XSSC/PBT for 10 min and finally PBT for 10 min. The embryos were then blocked in 2%

sheep serum / 2mg/ml BSA / PBT at room temp for several hours. The Anti-DIG alkaline phosphatase conjugated fab fragment antibody (1:2500, Roche) was then added and left overnight at 4°C. The following day embryos were washed with PBT at room temp six times for 15 min each, before being washed three times for 5 min each in staining buffer (100mM Tris-HCL pH 9.5, 50mM $MgCl_2$, 100mM NaCl, 0.1% Tween 20). Embryos were then placed in glass staining dishes and the colour developed with a 100 mg/ml NBT (Nitro blue tetrazolium) /50 mg/ml BCIP (5-Bromo-4-chloro-3-indolyl phosphate dipotassium salt) solution. The colour reaction was stopped by washing embryos in stop solution (1 XPBS pH 5.5, 2mM EDTA) for 3 min and re-fixing in 4% PFA for 20 min.

2.8 Extraction of genomic DNA from embryos and adult zebrafish

2.8.1 Extraction of DNA from 5 dpf embryos

Embryos were fixed overnight in 100% methanol at 4°C, then individual embryos were placed in PCR tubes and the excess methanol was evaporated by placing the tubes at 42°C for 5 min. 50µl of lysis buffer (10mM Tris-HCL pH8, 1mM EDTA, 0.3% Tween 20, 0.3% Igepal CA-630) was then added to each well and embryos were incubated for 10 min at 98 °C, prior to digestion with Proteinase K (1mg/ml) at 55°C overnight. The Proteinase K was inactivated by incubation at 98°C for 10 min. and the DNA was subsequently diluted (1: 25) before use in PCR reactions.

2.8.2 Extraction of DNA from adult zebrafish

Adult zebrafish were anaesthetized in 0.02% 3-amino benzoic acidethylester (tricaine), approximately 0.5cm of the tip of the tail fin was cut and the tissue was digested in 400µg/ml of Proteinase K/TE(10mM Tris-HCL pH8, 1mM EDTA) at 55°C overnight. The Proteinase K was

inactivated by incubation of the sample at 98^{o}C for 10 min. A 1 in 50 dilution of each DNA sample was used in subsequent PCR reactions.

2.9 Primer design and PCR

2.9.1 Primer design

All primers were created by Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) using sequence obtained from the Ensembl zebrafish genome browser (Zv4, Zv5 and Zv6) (Hubbard et al., 2007). Indel primers were generated from indel regions annotated onto the Ensembl Zv5 DAS (distributed annotation system) track. Indels are regions of insertions or deletions in the genome that are polymorphic between two individual Tübingen zebrafish.

2.9.2 Genomic DNA PCR for genotyping of adult fish by sequencing

Nested PCR was performed to amplify the region containing the mutation for sequencing. The second set of inside primers were extended with M13F and M13R sequence for sequencing purposes. Each PCR was performed in a total vol of 10µl and contained 5µl of genomic DNA (diluted 1 in 50), 0.6µM forward and reverse primers, 1 X genotyping PCR buffer (10mM Tris-HCL pH8.5, 50mM KCL, 2mM $MgCl_2$, 0.01% Tween 20), 0.6mM dNTPS, 0.4U Taq polymerase. The PCR conditions were as follows. 94^{o}C for 3 min, 35 cycles of 94^{o}C for 45 s, 64^{o}C for 45 s, 72^{o}C for 90 s and finally 72^{o}C for 10 min. The second PCR had the same conditions as the first PCR, however, the genomic DNA was replaced with 180nl of the first PCR product (transferred by a Matrix QRep 96 pin replicator).

Prior to sequencing excess nucleotides were removed from the PCR product by addition of EXOSAP (0.5U of Shrimp alkaline phosphatase, 1U of Exonuclease I, and 1 X SAP buffer (20mM Tris-HCL-pH8, 10mM $MgCl_2$), samples were incubated at 37^{o}C for 1 hr followed by 80^{o}C for 15 min. The amount of PCR product was quantified using the Invitrogen Quant iT™ PicoGreen® dsDNA kit. PicoGreen® reagent (200nl) and TE buffer (10mM Tris-HCL pH7.5, 1mM EDTA) (100µl) was added to PCR product (2µl) and provided DNA standards, and the luminescence was detected in a fluorescence microplate reader (Perkin Elmer Fusion™) at 480nm. The concentration of DNA was calculated from the graph of the standards.

2.9.3 SSLP PCR from embryos

Simple sequence length polymorphisms (SSLPs) were detected by performing the PCR in a total vol of 15µl with 1µl of genomic 5 days post fertilization (dpf) embryonic DNA, 1 X PCR buffer (2mM $MgCl_2$, 13.6mM Tris-HCL, 68mM KCL, 0.00126% gelatin, 136.36µg/ml of BSA, 200µM of dNTPs), 0.3 µM of forward and reverse primers, and 0.4U of Taq polymerase. PCR conditions were as follows: 94^{o}C for 2 min, 35 cycles of 94^{o}C for 30 s, 60^{o}C for 30 s, 73^{o}C for 30 s and finally 73^{o}C for 5min. PCR products were run on a 2.5-3% agarose gel (with 0.75 µg/ml of ethidium bromide) in a Maxi-Plus horizontal electrophoresis unit (Sigma-Aldrich) at 200-250 V for 90 min, and visualized on a BIO RAD UV imager.

2.9.4 RT-PCR

cDNA was synthesized from 2µg of total RNA using the SuperScript™First-Strand Synthesis System (Invitrogen). RNA was mixed with random primers (0.25µg/µl) and incubated for 10 min at 70^{o}C. Then 1 X First Strand buffer, 0.5mM DTT and 10mM dNTPs were added to the RNA and incubated for 2 min at 42^{o}C, prior to the addition of the SuperScript reverse

transcriptase (10U/µl). The sample was subsequently incubated at 42°C for 1 hr, 70°C for 15 min then placed on ice for 10 min. The resulting cDNA was diluted 1 in 10 and 2 µl was used in a 20µl KOD polymerase PCR.

2.9.5 KOD Polymerase PCR

Blunt ended high fidelity PCR products were generated using Novagen® KOD Hot Start polymerase according to the manufacturer's protocol. The PCR was performed in a total vol of 20µl using 2µl of cDNA diluted 1 in 10, 1 X PCR buffer, 1mM $MgSO_4$, 2mM of dNTPs, 2µM of forward and reverse primer and 0.75U of KOD Hot Start polymerase. PCR conditions were as follows: 94°C for 2 min, 35 cycles of 94°C for 15 s, 60°C for 1 min, 72°C for 90 s and finally 72°C for 10 min. PCR products were run on a 1-2% agarose gel (with 0.75 µg/ml of ethidium bromide) and visualized using the BioRad UV imager.

2.10 Construction of the capZα1-GFP expression plasmid

The capZ α1-GFP pCS2+ construct was designed with GFP attached to the C-terminus of capZα1 by a 4 amino acid glycine linker (see appendix, Fig.1). Restriction sites and the sequence of the glycine linker were incorporated into the primers to facilitate the formation of the fusion construct and insertion into pCS2+. The open reading frame of capZα1 was amplified from cDNA (derived from 24 hpf wild type embryos) by KOD polymerase PCR using the CapZORFF and GFPR1 primers (Table 2.3). The GFP open reading frame was also amplified by KOD polymerase PCR (from an eGFP-pCS2+ plasmid) using GFPF2 and GFPR2 primers (Table 2.3). The capZα1-GFP fusion product was subsequently generated by an additional KOD polymerase PCR using the individually amplified capZα1 and GFP products (3ng) and capZORFF and GFPR2 primers. The fusion product was digested with BamHI and Xho I prior to ligation with

linearised pCS2+ (linearised with the same enzymes). The sequence of each clone was checked before RNA injection into the embryo.

Table 2.3. Table of primers used in generation of the capZ α1-GFP fusion construct. The BamHI and XhoI restriction sites are incorporated into the capZORFF and GFPR2 primers respectively. The sequence for the glycine linker is incorporated into the GFPR1 primer.

Primer name	Sequence 5′-3′
capZORFF	TCGAGTGGATCCATGACCGACTTTGAGGAGCCG
GFPR1	CTCCTCGCCCTTGCTCACGCCACCTCCGCCAGCGTTTTGCATCTCTTTACC
GFPF2	GTGAGCAAGGGCGAGGAGCTGTTCACCG
GFPR2	CCAGGCCTCGAGTTACTTGTACAGCTCGTCCAT

2.11 Immunohistochemistry

2.11.1 Phalloidin staining

Embryos at 1, 2, 3 and 5 dpf were fixed in 4% paraformaldehyde (PFA) for 2 hrs, permeabilized in 2% TritonX-100/1X PBS for 2 hrs, then incubated in Alexa Fluor® 488 phalloidin (Molecular Probes, 2.5 U/ml) overnight at 4^{o}C. The following day the embryos were washed three times for 10 min each in PBST (1 X PBS, 0.2% BSA, 0.1% TritonX-100), re-fixed for 20 min in PFA and washed again in PBST three times for 10 min each, before being mounted for visualization under the confocal microscope.

2.11.2 α-Actinin antibody staining

Embryos at various stages were fixed in 4% PFA for 2 hrs, washed twice in PBST and subsequently re-fixed at -20^{o}C in acetone for 20 min. They were then permeabilized in Proteinase K (5µg/ml), blocked in PBDT (1 X PBS, 1% BSA, 1% DMSO, 0.5% TritonX-100) and incubated with the primary antibody, mouse monoclonal anti-alpha-actinin 1:200 (Sigma A7811)

overnight at 4°C. After washing in PBDT several times the embryos were incubated in Fluorescein Isothiocyanate (FITC) conjugated, donkey anti-mouse antibody (1:400) overnight at 4°C. Embryos were washed in PBST and the staining pattern was subsequently observed by confocal microscopy.

2.11.3 CapZα1 antibody staining

Embryos were fixed in 100% methanol overnight at -20°C, they were then rehydrated into PBT with 5 min washes in 75% methanol/ PBST, 50% methanol /PBST, 25% methanol /PBST and finally 100%PBST for 30 min. Embryos were then permeabilized for 8 min in Proteinase K (10μg/ml). After three x 5 min washes in PBST they were then blocked for 2 hrs in 10% goat serum / PBST. The chicken CapZα1 primary antibody (1:200, Abcam) was left on overnight at 4°C. On the second day embryos were washed with PBST for 6 x 15 min, and blocked in the 10% goat serum/PBST for an hour before the secondary antibody was left on overnight (donkey anti-chicken conjugated to FITC, 1:200). On the third day the embryos were washed again with three 10 min washes in PBST, before being mounted onto slides and visualized by confocal microscopy.

2.11.4 Desmin antibody staining

Embryos were fixed on day 5 in 4%PFA for 1 hr at room temp, they were then washed in 0.5% Triton X-100/PBS three times for 5 min each. The primary antibody (1:100 dilution of rabbit anti-desmin antibody, Sigma D8281) was left overnight at 4°C. The following day the embryos were washed again in 0.5% Triton X-100/PBS three times for 5 min each before being incubated in the secondary antibody (1:200, anti-rabbit conjugated to FITC) for 1 hr at room

temp. The embryos were then washed in PBST, before they were mounted on slides and the immunostaining observed with the confocal microscope.

2.12 Image capture of live and fixed embryos

Images of live embryos and whole mount *in situ* hybridized specimens were taken with a Leica MZ16 FA dissecting scope or a Zeiss AXIO imager M1 compound microscope using a Zeiss Axiocam digital camera. The live embryos were initially anaesthetized in 0.02% tricaine and mounted in 2% methyl cellulose. The fixed and stained embryos were washed in increasing concentrations of glycerol (25%, 50% and 75%) before images were taken of embryos mounted in 100% glycerol. Confocal images of immunostained skeletal muscle were taken using a BioRad Radiance 2100 system with a Nikon E800 Eclipse microscope. The head and yolk of each embryo was removed before being mounted in VECTA SHIELD®.

2.13 Protein extraction from embryos

Chorions were removed from embryos of various stages, before embryos were added to lysis buffer (50mM Tris-HCL pH7.5, 150mM NaCl, 1mM EDTA, 1% Triton-X 100, 0.1% SDS, 2.5% Igepal CA-630) and 1 X Complete protease inhibitor (Roche). The embryos were then macerated in an eppendorf with a pestle. The homogenized tissues were centrifuged (16168 xg) for 10 min at 4^{o}C and the supernatant was recovered and snap frozen on dry ice prior to storage at -80^{o}C.

2.14 Western blotting

Each protein sample was mixed with 1 X NuPAGE®LDS sample buffer (Invitrogen) and 50mM DTT before being heated to 70^{o}C for 10 min, loaded onto a NuPAGE® Novex 4-12% Bis-Tris gel in 1 X MOPS SDS running buffer and run at 200 V for 1 hr. The protein on the gel was then transferred to a PVDF (polyvinylidene difluoride) membrane (Hybond™-P, Amersham Biosciences) in transfer buffer (20% methanol, 0.192M glycine, 0.025M Trizma base pH 8.3), using the Invitrogen Xcell II™ Blot Module for 90 min at 30 V. The membrane was then blocked in blocking buffer (5% Marvel skim milk in 0.05%Tween/PBS) for 2-3 hrs at room temp. The primary antibody (mouse IgG, anti-human CapZα, BD biosciences) was diluted (1:200) in antibody blocking buffer (2% Marvel in 0.05% Tween/PBS) and left to incubate with the membrane overnight at 4^{o}C. The membrane was then washed three times for 10 min with 0.05% Tween/PBS prior to incubation with the secondary antibody (1:2000 dilution of goat anti-mouse, IgG (H + L) HRP conjugated, diluted in blocking buffer) for 1 hr at room temp. The membrane was washed again in 0.05% Tween/PBS three times for 10 min. The secondary antibody was subsequently detected by chemiluminescence using the Amersham ECL plus Western Blot Detection System according to the manufacturer's protocol. Solution A (8 ml) was mixed with 200µl of Solution B. This mixture was then poured onto the membrane and incubated in the dark for 5 min before the membrane was transferred to a cassette, exposed to film (Amersham Hyperfilm™ ECL) and the image on the film detected on the developer (Compact X4, Xograph Imaging systems).

2.15 Transmission electron microscopy

Transmission electron microscopy (TEM) was performed by David Goulding. Embryos were dissected under fix at 4°C, separating the tails by transverse section just before the yolk sac. Primary fixation with 2.5% gluteraldehyde and 2% PFA in PBS (0.01M at pH7.2) was continued for a total of 2 hrs and followed by three rinses in PBS and two rinses in sodium cacodylate buffer (0.1M at pH 7.42) for 5 minutes each, all at 4°C. All further steps were conducted at room temp. Secondary fixation in cacodylate buffered 1% osmium tetroxide for 1 hr was followed by a brief rinse in the same buffer, immersion in buffered 1% tannic acid for 30 min and 1% aqueous sodium sulphate for 10 min. Specimens were dehydrated in an increasing series of alcohols and propylene oxide (staining *en bloc* at the 20% ethanol stage with 2% uranyl acetate for 20 min), infiltrated with Epon overnight (TAAB Laboratories) and then cured at 60°C for 24 hrs. Longitudinal 70nm ultrathin sections along the embryo tail were cut on a Leica EM UC6 ultramicrotome, mounted onto 200 mesh copper support grids and contrasted with uranyl acetate and lead citrate. Imaging was performed on a Tecnai Spirit Biotwin TEM at 120kV using a Tietz F4.15 CCD camera.