Chapter Two

Materials and Methods

2 Materials and Methods

2.1 Embryo collection

Zebrafish (*Danio rerio*) female and male pairs were placed in tanks together in the evening before eggs were required. Eggs are usually laid and fertilised the following morning shortly after the lights are turned on (lights on occurs regularly at 8:30am), though on occasion when embryo collection was desired later, males were kept physically separated from the females within the same tank and released when eggs were required. Embryos were collected in Embryo Water (red sea salt 180mg/l, methylene blue 2mg/l) shortly after having been laid. Embryos were raised from the day of collection up to 5 days at 28°C in Embryo Water. Embryos were staged according to the morphological criteria provided in (Kimmel et al., 1995). Zebrafish embryos collected for staining procedures were fixed at least overnight in 4% PFA in phosphate buffered saline (PBS: 137mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_4.7H_2O$, 1.4mM KH_2PO_4) at 4ºC. Embryos of 24 hpf or older were de-chorionated prior to fixation, to prevent fixation of the embryos with curled trunks, whereas embryos younger than 24 hpf were de-chorionated after fixation and before dehydration. Following fixation, embryos were dehydrated in increasing concentrations of MeOH in PBS (25%, 50%, 75% then 100%). Dehydrated embryos were stored in 100% methanol at -20 °C until required.

2.2 General molecular biology techniques

2.2.1 Small scale preparation of DNA

The Qiagen Spin miniprep kit (Quiagen) was used for all small-scale plasmid preparations, according to the manufacturer's protocol.

DNA and RNA were quantified by spectrophotometry at 260 nm (an OD of 1 was equated to 50 μg/ml double stranded DNA, 35 μg/ml single stranded DNA and 40 μg/ml RNA). The ratio between the readings at 260 nm and 280 nm provided an estimate of the purity of the nucleic acid preparation (pure preparations of DNA and RNA should have OD_{260}/OD_{280} values of 1.8 and 2.0, respectively).

2.2.2 Gel extraction of DNA

For the extraction of DNA from agarose gels, the QIAquick Gel Extraction Kit (Qiagen) was used according to manufacturers protocol. Samples were eluted in 30μl of water, 4μl of this was used for TOPO cloning or standard ligation reactions and 5μl was used in a standard 50μl PCR reaction.

2.2.3 Phenol/Chloroform extraction

To remove proteins from nucleic acid solutions, a mixture of phenol:chloroform:isoamyl-alcohol (25:24:1 volume ration) was added in a 1:1 volume ratio to the DNA solution and shaken for 1 minute. The sample was then centrifuged for 5 minutes and the upper (aqueous) layer was transferred into a new microcentrifuge tube, a further extracted with an equal volume of chloroform was performed to remove traces of phenol.

2.2.4 Ethanol Precipitation

Ethanol precipitation was carried out by adding 3 M NaOAc pH 5.5 (to a final concentration of 0.3 M) and 3 volumes of ice-cold 100% ethanol to the DNA

solution, which was then left on dry ice for approximately 20 minutes. Centrifugation at 20,000g for 5 to 20 minutes was performed and the DNA pellet was then washed in 70% ethanol, dried and re-suspended in TE or distilled water.

2.2.5 TOPO cloning

The cloning of PCR products was performed using the TOPO TA Cloning®kit (Invitrogen). The cloning reaction was performed according to the following conditions: 4 µl fresh PCR product or 4µl of gel purified product, 1µl of 1.2M NaCl solution and 0.5 µl pCR®-TOPO® vector. These were mixed gently and incubated for 5 minutes at room temperature.

2.2.6 Transformation of chemically competent bacteria

Transformation of the ligated vector was performed using chemically competent TOP10 cells (Invitrogen). Briefly, 2µl of TOPO ligation mix was added to 25µl cells and incubated on ice for 30mins. Cells were then heat shocked for 30s at 42^{o}C, then immediately transferred to ice, incubated for 2mins before adding 250µl SOC added. Cells were then incubated at 37^{o}C for 1hr and an aliquot of 10 to 200 µl from each transformation was spread onto a selective agar plate (100 mg/ml of ampicillin) and incubated overnight at 37°C. 40 µl of X-Gal (20 mg/ml in dimethylformimide) and 40 µl of IPTG (200 mg/ml) were used per plate for selection.

2.2.7 Restriction digestions

Restriction enzyme digests were performed at the recommended temperature for approximately 2 hours using commercially supplied restriction enzymes and buffers (Boehringer Mannheim, Promega, New England Biolabs). The enzyme

component of the reaction never comprised more than 10% of the reaction volume. For enzyme digests using more than one restriction enzyme, the buffer suggested by the manufacturer was used.

2.2.8 DNA Sequencing

DNA sequencing was performed using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit, according to the manufacturers instructions, in an ABI 377 automatic sequencer.

2.3 Bioinformatics and Genomics

DNA sequence manipulation and analysis was performed with either Sequencher, DNAStrider or DNASTAR software. Protein alignments were performed with the Clustal method in MegAlign (DNASTAR). Open reading frames were examined using ORFinder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).

2.3.1 Identification of zebrafish orthologues of mouse or human proteins

Zebrafish ESTs corresponding to orthologues of yeast, mouse or human genes were sought either by name in the nucleotide databases or by probing the zebrafish EST database with a defined protein sequence using the tblastn algorithm at http://www.ncbi.nlm.gov/BLAST/. Sequences found were clustered using Sequencher or SeqMan software. When required, ESTs were ordered to obtain DNA templates for riboprobe synthesis (from the Integrated Molecular Analysis of

Genomes and their Expression, IMAGE or Resource Centre/Primary Database, RZPD).

To define further sequence beyond EST data, the zebrafish genomic database was searched at http://www.ensembl.org/Multi/blastview?species=Danio_rerio. In all cases, consensus sequences were used to probe the protein databases using the blastx algorithm, at http://www.ncbi.nlm.gov/BLAST/, to confirm gene identity (Altschul et al., 1990).

2.3.2 Analysis of Identified Genes

The mRNA and protein sequence of genes identified from genomic sequence were examined using appropriate blast programmes to identify homologues. Zebrafish EST data was probed with the full length protein sequence of identified homologues using tblastn to extend zebrafish sequence and provide full length sequence data. Sequence data was clustered using SeqMan and protein sequence compared to homologues using MegAlign.

2.3.3 Oligonucleotide design

PCR primers were designed using the program Primer3 (Whitehead Web Page) at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi, from regions where reliable DNA sequence was available (Rozen and Skaletsky, 2000).

To disrupt mRNA translation, MOs were designed to target the region around the ATG start of translation. In general, the translation start site of zebrafish genes was identified by alignment of orthologues protein sequences belonging to several species and by examination with ORFinder. Splice MOs were designed against

splice sites identified through analysis of genomic and cDNA sequence. Sequence encompassing the target was sent to Gene Tools LLC, which designed the MO to be purchased. All MOs were 25-mers of approximately 50% G/C content, with less than 36% G content and no more than two consecutive Gs, and forming no more than 4 contiguous internal base pairing. Each design sequence was tested for representation elsewhere in the genome. The MOs designed for this work are listed in **Table 2.1**.

Targeted gene	MO sequence (5'-3')
Control	CCTCTTACCTCAGTTACATTTATA
IRE1	AGCAAACCAGCAACAGCAGCATCTG
XBP1	GTCCCTGCTGTAACTACGACCATTT
ATF-6	AACATTAAATTCGACGACATTGTGC
PERK	CTCCGTCCAGAGAGGGAATGAACAT
BiP	GCAAAAACAGGCAAAGCAACCGCAT
Cadherin-13	CTGGTCCAGTAAGTCATTGTGACAC
doc 1	GAGTTCCACTTGAAAGAAATGTCAT
doc 2	TGTGTCTTCTGCCATCGTGATACTT

Table 2.1 MOs used in this thesis.

2.4 Preparation of genomic DNA from Adult Fish

Whole tail fin from adult fish was dissected and placed in 0.5ml of extraction buffer (0.5% SDS, 0.1 M EDTA pH.8.0, 10mM Tris pH.8, 100 µg/ml Proteinase K) for 5 hours at 55°C. Phenol chloroform extraction and ethanol precipitation were carried out, and the final pellet was resuspended in 30µl TE. A 1:100 dilution of this was used PCR template. 5µl of 10-50 ng/µl adult fish DNA was used in a 20µl reaction.

2.5 Preparation of genomic DNA from embryos

Embryos were dehydrated in methanol, placed in individual wells of 96-well plates and the methanol evaporated. The digestion of each single embryo was done by incubating in 100 µl 100 µg/ml Proteinase K for 5 hours at 55ºC and the Proteinase K denatured by incubation at 95ºC for 5 minutes. 1µl of this crude solution was then used in a 20µl PCR reaction.

2.6 Polymerase Chain Reaction

2.6.1 SSLP mapping

All PCR reactions were performed in 96-well plates with a final reaction volume of 20 µl. For a 20µl total volume PCR reaction we used 4µl of 5x PCR buffer (0.25M KCl, 50mM Tris pH8.4, 12.5mM $MgCl_2$, dNTPs at 1mM and BSA 0.85µg/ml), 2µl 10mM forward and reverse primer solution, 0.2 µl of Taq polymerase (5u/µl) and an appropriate volume of template was used. Sterile H_2O was used to make the reaction volume up to 20µl. Plates were covered with

Microseal 'A' Film (Cat# MSA-5001, MJ Research, Inc) and sealed with heated lid PCR machines. The PCR conditions were as follows:

94ºC for 3 min

35 cycles:

92ºC for 30secs

62ºC for 30secs

72ºC for 30secs

72ºC for 10 min

This PCR program was also used for all RH mapping and for various PCR amplifications, though the extension time was varied accordingly where 1kb of DNA was allowed 1minute extension at 72ºC. On occasion, PCR reactions were amplified across a gradient range (55ºC-70ºC) of primer binding temperatures to determine optimum conditions.

2.6.2 Radiation hybrid panels

96 PCR reactions were performed using the LN54 panel (Hukriede et al., 1999). 20µl total reaction using 0.2µl of AmpliTaq Gold (Perkin Elmer), 2µl of each primer (10µM), and 4µl PCR buffer (5X). The SSLP PCR program was used (see previous section). Analysis of the results and the calculation of linkage and LOD scores was carried out at http://mgchd1.nichd.nih.gov:8000/zfrh/beta.cgi.

2.6.3 dCAPS Analysis

Primers for dCAPS analysis were designed using the web-based dCAPS finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html) with wild type and mutant genomic sequence for COPβ and COPβ' (Neff et al., 2002). The primers used for PCR were:

COPβ forward: 5'-GTTGAGAATGAGTTGAAGAAGGAGGCTCGA-3'

COPβ reverse: 5'-GAAACGTCAGTGTGGACGTG-3'

Which generated an XhoI polymorphism in the wild type sequence.

COPβ' forward: 5'-AGGCAGCCTTTCTGCCCGCACAGA-3'

COPβ' reverse: 5'-CGCACTGCTCTCTAGGGTTT-3'

Which generated an XhoII polymorphism in the wild type sequence.

2.6.4 RT-PCR

For total RNA extraction, embryos were pooled and homogenized in 0.5ml of TRIzol reagent (GibcoBRL) and left at 37ºC for 5mins. 100µl of chloroform was then added, samples were mixed by hand for 15s and then centrifuged at 13000rpm for 10mins at 4^{o}C. The aqueous phase was transferred to a fresh tube and RNA was precipitated with isopropyl alcohol, and then centrifuged for 30mins. The resulting pellet was washed with 70% ethanol and re-suspended in DEPC treated water and the concentration of RNA determined. First strand cDNA was synthesized using superscript reverse transcriptase (GibcoBRL) as manufacturers instructions. Briefly, 1µg of total RNA was used in a 20µl reaction with either random hexamer, polyT or gene specific primers, cDNA was amplified for 1hour at 42ºC and diluted 1:10. 5µl of this template solution was used in a 50µl PCR reaction. On occasion, large fragments or fragments cloned for sequencing were amplified using the KOD Hot Start kit (Novagen) as per the suppliers directions.

2.6.5 Rapid amplification of cDNA ends (RACE)

RACE was performed using the GeneRacer cDNA amplification kit (Invitrogen). mRNA was isolated from total RNA using TRIzol (as described above). The synthesis of the cDNA modified with the Generacer 5' and 3' adaptors was performed as per the manufacturer's protocol, using either random primers or poly-T primer in the reverse transcription reaction. All RACE reactions were performed by nested PCR. The GeneRacer 5' primer (CGACTGGAGCACGAGGACACTGA) or the GeneRacer 3' primer (GCTGTCAACGATACGCTACGTAACG), when performing 5' or 3' RACE reactions, respectively, and a gene-specific primer (GSP 1) designed using Primer3 software (see above), were used in a first round of PCR, for 15 cycles. The product of this round of PCR was diluted 1/40 and 5 µl of this dilution was used as template for the second round of PCR. In the latter, the GeneRacer 5' nested primer (GGACACTGACATGGACTGAAGGAGTA) or the GeneRacer 3' nested primer (CGCTACGTAACGGCATGACAGTG), when performing 5' or 3' reactions, respectively, and a second nested gene-specific primer (GSP 2) were used for 25 cycles. The Touchdown PCR program was used in all cases with the extension time varying according to the size of the expected product (2min per kb).

94°C for 3 minutes

5 cycles:

94°C for 30s

72°C for 4 mins*

5 cycles:

94°C for 30s

70°C for 4 mins*

25 cycles:

94°C for 20s

68°C for 4 mins*

*(extension time in min was varied according to the expected length of the cDNA; e.g. 1kb = 2mins, 2kb = 4min etc.).

2.6.6 Agarose Gel Electrophoresis

Analysis of polymorphic markers in meiotic mapping and examination of nucleic acid size was performed by agarose gel electrophoresis. Gels were prepared by dissolving agarose in 1x TAE (20 mM TRIS acetate, 1 mM $Na_2EDTA.2H_2O$ (pH 8.5)) to a final concentration of 3-4% for polymorphic analysis and 0.8–2% (w/v) for size determination, depending on the expected size of the DNA fragments, and 0.4% ethidium bromide. Nucleic acid samples were mixed with 5x gel loading buffer (Orange G: 4g Sucrose and 0.025g Orange G in 10ml water) and, in the case of RNA, with RNase inhibitor. Electrophoresis was performed at 5–20 V/cm gel length until appropriate resolution was achieved. Ethidium bromide-stained nucleic acid was visualised using ultraviolet light ($\lambda \approx 302$ nm) and fragment size was estimated by comparison with the 1kb or 100b ladder molecular weight markers (Promega) run in at least one of the gel lanes.

2.7 Whole-mount *in situ* hybridisation

For the synthesis of riboprobes, template (plasmid) DNA was linearised for 2 hours, phenol/chloroform purified, ethanol precipitated and then resuspended. Approximately 2μg of linearised plasmid was used in the synthesis of probe. In all cases, digoxigenin (DIG)-labelled uracil triphosphate (UTP) (Boehringer Mannheim)

was incorporated during RNA transcription, as per the manufacturer's instructions. After synthesis, riboprobes were treated with 20 U DNase I (Boehringer Mannheim) at 37 °C for 15 min to remove DNA template and were purified by size-exclusion chromatography through a DEPC water column (Clontech Chroma Spin-100). All riboprobes were electrophoresed on a 1% agarose gel to check size and integrity prior to use. Riboprobes were added to Hyb shortly after synthesis and were stored at -20°C. **Table 2.3** has a list of all cDNAs used as templates for anti-sense RNA probes used in this work, as well as the respective origin.

Whole-mount *in situ* hybridisations were performed essentially as described by Thisse (Thisse et al., 1993). Embryos fixed with 4% paraformaldehyde/PBS at 4°C were dehydrated with methanol at -20°C and rehydrated by soaking for 5 minutes each in 75% methanol/PBT (1x PBS + 0.1% Tween 20); 50% methanol/PBT; 25% methanol/PBT and then 4 times 5 minutes in 100% PBT. All embryos >36 hpf were digested with proteinase K (10 µg/ml) for 5 minutes and then refixed in 4% paraformaldehyde for 20 minutes at room temperature and then washed in PBT 5 times 5 minutes. They were then transferred to hybridisation buffer (50% formamide, 5X SSC (pH7.0), 500 µg/ml type VI torula yeast RNA, 50 µg/ml heparin, 0.1% Tween 20, 9 mM citric acid to pH 6.0-6.5) for 2-5 hours at 70°C (prehybridisation). The hybridisation buffer (Hyb) was then replaced with the mixture containing 150 ng of DIG-labelled RNA probe in 200 µl of preheated hybridisation solution and the embryos were incubated at 70°C overnight. Washes were performed at the hybridisation temperature with preheated solutions for 15 minutes each with 75% Hyb/2X SSC; 50% Hyb/2X SSC; 25% Hyb/2X SSC; 100% SSC and finally 2 times 30 minutes in 0.2X SSC. A series of washes were performed at room temperature for 10 minutes each in 75% 0.2X SSC/PBT; 50%

0.2X SSC/PBT; 25% 0.2X SSC/PBT and 100% PBT. Embryos were blocked in 2 mg/ml BSA, 2% goat serum in PBT for several hours and then incubated with alkaline-phosphatase (AP)-conjugated anti-DIG Fab fragments diluted 1:5000 in 2 mg/ml BSA, 2% goat serum in PBT at 4°C overnight with agitation. After washing at least 8 times for 15 minutes with PBT, the embryos were rinsed 3 times 5 minutes in NTMT reaction buffer (0.1 M Tris-HCl pH9.5; 50 mM $MgCl_2$; 0.1 M NaCl; 0.1% Tween 20). Detection was performed using NBT/BCIP (112.5 µl of 100 mg/ml NBT in 70% dimethylformamide and 175 µl of 100 mg/ml BCIP in 70% of dimethylformamide 1added to 50 ml of NTMT). After stopping the reaction with 100% PBS (pH 5.5), the embryos were refixed in 4% paraformaldehyde/PBS. Embryos were cleared with 20% glycerol/80% PBS, 50% glycerol/50% PBS and stored at 4°C in 80% glycerol/20%PBS.

2.8 Morpholino injection

Morpholino oligonucleotides (MOs) were obtained from Gene Tools, LLC. MOs work through an RNase-H independent process, via binding directly to target RNA sequence to either block translational initiation at the ATG or prevent correct splicing. Control MO was that suggested by Gene Tools, LLC.

Lyophilised MO was re-suspended in 60µl of deionised water. 1µl of this solution was diluted in 799µl 0.1NHCl and O.D measured at 265nm. Precise concentration was determined the specific absorbance and molecular weight as provided by Gene Tools. Prior to microinjection, the MOs were diluted using MO buffer to titrate the dose (5 mM Hepes pH7.2, 0.2 M KCl and 2.5 mg/ml phenol red).

A volume of 1.4nl was injected through the chorion and into the yolk of 1 to 4-cell stage embryos to deliver the desired dose of MO.

Injection needles were prepared by pulling filament-containing borosilicate glass capillaries (Word Precision Instruments, 1B100F-4, outside diameter 1.0mm, inside diameter 0.75mm) with a vertical pipette puller (David Kopf Instruments), cutting the edge with a razor blade, and calibrating under the microscope with a millimetre ruler. Injection system consisted of a needle holder (Word Precision Instruments), carried by a 3-axis micromanipulator (Narishige), connected to a compressed air flow controlled by a control panel (World Precision Instruments) and triggered by a foot pedal. Zebrafish embryos were injected at the 1 to 4-cell stage with 1.4 nl of the desired solution. Embryos were aligned on the side of a glass slide in a sterilin Petri dish under relatively dry conditions.

2.9 Confocal Microscopy

Calreticulin-CFP (Cal-CFP) and 1,4-galactosyltransferase-YFP (GalT-YFP) plasmids were obtained from BD Biosciences (Clontech Living Colors pECFP-ER Cat. #6907-1 and pEYFP-Golgi Cat. #6909-1). The plasmids were linearised with StuI and Co-injected into 1-cell stage embryos in a volume of 1.4nl at a concentration of 10ng/µl each. These embryos were injected with either 3.5ng COPα MO or 3.5ng of control MO in a 1.4nl volume. Confocal microscopy was performed on BioRad Radiance 2100 system with a Nikon E800 Eclipse microscope.

Embryos used for confocal microscopy were collected, injected and incubated in 0.3x Danieau's solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$,

0.6 mM $Ca(NO_3)_2$, 5 mM HEPES (pH 7.6)) to prevent any residual fluorescence from the methyl blue in zebrafish blue water.

2.10 Photomicrography

High-power images from both live and fixed embryos were obtained using a Zeiss Axiophot microscope fitted with either a Kodak DCS420 digital camera, or a Jenoptik Jena system that used Openlab 3.1.2 software. Living zebrafish embryos were photographed in 3% methylcellulose (Sigma). Images were treated with AdobePhotoshop.

Solution	Formulation
1X PBS	137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4
1X PBT	1X PBS, 0.1% Tween 20
1X TAE	40 mM Tris.Acetate, 2 mM $Na_2EDTA.2H_2O$ (pH 8.5)
1X TE	1 mM EDTA, 10 mM Tris.HCl pH 8.0
20X SSC	3 M NaCl, 0.3 M Na_3citrate.$2H_2O$, adjust pH to 7.0 with 1 M HCl
5x PCR buffer	0.25M KCl, 50mM Tris pH8.4, 12.5mM $MgCl_2$, dDTPs (A, T, G, C) at 1mM and BSA 0.85µg/ml
1x Danieau's solution	58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES (pH 7.6)
Orange G (5x)	4g Sucrose, 0.025g Orange G in 10ml H_2O

Table 2.2 Formulation of frequently used solutions.

Protein	Origin	Enzyme	Pol
COPβ	RT-PCR product (this thesis)	EcoRV	SP6
COPβ'	RT-PCR product (this thesis)	BamHI	T7
COPε	RT-PCR product (this thesis)	XbaI	SP6
COPδ	RT-PCR product (this thesis)	XhoI	SP6
COPγ2	RT-PCR product (this thesis)	HindIII	SP6
COPζ	RT-PCR product (this thesis)	HindIII	T7
COPζ2	RT-PCR product (this thesis)	EcoRV	SP6
Echidna hedgehog	(Currie and Ingham, 1996)	EcoRI	SP6
BiP	RT-PCR product (this thesis)	SalI	SP6
doc	RT-PCR product (this thesis)	HindIII	T7

Table 2.3 In situ hybridisation probes used in this thesis.