Chapter 2

Materials and Methods

Summary

In this chapter I describe the methods used to identify and clone zebrafish *jam* paralogues; analyse homology and evolutionary relationships; characterize gene expression and protein localization; maintain, genotype and characterize *jamb* and *jamc* mutant fish; produce recombinant forms of zebrafish Jam proteins and test interactions between them using surface plasmon resonance.

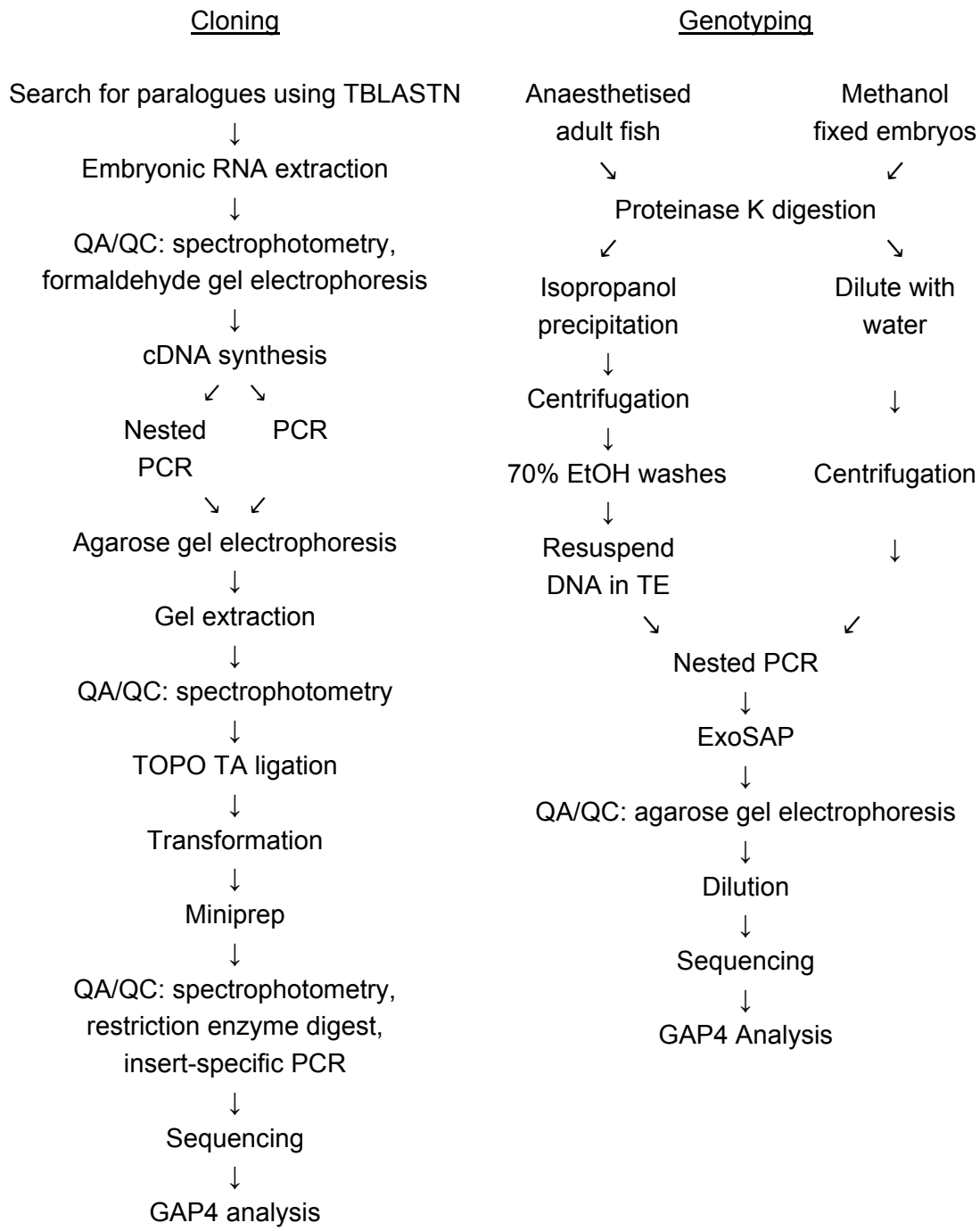

Figure 2.1 Flow diagram of cloning and genotyping methods.

Flow diagram of methods used to clone novel *jam* family paralogues and genotype zebrafish mutant adults and embryos, outlined in sections 2.1 and 2.2.

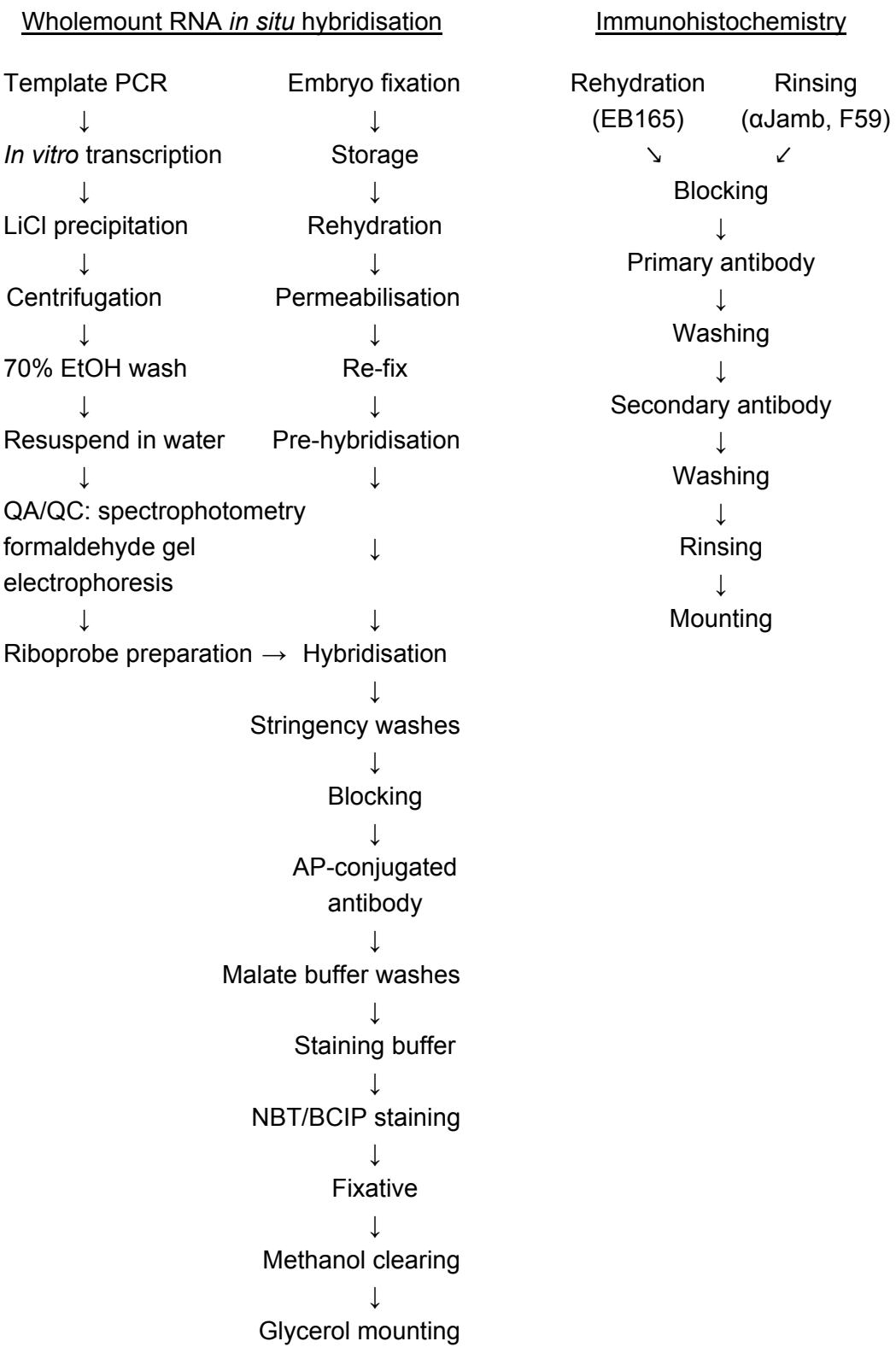

Figure 2.2 Flow diagram of RNA and protein expression detection methods.

Flow diagram of methods used to detect RNA and protein expression in wild-type and mutant embryos, outlined in section 2.3.

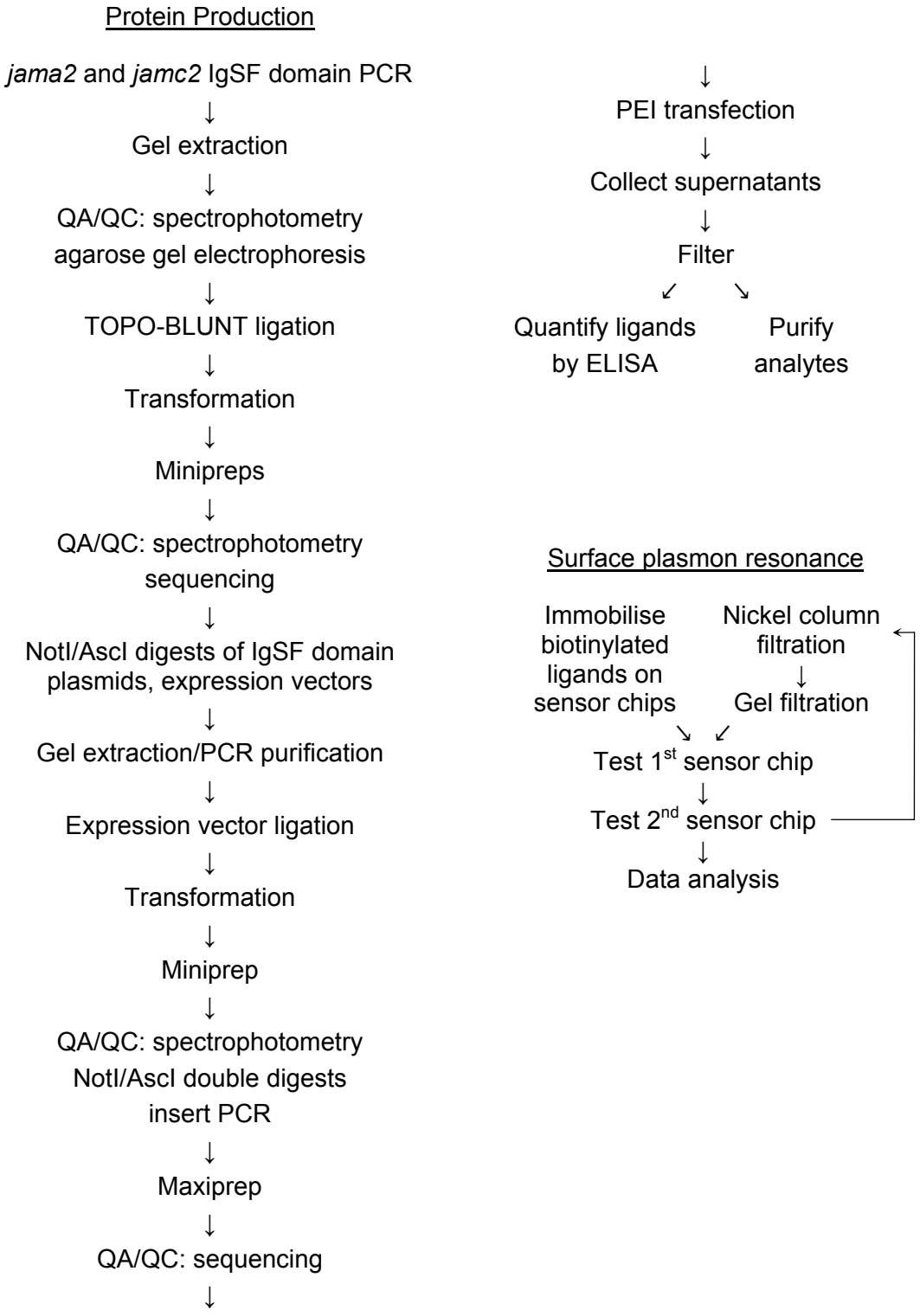

Figure 2.3 Flow diagram of protein production and biochemistry methods.

Flow diagram of methods used to produce recombinant Jam immunoglobulin superfamily domains and test biochemical interactions between the proteins, outlined in section 2.6.

2.1 Cloning and homology

Novel zebrafish *jam* family genes, *jama2* and *jamc2*, were identified by TBLASTN searching in the zebrafish genome sequence (Zv6; Hubbard *et al*, 2007) at Ensembl (www.ensembl.org) using the primary amino acid sequence of the extracellular immunoglobulin domains of Jama and Jamc. Both genes were subsequently cloned through 3′ RACE and RT-PCR (see below and figure 2.1).

2.1.1 Cloning of *jama2* by 3′ RACE

The full-length sequence of *jama2* was subsequently determined by sequencing of cloned 3′ RACE products amplified from cDNA prepared from RNA extracted from 24 hours post fertilisation (h. p. f.) wild-type embryos, as follows:

Zebrafish embryonic RNA was extracted from approximately 30-50 wild-type embryos fixed in methanol, using the Nucleospin RNA II kit (Macherey-Nagal) according to the manufacturer's instructions. The RNA was assessed for purity and quantity by absorbance at 260 and 280 nm using a spectrophotometer and formaldehyde gel electrophoresis, described below (see section 2.1.3).

First strand cDNA was then synthesised from RNA using the SMART RACE kit (Clontech) as per manufacturer's instructions (+RT cDNA). A negative control synthesis without reverse transcriptase (-RT cDNA) was prepared in parallel.

Gene-specific nested 5′ primers (0511, 0512, 3565, 3566; table 2.1) and SMART RACE universal primers (0141 and 0142; table 2.1) were used to amplify *jama2* and a positive control, *igsf11*, by touchdown PCR with Advantage II Polymerase Mix (Clontech). The same reactions were performed in parallel using the negative control -RT cDNA preparation in place of the +RT cDNA template.

The PCR products were analysed by agarose gel electrophoresis, described below (see section 2.1.3). A single 1.5 kbp band, present only in the +RT cDNA nested PCR reaction, was purified from an agarose gel using the QIAquick Gel Extraction kit (QIAGEN) as per manufacturer's instructions. The purified product was assessed for quantity and quality by absorbance at 260 and 280 nm using a spectrophotometer.

The purified *jama2* 3′ RACE product was cloned into the pCR4-TOPO (Invitrogen) by incubation with different concentrations of the vector-topoisomerase complex. TOP10 chemically competent bacteria (Invitrogen) were transformed with each ligation reaction by preincubation on ice, followed by a 30 second heat shock at 42°C. The bacteria were allowed to recover in SOC media (2% tryptone, 0.5% yeast extract, 8.55 mM NaCl, 20 mM $MgSO_4 \cdot 7H_20$, 20 mM dextrose monohydrate;

Invitrogen) by shaking at 37°C, 200 r. p. m. for 1 hour, before plating onto pre-warmed LB agar plates containing kanamycin (50 µg/ml). Inoculated plates were incubated overnight at 37°C.

Colonies that had grown on the selective plates were counted to assess transformation efficiency: approximately 5.0 x 10^5 colony forming units. Six different colonies were transferred to 3 ml 2 x TY (1.6% tryptone, 1% yeast extract, 85.5 mM NaCl) cultures supplemented with kanamycin (50 µg/ml). The cultures were incubated at 37°C, 200 r. p .m. overnight.

Plasmids were purified from the overnight cultures using the QIAprep Miniprep Spin kit (QIAGEN) as per manufacturer's instructions. Each plasmid preparation was assessed for quantity and quality by absorbance at 260 and 280 nm using a spectrophotometer.

The ligated plasmids were tested for the correct insert by digestion of a small sample with the restriction enzyme EcoRI (NEB), used according to the manufacturer's instructions. Also, the vector insert was amplified from each plasmid by touchdown PCR using insert-specific primers (0142 and 3566; table 2.1). The restriction digests and PCR products were analysed by agarose gel electrophoresis. All plasmids contained an insert of expected size.

Each plasmid was sequenced 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. Four primers (M13F, M13R, 3566, 0142; table 2.1) were used to direct sequencing of both strands of the plasmids.

The sequences from each successful sequencing reaction were aligned and compiled into a GAP4 database for analysis.

2.1.2 Cloning of *jamc2* by RT-PCR

The near-complete sequence of *jamc2* was determined by sequencing of PCR products from cDNA prepared using RNA extracted from 24 h. p. f. wild-type embryos (described above) as follows:

First strand synthesis of cDNA from extracted zebrafish RNA was performed using a T_{20}VN oligomer and Superscript III reverse transcriptase (Invitrogen). Negative control synthesis reactions without reverse transcriptase (-RT cDNA) were performed in parallel.

Different combination of *jamc2*-specific primers (3503, 3504, 3505, 3506, 3533; table 2.1) were then used in touchdown PCR reactions with Advantage II Polymerase

Mix (Clontech). Additional positive control primers directed against *ef1α* and *jamb* (3408, 3409, 3489, 3490; table 2.1) and an -RT cDNA template negative control were used in parallel reactions. The products were analysed by agarose gel electrophoresis.

The presumed full-length *jamc2* PCR product (3506 and 3533; table 2.1) was purified from an agarose gel, cloned into pCR-TOPO4 (Invitrogen), verified by EcoRI digestion and insert-specific touchdown PCR (3506 and 3533; table 2.1) and sequenced as previously described.

2.1.3 Gel electrophoresis of DNA or RNA

DNA samples were diluted with sample loading buffer (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF) and loaded onto 1.5% agarose, TAE-buffered (40 mM tris, 20 mM acetic acid, 1 mM EDTA) gels containing ethidium bromide (0.1 µg/ml). The samples were run through the gel with a current of 120 V for varying times, and visualised on a UV transilluminator (BIORAD).

RNA samples were diluted in sample loading buffer (30% formamide, 20% glycerol, 80 mM MOPS free acid, 20 mM sodium acetate, 8 mM EDTA, 2.5% formaldehyde, 0.2% bromophenol blue, pH 7) and loaded onto 1.5% agarose, formaldehyde-buffered (20 mM MOPS free acid, 5 mM sodium acetate, 1 mM EDTA, 0.7% formaldehyde) gels containing ethidium bromide (0.1 µg/ml). The samples were run through the gel with a current of 120 V for varying times and visualised on a UV transilluminator (BIORAD).

2.1.4 Homology and molecular genetics analysis

Amino acid sequences for mouse and human JAM-A, JAM-B, JAM-C, ESAM, CAR, A33 and JAM4 were retrieved from the NCBI database (www.ncbi.nlm.nih.gov). Alignments of nucleotide and amino acid sequences were performed using ClustalW (www.ebi.ac.uk/ClustalW). A neighbour-joining tree was drawn from this alignment using the Poisson Distribution model in MEGA (v3.1; Kumar *et al*, 2004) using 500 bootstrap replicates.

Signal peptide cleavage sites and transmembrane domains were predicted from amino acid sequences using SignalP (v3.0; Bendsten *et al*, 2004) and TMHMM (v2.0; Krogh *et al*, 2001).

A detailed comparison of *jamc* and *jamc2* genomic loci was performed using zPicture, an interactive Blastz web tool (zpicture.dcode.org; Ovcharenko *et al*, 2004), and zebrafish genome sequence data (Zv6; Hubbard *et al*, 2007).

2.2 Zebrafish husbandry and genotyping

2.2.1 General husbandry and embryo collection

Embryos heterozygous for the *jamb*^{HU3319} allele were kindly provided by the Dr. Edwin van der Cuppen of the Hubrecht Laboratory, Utrecht, Netherlands. Embyros heterozygous for the *jamc*^{sa0037} allele were kindly provided by the Wellcome Trust Sanger Institute Zebrafish Mutation Resource, Hinxton, Cambridge. The mutants were inbred to generate homozygous lines, and maintained as outcrossed heterozygous lines. All fish were maintained according to Institute and Home Office regulations.

Fixed *prdm1*^{tp39} embryos were kindly provided by Dr Stone Elworthy.

For breeding, male and female pairs were put into breeding tanks with a mesh divider designed to separate adults from eggs released during mating. Mating pairs spawned after light cycle activation at 8.30 AM. Any embryos were collected into egg water (0.18 g/l sea salt, 2 mg/l methylene blue) and raised at 28°C. Embryos were staged accordingly to morphology, as outlined by Kimmel *et al* (1995).

2.2.2 Genotyping zebrafish adults and embryos

To genotype adult and embryonic zebrafish I extracted DNA from amputated fin tissue or whole embryos, respectively, using a proteinase K digestion method, followed by nested PCR and sequencing (figure 2.1).

Zebrafish adults, no younger than three months old, were anaesthetized in 0.02% 3-amino-benzoic acid ethyl ester (tricaine), before amputation of the tip of the tail fin. Adult fish were subsequently placed in individual tanks until genotyping was completed. Fin tissue was digested in 100 µg/ml of proteinase K (Invitrogen) in lysis buffer (100 mM tris-HCl, 200 mM NaCl, 0.2% SDS, 5 mM EDTA, pH 8) at 55°C overnight, followed by vortexing to ensure disruption of the tissue. The proteinase was inactivated by incubation of the sample at 80°C for 30 min. DNA was purified from the lysed tissue by precipitation upon the addition of 300 µl isopropanol. After repeated inversion of the sample to ensure mixing, the precipitant was collected into a pellet by centrifugation for 40 minutes at high speed (96 well plates: 3220 x *g*; eppendorf tubes: 16100 x *g*). Precipated DNA was washed twice with 70% ethanol, allowed to dry and then dissolved in 500 µl TE (10 mM tris-HCl, 1 mM EDTA, pH 8).

Whole zebrafish embryos were fixed at an appropriate stage in methanol overnight at -20°C. Individual embryos were placed in each well of a 96-well plate and any remaining methanol allowed to evaporate. The embryos were then digested

in 25 µl of 1.5 mg/ml proteinase K in TE for at least 4 hours at 55°C. The enzyme was inactivated at 80°C for 10 minutes and samples allowed to cool before diluting with 75 µl sterile water. Before use of the samples in PCR reactions, any undigested debris was collected at the bottom of each well by centrifugation.

Purified adult DNA or digested embryos were subsequently used in nested touchdown PCR reactions using primers specific to exon 3 of *jamb* (B3-1, B3-2, B3-3, B3-4; table 2.1) or exon 5 of *jamc* (C5-1, C5-2, C5-3, C5-4; table 2.1). The PCR products were treated with exonuclease I (0.1 units/µl, NEB), to remove excess primers, and shrimp alkaline phosphatase (0.05 units/µl, NEB) to dephosphorylate PCR products, in buffer (20 mM tris-HCl pH 8, 10 mM $MgCl_2$) at 37°C for 1 hour. The enzymes were inactivated at 80°C for 20 minutes. Each PCR reaction was checked for quality by agarose gel electrophoresis and then diluted 1:2 with sterile water. The nested primers (B3-2, B3-3 and C5-2, C5-3) have M13 forward and reverse sequence tails, allowing all products to be sequenced with generic primers (M13F and M13R; table 2.1) as previously described. The sequence data was compiled into a GAP4 database for analysis.

2.3 Protein and RNA expression detection

2.3.1 Embryo fixation

Embryos collected from mating pairs were allowed to develop to an appropriate stage according to their morphology, as outlined by Kimmel *et al* (1995) and subsequently fixed according to use. For immunohistochemistry with Jamb (Everest Biotech), F59 (Developmental Studies Hybridoma Bank, University of Iowa, U. S. A.) antibodies or Alexa-488 conjugated phalloidin (Molecular Probes), embryos were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS; 135 mM NaCl, 1.3 mM KCl, 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, pH 7.4) at room temperature for 2 hours or overnight at 4°C. For immunohistochemistry with EB165 antibody (Developmental Studies Hybridoma Bank) or wholemount RNA *in situ* hybridisation, embryos were fixed with 4% paraformaldehyde in PBS at 4°C for 15-30 minutes and then stored in methanol at -20°C overnight or longer for storage.

2.3.2 Wholemount RNA *in situ* hybridisation

The expression patterns of *jam* family genes during development were characterised by wholemount RNA *in situ* hybridisation (figure 2.2) using antisense riboprobes transcribed from the immunoglobulin superfamily domain-encoding regions of each gene. The *kirrel* riboprobe was transcribed from the extracellular domain-encoding region, which had been cloned into protein expression vectors

previously.

Antisense hapten-labelled riboprobes were prepared by *in vitro* transcription of PCR templates amplified from expression plasmids described below (see 2.6.1), using touchdown PCR and flanking vector specific primers (3268 and 3269; table 2.1). The antisense primer (3268) contains a T7 polymerase binding site. PCR products were analysed by agarose gel electrophoresis, then purified using QIAquick PCR Purification kit (QIAGEN) as per manufacturer's instructions. The purified templates were assessed for quality and quantity by absorbance at 260 and 280 nm using a spectrophotometer. The templates (50 ng/µl) were transcribed using T7 polymerase (Roche; 1 units/µl) and a NTP labelling mix spiked with digoxygenin-11-UTP (1 mM ATP, CTP, GTP, 0.65 mM UTP, 0.35 mM digoxygenin-11-UTP) in transcription buffer (Roche; 40 mM tris-HCl, 6 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM spermidine) with RNAseOUT ribonuclease inhibitor (Invitrogen; 2 units/µl) for 1 – 2 hours at 37°C. Transciption was stopped by addition of DNAseI (1 units/ µl) to degrade the template and ethylenediaminetetraacetic acid (EDTA; 16 mM). Transcribed riboprobes were precipitated used lithium chloride (0.1 M) and cold ethanol. The samples were inverted to ensure mixing, incubated at -80°C for 30 minutes then the precipitated RNA was collected by centrifugation at 13000 x *g* at 4°C for 15 minutes. The pellet was washed with 70% ethanol and allowed to partially dry in air before resuspension in diethylpyrocarbonate-treated (DEPC) water. The riboprobes were assessed for quality and quantity by formaldehyde gel electrophoresis and absorbance at 260 and 280 nm using a spectrophotometer. Riboprobes were stored at -80°C until use.

Wholemount RNA *in situ* hybridisation was performed essentially as described in Thisse and Thisse (2007). Briefly, embryos were rehydrated through a methanol series, rinsed in PBST (PBS, 0.1% tween-20), and permeabilised in proteinase K (10 µg/ml in PBST) according to stage: shield – 1 minute, 1-10 somites – 2 minutes, 21 somites – 8 minutes, 24 h. p. f. – 10 minutes, 48 h. p. f. – 25 minutes. Embryos were rinsed in PSBT then fixed in 4% paraformaldehyde in PBS for 20 minutes, rinsed in PBST again and then placed in hybridisation buffer (50% formamide, 5 x SSC, 50 µg/ml heparin, 0.5 mg/ml RNAse free torula yeast tRNA, 10 µM citric acid, 0.1% tween-20, pH 6; 5 x SSC: 75 µM sodium citrate, 750 µM NaCl) for 5 minutes at 68°C. The buffer was replaced with fresh prewarmed hybridisation buffer and left to incubate at 68°C for two hours. Riboprobes were prepared before use by heating 100 ng of each probe in 100 µl of hybridisation buffer to 80°C for 5 minutes, followed by storage on ice. Hybridisation buffer was removed from the embryos, without allowing

them to touch the air, and replaced with the prepared riboprobes and then incubated at 67°C overnight. The riboprobe was then removed and embryos were incubated with pre-warmed 50% formamide, 2 x SSCT (2 x SSC, 0.1% tween-20) for 30 minutes at 68°C, twice, then pre-warmed 2 x SSCT for 15 minutes at 68°C, then pre-warmed 0.2 x SSCT for 30 minutes at 68°C, twice. The embryos were placed at room temperature, rinsed in malate buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) then blocked for two hours in 2% Boeringher blocking reagent in malate buffer on a rotating wheel at room temperature. After blocking, the embryos were incubated with an alkaline phospatase-conjugated anti-digoxygenin antibody (Roche) diluted 1:5000 in 2% blocking solution overnight at 4°C on a rotating wheel. The antibody solution was removed and embryos were washed six times with malate buffer for 20 minutes, rinsed with freshly prepared staining buffer (100 mM tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) then incubated with nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-indoyl phosphate (BCIP) in staining buffer (Roche; 0.4 mg/ml NBT, 0.19 mg/ml BCIP). The colour development was stopped, as required, by rinsing in PBST and fixation in 4% paraformaldehyde for 20 minutes at room temperature or overnight at 4°C. The embryos were rinsed with PBST, cleared through a methanol series (25% – 100% – 25%) rinsed with PBST and mounted in glycerol, beginning at 50% glycerol PBST and gradually transferred to 100% glycerol. Embryos were then stored at 4°C.

2.3.3 Immunohistochemistry

Immunohistochemistry was used to characterise the subcellular localisation of Jamb protein and the differentiation of fast and slow muscle in mutant embryos (figure 2.2). Prior to commencement of this project, a polyclonal antibody was raised against the recombinant extracellular domain of Jamb in goats, tested for activity against Jamb and Jamc by enzyme-linked immunosorbent assay (ELISA) and then subsequently affinity purified by Everest Biotech.

Fixed embryos were rinsed in PBSTri (phosphate buffered saline and 1% triton X-100) and then incubated in a blocking solution of 10% normal donkey serum in PBSTri for 2-3 hours at room temperature on a rotating wheel. Embryos were then incubated in anti-Jamb antibody, diluted 1:4 and preincubated in blocking solution, at 4°C on a rotating wheel, followed by six, 20 minute washes with PBSTri. The primary antibody was detected by either Alexa-488 or Alexa-568-conjugated anti-goat secondary antibody raised in donkeys (Molecular Probes), diluted 1:1000 and pre-incubated in blocking solution, overnight at 4°C on a rotating wheel. The embryos were washed six times for 20 minutes in PBSTri, then rinsed in PBS and mounted

in SlowFade Gold with 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes).

Slow muscle-specific myosin heavy chain (sMyHC) was detected with the mouse monoclonal antibody F59 (Developmental Studies Hybridoma Bank) using the same protocol, with the exception of blocking with 10% normal goat serum in PBSTri, dilution of the antibody by 1:200 in blocking solution, an Alexa-488 or Alexa-568-conjugated anti-mouse antibody raised in goats (Molecular Probes) diluted 1:1000 in blocking solution.

Fast muscle-specific myosin heavy chain (fMyHC) was detected with the mouse monoclonal antibody EB165 (Developmental Studies Hybridoma Bank) as with the F59 antibody, with the exception that the methanol-fixed embryos are rehydrated into PBS.

F-actin was detected by Alexa-488-conjugated phalloidin (Molecular Probes), diluted 1:40 – 1:80 in PBSTri and incubated for one hour at room temperature on a rotating wheel, rinsed in PBS and mounted in SlowFade Gold with DAPI (Molecular Probes).

2.3.4 Microscopy and image processing

Images of wholemount RNA *in situ* hybridisation embryos were taken using either a Leica MZ16FA dissecting microscope or a Zeiss AXIO Imager M1 microscope with a Zeiss AxioCam HRc digital camera and Zeiss AxioVision (v4.5) software. Images of wholemount fluorescent immunohistochemistry or mRFP-labelled embryos were captured using either a Zeiss Axioplan 2 microscope with a Hamamatsu ORCA-ER digital camera and Improvision Volocity (v4.2.0) software, or a Leica TCS SP5/DM6000 confocal microscope with Leica Application Suite Advanced Fluorescence (v2.0.0 build 1934) software.

Images were globally adjusted for dynamic range and resampled to a consistent resolution of 300 dots per inch. Colour images of wholemount RNA *in situ* embryos were corrected for colour balance. All figures and image processing were performed using Adobe Photoshop CS2 (v9.0).

2.4 Characterisation of loss-of-function mutants

2.4.1 Morpholino injections

1- and 2- cell stage embryos were injected with approximately 4 nl of translation blocking morpholinos (approximately 200 μM, 5-7.5 ng per embryo) diluted in sterile water with 0.1% phenol red (Sigma). Translation blocking morpholino sequences were as follows: *jamb*: GCA CAC CAG CAT TTT CTC CAC AGT G; *jamc*: TTA ACG

CCA TCT TGG AGT CGG TGA A.

2.4.2 Labelling cell membranes with membrane-targeted RFP

To label all cell membranes, embryos were injected with mRNA encoding red fluorescent protein (RFP) fused to two N-terminal Lyn kinase myristolation sites. Briefly, capped membrane-targeted red fluorescent protein (mRFP) mRNA was transcribed from a NotI linearised plasmid, kindly provided by Dr Mariella Ferrante, using the mMessage mMachine kit (Ambion) and SP6 polymerase. Transcription was stopped by addition of stop solution (0.5 M ammonium acetate, 10 mM EDTA) and the template was degraded by DNaseI for 30 minutes at 37°C. Transcribed mRNA was purified by addition of 1 volume of water saturated phenol/chloroform, mixing by inversion and centrifugation at high speed (13000 x *g*). The aqueous phase was mixed with 1 volume of chloroform followed by centrifugation (13000 x *g*). The mRNA was then precipitated from the aqueous phase with 1 volume of cold isopropanol, mixed by inversion and incubated at -20°C for 30 minutes. The mRNA was collected into a pellet by centrifugation (13000 x *g*) for 30 minutes at 4°C, then washed with 70% ethanol, allowed to partially dry in air, resuspended in DEPC-treated water and stored frozen at -80°C. 1-2 cell stage embryos were microinjected with approximately 4 nl of mRNA (25 ng/µl) diluted in sterile water, 0.1% phenol red (Sigma-Aldrich). The injected embryos were fixed with 4% paraformaldehyde overnight at 4°C, rinsed with PBSTri, mounted in SlowFade Gold with DAPI (Molecular Probes) and observed by confocal microscopy.

2.4.3 Quantification of fast muscle fibres

Wild-type and mutant embryos labelled with mRFP (see 2.4.2) were fixed at 24, 32 or 48 h. p. f. in 4% paraformaldehyde, overnight at 4°C. Fixed embryos were treated with Alexa-488-conjugated phalloidin and mounted in SlowFade Gold with DAPI (see 2.3.3). Z-stacks of confocal microscopy images were taken between myotomes 10-15 of mRFP, Alexa-488-conjugated phalloidin-labelled embryos. Optical cross-sections were computed from the microscopy data using Leica Application Suite Advanced Fluorescence software. Fibres were manually counted in each cross-section; superficial slow muscle fibres were excluded from analysis. Statistical significance between wild-type and mutant fibre counts was determined by one-tailed Student's t-test, modified to take unequal sample size and variance into account:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{s_{\bar{X}_1 - \bar{X}_2}}; \quad s_{\bar{X}_1 - \bar{X}_2} = \sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}$$

and degrees of freedom are calculated by:

$$d.f. = \frac{(s_1^2/n_1 + s_2^2/n_2)^2}{\frac{(s_1^2/n_1)^2}{(n_1 - 1)} + \frac{(s_2^2/n_2)^2}{(n_2 - 1)}}$$

where $\bar{X}_i$ is the mean, s_i is the standard deviation and n_i is the number of embryos in the i-th population.

2.4.4 Acridine orange assay

Dechorionated wild-type and mutant embryos were incubated in staining solution (17 µg/ml acridine orange, 0.18 g/l sea salt, 50 units/ml penicillin, 50 µg/ml streptomycin) for 30 minutes in the dark at room temperature. The embryos were washed with buffer (0.18 g/l sea salt, 50 units/ml penicillin, 50 µg/ml streptomycin) and analysed with a fluorescent dissecting microscope.

2.5 Transplant experiments

Transplants were performed essentially as described by Xu *et al* (2008). Briefly, 1-2 stage donor embryos were injected with lysine-fixable fluorescein or rhodamine labelled dextran (10000 Da, 1% in sterile water; Molecular Probes). Donor and host embryos were immobilised in 2% methylcellulose (Sigma) on glass slides. Fluorescently-labelled donor cells were then transplanted into the marginal cells of unlabelled host embryos between high/sphere to approximately 30% epiboly stages. Transplanted embryos were maintained in embryo media supplemented with penicillin (50 units/ml) and streptomycin (50 µg/ml), fixed in 4% paraformaldehyde for 2 hours at room temperature, washed several times with PBSTri, mounted in SlowFade Gold with DAPI (Molecular Probes) and analysed by confocal microscopy.

2.6 Protein production and biochemistry

To test the biochemical interactions amongst Jam family proteins systematically, recombinant immunoglobulin superfamily domains of each protein were produced in mammalian cell culture and tested for interaction through surface plasmon resonance (figure 2.3).

2.6.1 Expression vectors

Jam family immunoglobulin superfamily domains, including the native signal peptide, were cloned into expression vectors based upon modified pTT vectors (Durocher *et al*, 2002) containing (figure 2.4): an in-frame CD4-d3&4 tag-encoding region, that improves the efficiency of protein production and is detectable by the monoclonal antibody OX68 (Brown and Barclay, 1994); either a hexa-histidine tag (6-

His) or a peptide substrate for a biotinylation enzyme, BirA; an Epstein-Barr virus origin for episomal replication of the plasmid; and regulatory elements (cytomegalovirus promoter, SV40 polyA signal). This had previously been performed for *jama*, *jamb*, *jamc* and *jamb2*.

Briefly, the immunoglobulin superfamily domains of *jama2* and *jamc2* were amplified from 3′ RACE and RT-PCR clones, described previously (see 2.1.1 and 2.1.2) by touchdown PCR using primers containing NotI and AscI restriction enzyme recognition sites (3595, 3596, 3597, 3598; table 2.1) and a proof-reading polymerase, Advantage II Polymerase Mix (Clontech). The PCR products were analysed by gel electrophoresis. Single bands corresponding to the predicted size were purified using QIAquick Gel Extraction kit (QIAGEN) as per manufacturer's instructions, and assessed for quantity and quality by spectrophotometry. The PCR products were ligated to pCR-BLUNTII-TOPO (Invitrogen) as per maufacturer's instructions. The ligation products were used to transform chemically-competent bacteria. Transformed clones were selected for on LB agar plates containing kanamycin and grown in 3 ml cultures of 2 x TY media with kanamycin. The *jama2/jamc2* insert-containing plasmids were purified from the cultures using the QIAprep Miniprep Spin kit (QIAGEN) as per manufacturer's instructions, assessed for quality and quantity by spectrophotometry and sequenced using vector-specific primers flanking the insert site (M13F and M13R; table 2.1).

Both histidine-tag and biotin-tag expression vectors and sequence-verified *jama2* and *jamc2* subcloning pCR-BLUNTII plasmids were incubated with NotI and AscI restriction enzymes (NEB) according to manufacturer's instructions. The enzymes were heat inactivated and the cleaved DNA products purified using either QIAquick PCR Purification kit (for PCR products) or QIAquick Gel Extraction kit (for vectors; QIAGEN), quantified by spectrophotometry. Digested vector and PCR products were mixed in different ratios and incubated with T4 DNA ligase (NEB) in ligation buffer (50 mM tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM dithiothreitol, pH 7.5) overnight. Chemically-competent bacteria were transformed with the ligation products and positive clones selected on LB agar plates containing ampicillin. Several clones were used to inoculate 3 ml cultures of 2 x TY with ampicillin which were incubated overnight. The *jama2*/*jamc2* histidine-tag and biotin-tag plasmids were purified from the cultures using the QIAprep Miniprep Spin kit (QIAGEN) as per manufacturer's instructions, assessed for quality and quantity by spectrophotometry and then tested for the correct insert through NotI and AscI double digest and PCR using primers flanking the insert site (178, 180, 3534, 3538; table 2.1).

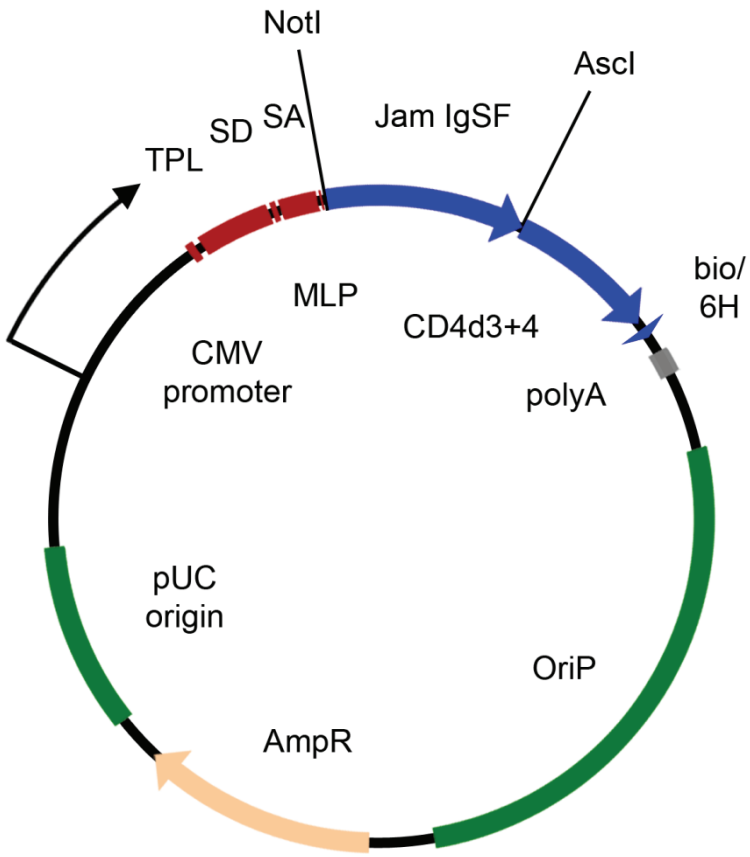

Figure 2.4 Genetic map of Jam protein expression vectors.

Diagram of genetic map of JAM protein expression vectors, drawn to scale. TPL – tripartite leader sequence, SD – splice donor, MLP – adenovirus major late promoter enhancer, SA – splice acceptor, NotI – NotI resctriction enzyme recognition site, Jam IgSF – sequence encoding Jam family immunoglobulin superfamily domains, AscI – AscI restriction enzyme recognition site, CD4d3+4 – CD4 domains 3 and 4 tag sequence, bio/6H – biotinylatable peptide tag or hexa-histidine tag sequence and stop codon, polyA – SV40 polyadenylation sequence, OriP – Epstein-Barr virus origin of replication, *AmpR* – β-lactamase gene, pUC origin – bacterial origin of replication, CMV promoter – cytomegalovirus promoter. Expression plasmid is approximately 6.5 kbp long without insert.

Verified *jama2* and *jamc2* expression vectors were used to transform chemically competent bacteria, which were selected for on ampicillin-containing LB agar plates. Single positive clones were used to inoculate 50 ml cultures of 2 x TY media supplemented with ampicillin. The cultures were incubated at 37°C with shaking at 200 r. p. m. overnight. Plasmids were purified from these cultures using the PureLink Hipure Plasmid Maxiprep kit (Invitrogen), quantified by spectrophotometry and diluted to 1 mg/ml in TE. The *jama2* plasmids were sequenced with vector-specific primers flanking the insert site (178 and 180; table 2.1). The *jamc2* plasmids were sequenced with insert-specific primers (3534 and 3538; table 2.1).

2.6.2 Transfection and purification

Protein production was based on an established system in our laboratory using polyethlyenimine-based (PEI) transfection of the HEK293E mammalian cell line (Durocher *et al*, 2002; Bushell *et al*, 2008). This cell line grows in suspension, allowing large quantities of protein to be produced from single transfections, and is kept under constant selection using G418 (also known as Geneticin) in order to maintain expression of the Epstein-Barr virus nuclear antigen 1 (EBNA1), allowing episomal replication of expression plasmids containing the Epstein-Barr virus origin. PEI forms polycationic complexes with the vector DNA, improving delivery of the plasmid to the cytoplasm of treated cells (Boussif *et al*, 1995).

HEK293E cells were maintained in Freestyle media (Invitrogen) supplemented with 1% fetal calf serum and G418 (50 µg/ml; Sigma), incubated at 37°C, 5% CO_2, 70% humidity with orbital shaking at 120 r. p. m. in baffled polycarbonate flasks (Corning). The cells were passaged to a density of 2.5 x 10^5 cells/ml in fresh media approximately every fourth day.

For transfection of histidine-tag expression vectors, cells were split into 50 ml of fresh media at a density of 2.5 x 10^5 cells/ml and allowed to recover for 24 hours. 50 µl of expression vector (1 mg/ml) was mixed with 110 µl linear 25 kDa PEI (1 mg/ml; Polyscience) in 2 ml of Freestyle media (without calf serum), left to rest for 5 minutes at room temperature, then added to the cell suspension. After six days of incubation, the supernatants are harvested from the cell suspension by centrifugation (3220 x *g*), filtered through a 0.22 µm filter and stored at 4°C. The same protocol was used for the transfection of biotin-tag expression vectors, with the exception of supplementing cell culture media with D-biotin (100 µM), and co-transfecting cells with a plasmid containing a secreted form of the *E. coli* biotin ligase *BirA* (5 µl, 1 mg/ml). The harvested supernatants were decanted into SnakeSkin dialysis tubing (molecular weight cutoff 10,000 Da; Thermo Scientific) and dialysed against PBS over 2-3 days

with approximately six changes of buffer, 25 to 30 L of buffer in total. The dialysed supernatants were filtered through a 0.22 µm filter and stored at 4°C.

Prior to use in surface plasmon resonance experiments, histidine-tag analyte proteins were purified from harvested supernatants using sepharose columns charged with nickel (HisTrap HP 1ml; GE Healthcare) and ÄKTAprime plus purification system (GE Healthcare) with real-time monitoring of flow-through absorbance at 280 nm. Briefly, a fresh nickel column was prepared by pre-elution with elution buffer (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 0.5 M NaCl, 0.4 M imidazole, pH 7.4, filtered and degassed under vacuum) and allowed to equilibrate in running buffer (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 0.5 M NaCl, 40 mM imidazole, pH 7.4, filtered and degassed under vacuum) at a flow rate of 1 ml/min. Meanwhile, imidazole (10 mM) and NaCl (100 mM) was added to approximately 150 ml of harvested supernatant, warmed to room temperature. The sample was passed over the equilibrated column at a flow rate of 1 ml/min, washed with 15 column volumes of running buffer and eluted with 10 column volumes of elution buffer. The eluant was collected into 0.5 ml fractions. The peak fractions were combined for gel filtration (between 1.5 and 2.0 ml of eluant).

Immediately before use in surface plasmon resonance experiments, the combined fractions from nickel column purification were further purified by gel filtration to remove aggregated and unfolded protein and buffer exchange. Briefly, the gel filtration column (GE Healthcare; Superose 6 prep grade resin, XK 16/70 column; 125 ml column volume, 62.6 cm bed height, 13,561 plates/m) was equilibrated with 2 column volumes of running buffer, HBS-EP (10 mM HEPES, 150 mM NaCl, 30 mM EDTA, 0.05% polyoxyethylenesorbitan 20, pH 7.4, filtered and degassed under vacuum) before applying the combined fractions to the column (approximately 1.5% of column volume), followed by washing with running buffer at a flow rate of 1 ml/min. Fractions (1.2 ml) were collected after approximately 45 ml of running buffer (equivalent to the void volume of the column) had passed through the column. The concentration of peak fractions was estimated by absorbance at 280 nm using *in silico* predicted extinction co-efficients (Gill and von Hippel, 1989).

2.6.3 Quantification by ELISA

Expression of recombinant proteins produced with a biotin ligase substrate peptide tag were quantified by ELISA using streptavidin-coated 96-well plates (Nunc Immobilizer) to capture biotinylated ligand and a monoclonal antibody that binds the CD4d3+4 tag, OX68.

Briefly, streptavidin-coated detection plates were rinsed briefly with PBST and

then blocked for at least 1 hour in 0.5% bovine serum albumin (BSA) in PBS. The detection plate was washed with PBS. Serial dilutions of each protein tested (in 0.2% BSA, PBS) were added to the detection plate in triplicate and left to incubate at room temperature for 30 minutes. The detection plate was washed repeatedly with PBS, followed by incubation with OX68 antibody (1:700, 0.2% BSA, PBS; Serotec) for 1 hour at room temperature. After repeated washing with PBS, the detection plate was incubated with alkaline phosphatase-conjugated anti-mouse antibody (1:5000, 0.2% BSA, PBS; Sigma) for 1 hour at room temperature. The detection plate was washed with PBS and then detected with *p*-nitrophenyl phosphate substrate (Sigma). Fluorescence was measured at 420 nm using a plate reader (PHERAstar plus; BMG Labtech).

2.6.4 Surface plasmon resonance

Surface plasmon resonance (SPR) was used to identify interactions between all six family members in both possible orientations of immobilised ligand and soluble analyte (figure 2.3).

Each biotinylated ligand was immobilised to a flow cell demarcated on a streptavidin-coated sensor chip (Series S Sensor Chip SA; GE Healthcare) in a molar equivalent amount to biotinylated CD4d3+4 immobilised to the control flow cell of the same chip (see Chapter 5, figure 5.3). The total amount of protein immobilised in each flow cell does not affect the kinetic parameters derived from collected data, but does influence the magnitude of response observed for an interaction. Both chips were stored in HBS-EP buffer at 4°C between experiments.

After purification, increasing concentrations of an analyte were passed over the flow cells of each of the two sensor chips at a high flow rate (100 µl/min), zebrafish physiological temperature (28°C), with real-time changes in surface plasmon resonance recorded at a frequency of 10 Hz using a Biacore T100 SPR machine (GE Healthcare). This process was repeated for each analyte.

2.6.5 Data analysis

An interaction was deemed to occur between the ligand and analyte tested if there was an increase in the response in the query flow cell during the injection phase, above that of the control flow cell tested in parallel. To determine this, the real-time SPR data of the control flow cell was subtracted from that of the query flow cell for each experiment, with correction for delay of sample delivery between flow cells.

In addition, the magnitude of change in response must increase with respect to rising analyte concentration, eventually reaching saturation, to demonstrate binding.

This was not possible for all interactions, likely because some interactions have a low K_D, *i.e.* to saturate binding required a higher concentration of analyte than was available.

To determine the dissociation rate constant (k_d) for each interaction, dissociation phase data for three mid-range concentrations of analyte were normalised and plotted as percent bound against time, with 100% bound at t = 0. The dissociation curves were averaged and then equations of the form:

$$y = Ae^{-k_d \cdot t}$$

were fitted to the data. The fitted curves demonstrated a R^2 > 0.9. The determined dissociation rate constants were used to calculate interaction half-lives, a concentration independent comparative measure:

$$t_{½} = \frac{\ln 2}{k_d}$$

Interactions in which a preliminary estimate of k_d was ≥ 6.9 were not considered for full analysis because of a lack of data. The half life of such an interaction is below the frequency of detection of the instrument used.

To determine if the quantified interactions were first order, the natural logarithm of the averaged normalised dissociation phase data for each interaction was plotted against time. First-order interactions are characterised by a straight line, with a gradient of $-k_d$.

All analysis was performed using Microsoft Excel 2007.

Table 2.1 Oligonucleotide sequences.

Name	Sequence
0141	CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA TCA ACG CAG AGT
0142	CTA ATA CGA CTC ACT ATA GGG C
0178	ACC TGG GGT ATC TGA AGG GT
0511	TGT ATG GAG GAG AGC ACA CAC GCA TCT
0512	AGT GGC AGT GGG CTC TAA AGG GGA AAA CAC
3268	GGA TCC TAA TAC GAC TCA CTA TAG GGA GGC CGT GAT GGA GGT CGA CGG CG
3269	ACA GGT GTC CAC TCC CAG GTC CAA G
3408	CCC CTG GAC ACA GAG ACT TCA TCA
3409	ACA CGA CCC ACA GGT ACA GTT CCA
3489	GTC AGC AGT CGC AAT CCT AAA GTG G
3490	TTT GTA CCA GGT GTA GAC GGC AGG T
3503	CTG GTG CTC TTC TAC TGG CTG TGT A
3504	AAC GCT TTC CCT GGT GCT CTT CTA C
3505	GCA TCC TCT TTC TTT ACC GAC CGG A
3506	GAT GAC GAA GGA GGA TTT GTG GCG A
3533	CTA AAC CTG CAT GTG AAA CAG CGG C
3534	ACC TTC TGA TAC TGA ACG CC
3538	CAG TTC AGT GCT TGA ACC CA
3565	GTG CGG ACC TAG CAA ATA AAC AGC TG
3566	CGG GTA ACA TTT GAA ACG CAT ACC G
3595	GCG GCC GCC ACC ATG GCG TTC GGC CGT CAA ACG CTT TCC CT
3596	GGC GCG CCC ACA ATG TCC AAG TCA TAC ACT TCC
3597	GCG GCC GCC ACC ATG GTG ACT TTA GTC TTT GTG TGT CTC TC
3598	GGC GCG CCA CTG CTG TCT ACA TCA TAA ACT TCC
M13F	TGT AAA ACG ACG GCC AGT
M13R	CAG GAA ACA GCT ATG ACC
B3-1	TTC TGT AAT TTG CTG CAA CG
B3-2	TGT AAA ACG ACG GCC AGT TGC TGA TGA CCG TTA AAC AC
B3-3	CAG GAA ACA GCT ATG ACC AGG GTT GGT GTC TTT CTC AG
B3-4	CCA TAG TAG ACG AAG GAC ACG
C5-1	GAG GAA ACC TCT GAA ACT GC
C5-2	TGT AAA ACG ACG GCC AGT ACT GAG TCG CTG TAA TGG TG
C5-3	CAG GAA ACA GCT ATG ACC AGC AGA TTC TCC TCA TGT CTG
C5-4	GGC ACT GAG TAC AAA TGG TG