Chapter 2

Materials and Methods

2.1 Embryology methods

2.1.1 Embryo Fixation

At different stages of embryo development 10 embryos were placed in Eppendorf tubes and the water replaced with 4% PFA in PBS. These were stored overnight at 4°C. If embryos were over 20 somites the chorions were removed before fixing in PFA. If the embryos were younger than 20 somites the chorions were removed after fixing. Fixed embryos were dehydrated by washing 3 times with 100% MeOH. They were then stored in 100% MeOH at -20°C.

2.1.2 In Situ Hybridization Analysis.

The embryos were rehydrated by serial incubations of PBT and methanol (75% MeOH /25% PBT for 5 min, 50% MeOH -/50% PBT for 5 min, 25% MeOH /75% PBT for 5 min, 100% PBT for 5 min twice). They were then placed in hybridisation mix (HM) for 5 min, the HM was changed and the embryos placed in the incubator at 68°C for no less than 1 hour. The HM was then removed and replaced with 200µl of HM containing 1 µg/ml of probe and left to hybridise overnight at 68°C.

The embryos were washed in 50% HM/50% 2×SSC for 5 min, 2×SSC for 15 min, and 0.2×SSC for 2 x 30 min at 68°C. They were then washed with 50% 0.2×SSC/50% PBT for 10 min and PBT for 10 min at room temberature (RT). The embryos were finally washed in fresh PBT/2% goat serum/2mg:ml BSA and left for several hours at RT. The PBT was then replaced with PBT/2% goat serum/2mg:ml BSA containing 1:2500 dilution of anti-dig solution (Roche) and left at 4°C overnight.

The PBT antibody solution was removed and the embryos washed with PBT (PBT quick wash, PBT for 3×5 min, PBT for 4×15 min all at RT). The embryos were then washed with staining buffer (2×5 min) incubated in staining solution (NBT/BCIP (Roche)) in the dark and monitored for expression. The staining reaction was stopped

by removing the staining solution and washing the embryos in PBS pH 5.5/2mM EDTA for 3 min. The embryos were then fixed in PFA for 20 min at RT.

2.1.3 Designing UTR or ATG Morphlino's

Antisense MO-modified oligonucleotides or MOs will be used for loss of function anlysis. In zebrafish these are both effective and specific translation inhibitors (Nasevicius and Ekker, 2000). The MOs were designed using the known sequences for each zebrafish *rab* and 25 bases of sequence unique to that gene designed either against the start codon (ATG) of the open reading frame or against the 5' UTR region. These were then sent for synthesis to Genetools (www.gene-tools.com). The MOs arrived back in a powdered form that was re-hydrated in distilled water. The MOs were quantified and the working doses achived by dilution with phenol red and MO buffer (see section 2.1.5). Phenol red was used as it is pH sensitive and therefore enables visualization of those embryos that were not correctly injected.

2.1.4 Designing Splice MO's

Splice MOs were designed by identifying an intron exon boundary of the gene of interest. A MO was then designed against 25 bases of this region with at least 10 bases in the intonic region and at least 10 bases in the exonic region. The sequence was then sent to Genetools for synthesis. The MO was then diluted as in section 2.1.5.

2.1.5 Resuspension, cleaning and dilution of MOs

The powdered MO was dissolved in 60µl of distilled water. 1µl of MO was diluted in 800µl of HCl 0.1M and quantified using a spectrophotometer. The concentration of MO was calculated as follows:

$$C(\mu g/\mu l) = (A \times 800 \times MW) / \text{molar absorbency}(\varepsilon)$$

Where A was the value from the spectrophotometer. The stocks were diluted in phenol red at 2ng, 4ng and 8ng per 1.4nl.

2.1.6 Microinjection of embryos at the 1- 8 cell stage

The embryos were injected using a pneumatic picopump (World Precision Instruments inc.) attached to nitrogen-filled tubing and holder. This was mounted on a Narishigne micro manipulator which was in turn mounted on a Kanetec stand. The injection needles used were filamented borosilicate glass capillaries (World Precision Instruments inc 1B00F-4) pulled using a Kopf needle puller (model 720 at solenoid level 3 and heat level 15) and the edge cut with a razor blade, and calibrated under the microscope with a millimetre ruler. The embryos were aligned for injection along a glass slide placed in a glass petri dish. 1.4nl of the desired solution was injected into the yolk of the embryo at the 1-4 cell stage under a Leica dissecting microscope.

2.1.7 Microinjection of embryos for 1 cell in 128

Embryos were de-chorionated on agarose in 1x danieau solution at the four cell stage. A ramp was made by placing a glass slide at a slight angle into a small petri dish containing 2% molten agarose (Sigma) in 1x Danieau solution. When the agarose was set the slide was removed and the dish filled with 1x Danieau solution. A filamented borosilicate glass capillarie (World Precision Instruments inc 1B00F-4) was pulled into a needle and the end cut with a razor blade. The needle was filled with the desired solution and calibrated to inject 100pl. When the embryos reached the 64 cell stage they were lined up on the ramp, when they reached the 128 cell stage a single cell in the animal pole was injected (see section 2.1.6 for apparatus used).

2.1.8 Visualizing biotinylated-dextran.

Embryos were fixed (see section 2.1.1), rehydrated (see section 2.1.2) and incubated for 30 minutes in 1:5000 avin in PBT. The embryos were then washed 3 times with PBT and soak for 30mins in 0.4mg/ml DAB/PBT (Sigma). The solution was then changed for DAB/PBT with 0.003% H_2O_2 and watched for 30mins. Once the desired staining was achieved the reaction was stopped by rinsing with PBT. The embryos were then fixed in PFA for 20 mins at RT.

2.1.9 Fixing Embryos for Protein and/or RNA extraction.

Embryos were transferred into a clean Petri dish and washed once in 1x Danieau solution. The required number of embryos were placed in a 1.5ml Eppendorf and washed again. All the Danieau solution was removed leaving. The tubes were then flicked so the embryos are arranged along the side of the tubes. The tubes were then snap frozen in dry ice and stored at -80.

2.1.10 Assaying epiboly movements

The embryos were de-chorinated at dome stage, 30% epiboly and shield stage in a petri dish containing 1% agarose and filled with 1x Danieau solution. The embryos were then placed in glass dishes contaning 5mg/ml of biotinylated-dextran (Molecular probes 10,000mw lysine fixable) in 1x Danieau solution for 20 minutes. The embryos were then washed 3 times over 15 minutes and fixed in 4% PFA.

2.1.11 Staging embryos

During the Rab screen in Chapter 3 the embryos were staged according to time post fertilization. Therefore, both the control MO injected embryos and the *rab* morpholino injected embryos are the same age but not necessarily the same developmental stage. This enables any developmental delay to be visualized. In contrast during both the characterization- and microarray analysis of *rab5a2* (Chapters 5 and 6) the experimental and control embryos were analyzed at the same developmental stage. Here, the time post fertilization differed due to the developmental delay seen in the experimental embryos. Staging in this way was necessary to compare the pathways affected by *rab5a2* at set developmental stages.

2.2 Molecular Methods

2.2.1 Synthesis of Probe

2µg of plasmid was linearised by digesting with the appropriate enzyme for 2hrs at 37°C. The total volume was then made up to 100µl. 100µl of phenol/chloroform/isoamylalcohol solution was added to the linearised DNA vortexed briefly and centrifuged for 2 minutes at 13000rpm. The top layer was removed and put in a clean Eppendorf. 100µl of chloroform/isoamylalcohol solution was added vortexed and centrifuged for a further 2 minutes. The top layer was again removed and put in a clean Eppendorf. 10ul of NaAc (3M pH 5.2) and 3x100% ethanol was added. This was left at –20°C for 1hour and then centrifuged at 13000rpm for 30 minutes. The ethanol was removed and 300µl of ice cold 70% ethanol added and then centrifuge at 13000rpm for 10 minutes. The ethanol was removed and the DNA pellet dried at 37°C. The DNA was then resuspended in 8.5µl of water.

The RNA was synthesized by adding 4µl of 5x transcription buffer (NEB), 2µl of 0.1M DTT, 2µl of NTP-DIG-RNA (Roche), 1.5µl of RNAse inhibitor (NEB) and 2µl of polymerase (NEB) to the resuspended DNA. This was incubated at 37°C for 2hrs. A 1µl aliquot of the RNA was removed and run on a 1% agarose/TAE gel to estimate the amount of RNA synthesized. The volume of the reaction was then increased to 50µl and 2µl of DNase I (ribonuclease-free) (Promega) was added. The reaction was then incubated at 37°C for 30 minutes to remove the DNA template and leave only RNA.

The RNA was recovered by centrifuge using a Chroma spin-100 column (Clontech). The column was first centrifuged to remove the water. The volume of RNA was increased to 100µl and added to the column which was spun at 3000rpm for 5 min to recover the RNA. The RNA was quantified in a spectophotometer and diluted to the desired concentration using RNAse free water.

2.2.2 PCR amplification of DNA

5µl of template DNA, 5µl of forward primer (10µM), 5µl reverse primer (10µM), 0.5µl polymerase (eg. Taq), 10µl 5x PCR buffer and 24.5µl H_2O were mixed together in a 0.2ml PCR tube. The tube was placed in the thermal cycler (MJ Research inc) at:

1) 94°C for 2min
2) 94°C for 15 seconds
3) Annealing temperature of primers for 30seconds
4) 72°C for 30 seconds
5) Goto 2 29 times more

To check the size of the PCR product 1µl of the reaction was run on a 2% agrose (Sigma) gel with an appropriate DNA ladder.

2.2.3 Cloning

2µl of PCR product, 1µl of salt solution, 1µl of TOPO cloning vector and 1µl of sterile water were mixed together in and Eppendorf tube and left to incubate at room temperature for 20 minutes. They were then placed on ice.

2.2.4 Transformation

2ul of the TOPO Cloning reaction was added to 20µl of One Shot TOPO 10 chemically competent E.Coli and mixed gently. The mixture was incubated on ice for 20minutes and then heat-shocked for 30 seconds at 42°C. The tubes were immediately transfered on to ice and left for 2 minutes. 250µl of room temperature SOC medium was added to the reaction, the tubes were caped tightly and shake horizontally (200rpm) at 37°C for 1 hour. Pre-warmed selective plates were spread with either 25µl or 200µl of the transformation reaction and incubated overnight at 37°C. Finally 3-5 colonies were picked and cultured in L-broth with a suitable selective antibiotic over night at 37°C. The culture was then centrifuged and the supernatant drained. The plasmid DNA was then recovered from the remaining pellet by the use of a Qiagen miniprep kit.

2.2.5 Recovery of Plasmid DNA from E.Coli.

This was done using a Qiagen mini prep kit using the protocol set out by the manufacturer.

2.2.6 5' capped RNA synthesis

5µl of linearised DNA, 10µl of 5x transcription buffer, 5µl of 0.1M DTT, 5µl of 5mM CAP (NEB), 5µl of 1mM GTP (NEB), 5µl of 5mM UTP (NEB), 5µl of 5mM ATP (NEB), 5µl of 5mM CTP (NEB), 2µl of RNAse inhibitor (NEB) and 3µl of polymerase (NEB) were mixed together in a 1.5ml Eppendof tube and incubateed at 37°C for 20mins. 4µl of 10mM GTP was added to the reaction which was incubate at 37°C for a further 2 hours. 1µl of the reaction was then run on a 1% agarose gel (Sigma) to check whether the RNA was synthesized. 3µl of RNAse free DNAse (Promega) was added and the reaction incubated at 37°C for a further 20 mins. 1µl of the reaction was then run on a 1% agarose gel. The volume of the reaction was increase to 100µl and the RNA recovered using chroma-100 spin columns (see section 2.2.1).

2.2.7 Agarose Gel Electrophoresis.

A 1% agarose gel was prepared by dissolving agarose in TAE and adding 5µl of ethidium bromide per 100ml. The gel was poured into a gel tray and combs placed in until set. Once set the combs were removed and the gel placed in the gel tank with TAE poured to the fill line on the tank. 10µl of ladder was loaded into the first lane with 1µl of the sample mixed with 9µl of orangeG load buffer loaded into subsequent lanes.

2.2.8 Extracting RNA from injected embryos

30 embryos were prepared as described in section 2.1.9. 100µl of Trizol was added to the frozen samples which were then homogenised (blue pestles from Eppendof). 20ul of chloroform was added and the tubes shaken vigorously for 15 seconds. The tubes were left at room temperature for 2-3minutes then centrifuged at 1200xg for 15minutes at 4°C. 70µl of the clear supernatant was removed and place in a clean 1.5

ml Eppendorf tube with 70µl of isopropanol. The samples remained at room temperature for 10 minutes and were then spun for 10minutes at 4°C. The isopropanol was removed leaving a pellet.The pellet was washed with 120µl of 70% ethanol and vortexed briefly. The pellet and ethanol were spun at 7,500xg for 10 minutes. The ethanol was removed and the pellet dried. The pellet was resuspended in 30µl of H_20 and 1µl run on a 1% agarose gel.

2.2.9 Making cDNA

1.5µg of RNA was placed in a clean Eppendorf and the volume made up to 12µl with water. 1µl of 0.5µM random primers were added to the RNA and the tube spun briefly. The samples were then incubated at 70°C for 10 minutes. 4µl of (5x) 1st strand buffer, 1µl of (10uM) DNTP's and 2µl of (0.1M) DTT was added and incubated at 42°C for 2minutes. 1µl Superscript RT (200U/ul) was added and the reaction incubated at 42°C for 1 hour. The reaction was heated to 70°C for 15 minutes and spun briefly. Finally the reaction was placed on ice for 10 minutes and stored at -20

2.2.10 Taqman RT-PCR

Using cDNA made from the protocol in section 2.2.9 a dilution plate was made. A control sample was diluted 1/10, 1/100 and 1/1000. All other samples were diluted 1/10.

2µl of the undiluted control was placed in the first well of a 96 well of an Applied Biosystems ABI optical plate, 2µl of 1/10 control dilution was placed in the second well, 2µl of of the 1/100 control dilution was placed in the third well and 2µl of the 1/1000 dilution placed in the fourth well. 2µl of all other samples were placed in the remaining wells. To each sample (including control dilutions) was added 10µl of Applied Biosystems TaqMan Universal PCR Master Mix, 1µl of TaqMan primer and 7µl of water. This was repeated using all the required primers and control primers

Each plate was then sealed with Applied Biosystems ABI optical adhesive covers (4311971). The plates were then placed in the RT-PCR (ABI prism) machine. Using the 7000system software the programme was set to absolute quantification for a 96 well plate and set for a 20µl reaction. The programme was then run and results saved for analysis.

2.2.11 Producing GFP-Nodal Fusion constructs

The cleavage sites for both Sqt and Cyc were identified (Figure 2.2.1) (Squint –$^{5'}$-CGGCGCCACAGAAGG$^{-3'}$,Cyclops–$^{5'}$-CTCAGGAGCCGCAGG$^{-3'}$) (Rebagliatia et al., 1998) and primers designed to isolate the pro and mature regions (Table 2.4.2)

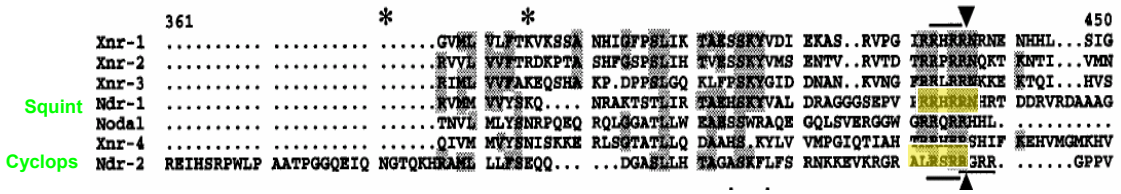

Figure 2.2.1: Cleavage site of Nodals in *Xenopus laevis* and zebrafish.
The yellow blocks identifiy the cleavage regions in zebrafish squint and cyclops.

The pro and mature regions of *sqt* and *cyc* were amplified from cDNA by PCR using KOD polymerase. A one percent agarose gel was run to check for the correct size product. The PCR products were cleaned using a Qiagen MinElute kit.1µl of Taq was added to 10µl of PCR product and 5µl of buffer and incubated at 72°C for 10 minutes. The products were cloned into the Invitrogen GFP cloning vectors (pcDNA3.1/CT-GFP TOPO and pcDNA3.1/NT-GFP TOPO) according to the Invitrogen protocol with the pro regions in the C-terminal linked GFP vector (pro-GFP) and the ligand regions in the N-terminal linked GFP vector (GFP-ligand).The clones were sent away for sequencing for conformation of correct insert.

The pro-GFP and GFP mature constructs were digested using the restriction enzymes BsrG1 and Xma1. The digests were then run on a gel and the large fragment from the pro-GFP construct cut from the gel and cleaned using a Qiagen gel extraction kit. This process was repeated for the smaller fragment of the GFP-ligand construct. The

large pro-GFP fragment was treated with SAP (Promega) as described by the manufacturer. The large pro-GFP fragments and the small GFP-ligand fragments were then ligated together (Figure 2.2.2) using T4 DNA ligase (Promega) as described by the manufacturer. The constructs were then transformed as described in section 2.2.4 and the DNA recovered using a Qiagen minprep kit.

The DNA was sequenced and the correct fusion constructs amplified using primers from Table 2.4.2 which add restriction sites to the amplified products. The amplified products and the insertion vector (pCS2+) were digested using EcoR1 and BamH1 and the products run on a gel. These were cleaned and ligated using T4 DNA ligase (Promega) as described by the manufacturer. The constructs were then transformed as described in section 2.2.4 and the DNA recovered using a Qiagen minprep kit. The constructs were sequenced and the correct fusion constructs used to produce capped RNA (see section 2.2.6) for injection (see section 2.1.6).

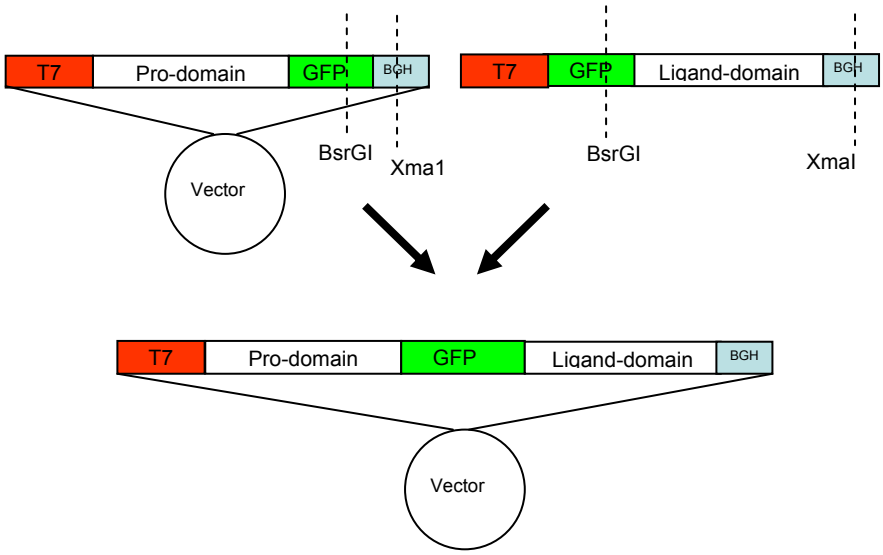

Figure 2.2.2: A diagrammatic representation of the methods used to make a GFP-nodal fusion construct

Showing the promoter domains and the restriction enzymes used and where they cut.

2.2.12 β-catenin stain

Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for four hours at 4°C. The embryos were then dechorionated and washed with PBS/ 0.1% Triton X-100 (PBS-Tr) five times. The embryos were then washed with

PBS/0.1% Triton X-100/% DMSO (PBS-DT) and blocked with 5% bovine serum in PBS-DT for one hour. After one hour the embryos were incubated with anti-β-catenin antibodies (1/500 dilution, Sigma C2206) overnight at 4°C. The embryos were then washed four times with PBS-Tr and incubated with secondary anti- rabbit antibody at 1/500 dilution, from the VECTASTIN ABC kit, for six hours at room temperature. The embryos were washed four times with PBS-Tr. Two drops of solution A and two drops of solution B (vectastain ABC kit) were added to PBT and left for 30min at RT after which time they were added to the embryos and left at RT for one hour. At the end of the hour the embryos were washed six times over the period of one hour. A diaminobenzidine (DAB, Sigma) tablet was dissolved in 33ml of PBT and any particulate spin out. 1ml of this was added to each of the goups of sample embryos which were left to incubate for 10minutes in the dark on glass. 1µ of 30% H_2O_2 is then added to the DAB and embryos these were then left in the dark and observed for staining. When it was time to stop the reaction the embryos were wahed in PBT.

2.2.13 Producing a dominant negative and constitutively active Rab5a2.

Primers were designed enabling a point mutation to be made in the GTP binding region of *rab5a2* producing resulting in the Rab being either in the GTP-bound conformation (constitutively active) or the GDP-bound conformation (dominant negative). The constitutively active primer produced an A to T mutation (shown in lower case in Materials section 2.4 Table 2.4.2) resulting in the amino acid at position 81 changing from Q to L. While the dominant negative primer also produced an A-T mutation (shown in lower case in Materials section Table 2.4.2) resulting in the amino acid at position 133 changing from N to L.

Using the Stratagene QuikChange Site-Directed Mutagenesis Kit 1µl of ds-DNA template (50ng) was added to a PCR tube containing 2.5µl of 10X QuickChange Multi reaction buffer (Stratagene), 18.3µl of double-distilled H_2O, 1.2µl of 10µM mutagenic primers, 1µl of dNTP mix (Stratagene) and 1µl of QuickChange Multi

enzyme blend (Stratagene). The reaction was placed in the thermal cycler (MJ Research inc) at:

1: 95°C for 1minute
2: 95°C for 1minute
3: 55°C for1minute
4: 65°C for 4minutes 45 seconds
5: Repeat from step 2 29 more times

The reaction was placed on ice for 2 minutes.1ul of *Dpn* I (Stratagene) restriction enzyme (10U/ µl) was added to the reaction which was pipetted up and down several times to mix the reaction. The reaction was spun for 1 minute then incubated at 37°C for 1hour.

45µl of the XL10-Gold (Stratagene) ultracompetent cells were transferred to a prechilled 14ml BD Falcon polypropylene round-bottom tube (BD biosciences Catalog 352059). 2µl of the β-ME mix (Stratagene) was added to the cells. The contents were swirled and incubated on ice for 10minutes swirling every 2 minutes. 1.5µl of the *Dpn* I –treated DNA was added to the ultracompetent cells which were then incubated on ice for a further 30 minutes. The cells were then heat pulsed in a 42°C water bath for 30 seconds and then incubateed on ice for 2 minutes.0.5ml of preheated (42°C) NZY+ broth was added to the reaction. The reaction was incubated at 37°C for 1 hour shaking at 225-250rpm. The DNA was then recovered using a Qiagen miniprep kit and sequenced to check for only the required mutation. All controls were performed as described in the Stratagene QuikChange Site-Directed Mutagenesis Kit

2.3 Expression Array

2.3.1 Total RNA extraction from tissue using Trizol.

The embryos were homogenised in 1.5ml Eppendorf tubes in 10 volumes of Trizol per embryo with a pestle (Eppendorf). The homogenate was then triturated six times with a 1ml filtered pipette tip then six times with a 200µl filtered pipette tip. The embryos were incubated for 5 minutes at room temperature. Phase Lock Gel-Heavy tubes were prespun at 1500rpm for 30seconds. The cell lysate was added to the Phase Lock Gel-Heavy and incubate at room temperature for 4 minutes. 0.2 ml of chloroform per 1 ml of TRIzol reagent initially used was added to the tubes. The tubes were capped and shaken vigorously for 15 seconds after which they were centrifuged at 12,000xg for 10 minutes at 2–8°C. The clear aqueous phase containing the RNA was transferred to a fresh 1.5ml Eppendorf tube. The RNA was precipitated by adding 0.5 mlof Isopropyl alcohol per 1ml Trizol reagent initially used. The samples were mixed by repeated inversion and allowed to incubate at room temperature for 10 minutes. The samples were centrifuged for 10 minutes at 12,000xg at 2–8°C. The supernatant was removed and the RNA pellet washed by adding 1ml of 75% ethanol per 1ml Trizol reagent initially used. The samples were vortexed and centrifuged at 7,500xg for 5 minutes at 2–8°C. The supernatant was removed and the pellet briefly air-dried. The RNA pellet was dissolved in 20µl of RNase free water and incubated at 55–60°C for 10 minutes. The RNA was quantitated using a Nano drop and 1 µl of each sample run on a 1% gel.

2.3.2 Generation of Amino Allyl-modified Amplified RNA (aRNA)

This protocol uses the Ambion Amino Allyl MessageAmp™ II aRNA Amplification Kit all reagents were from this kit unless otherwise stated.

2µg of RNA was prepared in 10µl of RNase-free water in a 0.5ml tube and placed on ice.1µl of T7 Oligo (dT) Primer (Ambion) and 1µl of 0.1X bacterial mRNA cocktail were added. The RNA oligo mixture was incubated at 70°C for 10 min and snap-chilled on ice. 2µlof 10x First Strand Buffer (Ambion), 4µl of dNTP mix (Ambion),

1µl of Rnase inhibitor (Ambion) and 1µl of ArrayScript (Ambion) were added to the mixture.The reaction mixture was spun briefly and incubated at 42°C for 2 hours and then chilled on ice for 2 minutes. 63µl of Nuclease-free water, 10µlof 10X Second Strand Buffer, 4µl of dNTP Mix, 2µl of DNA Polymerase and 1µl of Rnase H was added on ice to the reaction mixture and mixed gently. The samples were spun briefly and incubated at 16°C for 2 hours. 250µl of cDNA Binding Buffer was added to the samples and mixed by pipetting up and down 3-4times. The samples were transferred to a Filter Cartridge (Ambion kit) and centrifuge for 1 minute at 10,000xg. The flow-through was discarded. 500µl of wash buffer (Ambion kit) was added to the cDNA filter cartridge and centrifuged for 1 minutes at 10,000xg. The flow through was discarded. The cDNA Filter Cartridge was spun for an additional minute to remove trace amounts of buffer. 9µl of nuclease-free water (pre-heated to 55C) was added to the centre of the filter in the cDNA Filter Cartridge, left at room temperature for 2 minutes and then centrifuged for 1 minute at 10,000xg. A second 9µl of preheated nuclease-free water was added to the cartridge and spun as previously.

3µl of aaUTP (50mM), 12µl of ATP, CTP, GTP mix (25mM), 3µl of UTP Solution (50mM), 4µl of T7 10X Reaction Buffer and 4µl of T7 Enzyme Mix were added to the double-stranded cDNA at room temperature and mixed gently. The reaction mixture was spun briefly and incubated for 16 hours at 37°C. 60µl of room temperature nuclease-free water was added to the in vitro transcription reaction. 350µl of aRNA Binding Buffer and 250µl of 100% ethanol was added to each aRNA sample and mixed by pipetting the mixture up and down 3-4 times. The sample was pipetteed onto the centre of the filter in an aRNA filter Cartridge and centrifuged for 1minute at 10,000xg. The flow through was discarded. 650µl of wash Buffer was added to each aRNA Filter Cartridge and centrifuged for 1 minute at 10,000xg. The flow-through was disgarded and the aRNA Filter Cartridge spun for an additional 1 minute to remove trace amounts of wash buffer. 100µl of nuclease-free water preheated to 60°C was added to the centre of the filter. The filter cartridge was left at room temperature for 2 minutes and then centrifuged for 1 minute at 10,000xg. The aRNA in the elute was then quantitated.

2.3.3 Dye Coupling Amino Allyl-modified aRNA

Dye Coupling Reaction

The aRNA was spun for 5 minutes at 13000rpm. For each labelling reaction (2 per sample cy3 and cy5) 15µg of aRNA was removed and placed in a fresh tube. To this $1/10^{th}$ volume of 3M NaAc pH5.2 and 2.5 volumes of 100% ethanol was added and the mixture incubateed at -20°C for 30 minutes to precipitate. The precipitated aRNA was spun at 13000rpm for 5minutes. The pellet was washed with 500µl of 80% ethanol and spun at 13000rpm for 2-5minutes. The ethanol was removed and the pellet was resuspended thoroughly in 9µl of coupling buffer (Amino Allyl MessageAmp II aRNA Amplification Kit, Ambion). Light levels were reduced and activated CyDyes (CyDye Post-Labelling Reactive Dye Packs, Amersham) packs were left to equilibriate to room temperature. 11µl of DMSO (Amino Allyl MessageAmp II aRNA Amplification Kit, Ambion) was added per vial of dye pack. The resuspended CyDye was added to the aRNA in coupling buffer, mixed and incubate at room temperature in the dark for 45 minutes. 4.5ul of 4M hydroxylamine was added to the reaction mixture and incubated at room temperature for 15 minutes.

Purification of dye couples aRNA.

To each labelling reaction was added 75µl of RNase-free water and 350µl of buffer RLT from RNeasy mini kit (Qiagen). This was mixed thoroughly. 250µl of 100% ethanol was added and mixed thoroughly by pipetting. The sample was transfered to a RNeasy Mini column and spun for 1 minutes at 8000rpm. The column was removed to a fresh collection tube and 500µl of buffer RPE was added. The column was spun for 1 minute at 8000rpm.This was repeated twice. The column was placed in an elution tube and 50µl of water added. The column was incubated at room temperature for 2 minutes then spun at 8000rpm for 1 minute. Another 50µl of water was added to the column and incubated at room temperature for 2 minutes. The column was spun at 8000rpm for 1 minutes and the column discarded. The dye coupled aRNA was then quantified.

2.3.4 Competitive Hybridisation of labelled aRNA to Microarrays.

The labelled aRNA is spun for 5 minutes at 13000rpm. 2µg of labelled aRNA is removed and combined with those aRNAs to be compared on the array. 8µl of Cot-1 DNA (1ug/ul, Invitrogen), 4µl of polyA DNA (2ug/ul, Sigma), 25µl of salmon sperm DNA (10mg/ml, Ambion), 1/10th volume 3M NaAc pH5.2, 2.5 volumes of 100% ethanol were added to the combined aRNAs. The sample was incubated at 20°C for 30minutes and spun for 5 minutes at 13000rpm. The pellet was washed with 500µl 80% ethanol. The ethanol was removed and the pelley was dried for a few minutes at 70°C to remove any remaining ethanol. 10µl of water was then added and the sample incubated at 70°C for 5 minutes to dissolve the pellet. 50µl of RNA hybridisation buffer was added and mixed. The sample was the incubated at 70°C for 5 minutes and at room temperature for 10 minutes. The sample was then spun briefly, mixed and spun for 5 minutes at 13000rpm. 55µl of the hybridisation sample mixture was applied to a coverslip (25 x 60mm) placed on a flat surface. A microarray slide was gently lower onto the coverslip and placed DNA side up in the humid chamber to incubate at 47°C for 12-24 hours.

Array washes

The microarray was removed from the humid chamber and quickly placed in a slide rack submerged in 200ml of room temperature wash solution 1 (See Materials section 2.4). The slide was washed at room temperature for 5 minutes with gentle shaking. The slide rack was transfered to wash solution 2 and wash at room temperature for 15 minutes with vigorous shaking. This step was repeated with clean solution 2. The slide rack was then transfered to wash solution 3 and wash at room temperature for 5 minutes with vigorous shaking. This step was repeated with clean solution 3. After a final rinse in fresh wash solution 3, the slide rack is quickly transfered to a centrifuge and spun at 1000rpm for 1-2 minutes to dry the slides. The slides were then scanned using a laser-based scanner by PerkinElmer (ScanArray ExpressHT). The slides were analysed using image analysis software by PerkinElmer (version 3) which aligns the oligo spots and analyses the intensity of each of the dyes for each oligo spot on the array. The intensity data for each slide was then transferred into Agilent's Genespring software for analysis the difference in intensities between both dyes. This data was then grouped with the other replicates including the dye

swapped slides and analysed for differences between experimental and controls samples. The data was normalized using LOWESS (see Methods section 2.6) and finally the threshold for the fold change of gene expression between the experimental and control genes was set. For example only those genes that showed a 1.5 fold change in expression between the control samples and the experimental samples are listed to have changed. From the normalized data set those genes that show a significant change ($p<=0.05$) in a t-test were also listed to have changed. Therefore the genes that were finally selected to have changed were those that showed both a three fold change in expression and that were statistically significant. This ensures the minimum of false positive.

2.4 Materials

Phenol/chlorophorm/isoamylalcohol solution:

phenol:chloroform:isoamylalcohol = 25:24:1

Hybridisation Mix (HM):

	For 500 ml:
50% formamide	250 ml formamide
5×SSC	125 ml 20×SSC
0.1% Tween 20	500 µl Tween 20
pH adjusted to 6 with citric acid	4.6 ml citric acid 1M, pH6
50 µg/ml heparin	500 µl heparin 50 mg/ml 4×SSC
500 µg/ml Torula RNA	0.25 g Torula RNA

Staining buffer (NTMT):

	For 50 ml
100 mM tris HCl pH 9.5	5 ml 1M tris HCl pH 9.5
50 mM $MgCl_2$	2.5 ml 1M $MgCl_2$
100 mM NaCl	1 ml NaCl 5M
0.1% Tween 20	50 µl Tween 20

Staining solution:

NBT 100mg/ml - 112.5 µl

BCIP 50 mg/ml - 175 µl

Staining buffer - 50 ml

PCR buffer (5x):

2.5ml 2M KCL
1ml 1M Tris pH8.4
0.25ml 1M $MgCl_2$
200µl dATP 100µM
200µl dTTP 100µM
200µl dCTP 100µM
200µl dGTP 100µM
1.7ml BSA 10µg/ml

Phenol Red MO buffer:

1:4 25mg/ml phenol red : 5mM HEPES (pH7.2), 200mM KCl

Danieau Solution:

	30x
5M NaCl	1740mM
KCl	21mM
1M MgSO4	12mM
Ca(NO3)2	18mM
HEPES pH7.6	150mM

Expression Array:

Wash solution 1: 2X SSC filter sterilised
Wash solution 2: 0.1X SSC, 0.1% SDS, filter sterilised
Wash solution 3: 0.1X SSC, filter sterilised

RNA hybridisation Buffer:

50% formamide
5X SSC
0.1% SDS
0.1mg/ml BSA

Agarose gel for embryo manipulation

2% Agarose gel with 1x daneaus.

NZy+ Broth

10g of NZ amine (casein hydrolsate)

5g of yeast extract

5g of NaCl

Deionized H2O to a final volume of 1 liter

pH7.5

Autoclave then to 50mls of above was added:

625µl 1M MgCl2

625µl 1M MgSo4

1ml of 20% glucose (or 500µl of 2M glucose)

Gene name	MO Name	Type	Sequence	Clone
Rab3c1	zRab3c1	Splice	TGACATCAACTTACCAGTCCTGTAC	
Rab3c1	zRab3c1	ATG	TTGTCTTGCGTAGCAGCCATCTTCC	IMAGp998O1811847
Rab5a1	zrab5a1	ATG	GACAGTTGTCAATCACCCCGTCTTC	IMAGp998J226597
Rab5a2	zrab5a2	ATG	TCGTTGCTCCACCTCTTCCTGCCAT	IRAKp961M19104
Rab5b	zrab5b	ATG	CCTGCCTGTCCCACGGGTACTCATG	CHBOp575A2123
Rab5c	zrab5c	ATG	CGCTGGTCCACCTCGCCCCGCCATG	IRAKp961I04102
Rab1a3	MC14	UTR	GATTCATCGTGGACTGGACACTG	IRAKp961G04102
Rab11a1	MC3	UTR	TACGAACTCCGTGTTTTCAAATGTA	IMAGp998H109110
Rab20	MC5	UTR	AGACTCAACTCTCACAGGTAAACTC	IMAGp998N1514300
Rab3c1	MC7	UTR	CGTATAACTCCATTTGCTTTAGACA	IMAGp998O1811847
Rab11b1	MC9	UTR	ATTTTAGACAAGCCGCCCCGTCCTG	IMAGp998P2011849
Rab1a2	MC11	UTR	GTTCAGCAGGAGATCGGACTCTTTT	IRAKp961C23103
Rab11b2	MC13	UTR	ACCGCACTGAAATGTTGTTATTTAG	IRAKp961G01102
Rab6a	MC15	UTR	CAGACATGCTGCCGGTTCCACT	IRAKp961H19102
Rab28	MC17	UTR	GCTTCAGCTCGGCAGCGCGACAC	IRAKp961N08101
Rab11a2	MC19	UTR	ATCTCGATCAAAACAAAAGCGCAAA	LLKMp964G0315
Rab18(2)	MC21	UTR	ACCGGAAAATGCCTCTATGAGCAAA	MPMGp637E1023
Rab1a4	MC23	UTR	CATGACGGACAGCACGCGAAAATCC	Singapore 58G07
Rab1a1	MC25	UTR	AAAGGGCTTGTTATTGTTTGTCCAG	
Rab5a2	zRab5a2	Splice	ATGAAGCGTTTGTCTTACCTCCTAT	

Table 2.4.1 Experimental MOs showing sequence, type of MO, target gene and corresponding gene clone.

Primer Name	Sequence
Rab5a2 forward	CGGGATCCCGGTCATGGCAGGAAGAGG
Rab5a2 reverse	GGAATTCCGAGGAGCGTGGTTTAGGT
Constitutively active Rab5a2 forward	GGATACAGCTGGCCtGGAGCGCTACCACAG
Constitutively active Rab5a2 reverse	CTGTGGTAGCGCTCCaGGCCAGCTGTATCC
Dominant negative Rab5a2 forward	AGCTTTGGCTGGGAtCAAGGCAGACCTTGC
Dominant negative Rab5a2 reverse	GCAAGGTCTGCCTTGaTCCCAGCCAAAGCT
Forward Squint pro primer	ATGTTTTCCTGCGGGCTCCTGA
Reverse Squint pro Primer	TCCTTCTGTGGCGCCGA
Forward Squint ligand primer	AACCACAGAACTGATGATAG
Reverse Squint ligand primer	TCAGTGGCAGCCGCATTCT
Forward Cyclops pro primer	ATGCACGCGCTCGGAGT
Reverse Cyclops pro primer	CCCTGCGGCTCCTGA
Forward Cyclops ligand primer	GGCCGCCGGGGGCCA
Reverse Cyclops ligand primer	TCACAGGCATCCGCACT
pCs2 Cyc F	GGAATTCCATGCACGCGCTCGGAGT
pCs2 Cyc R	AAGGCCTTTCACAGGCATCCGCACT
pCs2 Sqt F	GGAATTCCATGTTTTCCTGCGGGCTCCTGA
pCs2 Sqt R	AAGGCCTTTCAGTGGCAGCCGCATTCT

Table 2.4.2: Experimental primers showing sequence, direction and target gene.

2.5 Equipment

2.5.1 Photography

Photographs were acquired using either a Zeiss Axioplan 2 compound microscope with the Axioplan camera and Axiovision 4 software or on a Leica dissecting scope with the Zeiss Axioplan camera and Axiovision 4 software.

2.5.2 Microscopy

General microscope work was done on a Leica (M295) dissecting microscope. For live embryos the microscopes own light was used. For *in situ*'s an external light source was used.

2.5.3 Confocal Microscopy

Embryos were placed in a depression slide in 1x danieau solution and covered with a 22mm x 40mm coverslip. The embryos were then viewed under a Biorad confocal microscope.

2.5.4 Optokinetic response apparatus

The optokinetic response apparatus consists of a spinning cylinder of alternating black and white colour. Inside this cylinder is a stable immobile platform on which the petri dish containing fish imobalised in methyl cellulose (Sigma) were placed (Figure 2.5.1 inset). The spinning cylinder is controlled by a dial which dictates the direction of cylinder movement and the speed (Figure 2.5.1). The apparatus was made by Loyd Stemple from technical Lego.

Figure 2.5.1: The optokinetic response apparatus for assaying embryos for blindness. Apparatus is seen placed under a under a Leica (M295) dissecting microscope. Inset picture shows white platform on which embryos in dish are placed and alternating white and black pattern of the spinning disk.

2.6 Statistical Analysis

The microarray data was analysed using Genespring (Agilent Technologies) which uses a standard t-test for analysis. The data is normalized to try and correct for systematic bias and remove non-biological influences. Genespring uses locally weighted scatterplot smoothing (LOWESS) for for global normaliztion. This adjusts for overall dye bias