Chapter 2 Materials & Methods

2.1 Zebrafish husbandry and genotyping

2.1.1 General husbandry

Adult zebrafish *nol9*$^{sa1022/+}$ and *las1l*$^{sa674/+}$ were kindly provided by the Wellcome Trust Sanger Institute (WTSI), Zebrafish Mutation Project, Hinxton, Cambridge. These adult zebrafish *nol9*$^{sa1022/+}$ were outcrossed to SAT wild-type and maintained as heterozygous lines. Fluorescent reporter lines *Tg(ins:mCherry)*^{jh2} (Pisharath et al., 2007) and *Tg(ptf1a:EGFP)*^{jh1} (Godinho et al., 2005), were kindly provided by Dr Elke Ober, MRC National Institute for Medical Research , Mill Hill, London . These two lines were incrossed to produce *Tg(ins:mCherry)*^{jh2}*;Tg(ptf1a:EGFP)*^{jh1}. The *tp53*^{zdf1} (Berghmans et al., 2005) was kindly provided by Dr Sebastian Gerety, Wellcome Trust Sanger Institute, Hinxton, Cambridge. The *nol9*$^{sa1022/+}$ adult zebrafish obtained from WTSI was outcrossed to *Tg(ins:mCherry)*^{jh2}*;Tg(ptf1a:EGFP)*^{jh1} and *tp53*$^{zdf1/+}$ to generate the lines *Tg(ins:mCherry)*^{jh2}*;Tg(ptf1a:EGFP)*^{jh1}*;nol9*$^{sa1022/+}$ and *nol9*$^{sa1022/+}$*;tp53*$^{zdf1/+}$ respectively. The *las1l*$^{sa674/+}$ was outcrossed to *Tg(ins:mCherry)*^{jh2}*;Tg(ptf1a:EGFP)*^{jh1} to produce *Tg(ins:mCherry)*^{jh2}*;Tg(ptf1a:EGFP)*^{jh1}*;las1l*$^{sa674/+}$. The *nol9*$^{sa1022/+}$*;tp53*$^{zdf1/+}$ was outcrossed to *Tg(ins:mCherry)*^{jh2}*;Tg(ptf1a:EGFP)*^{jh1} to generate the line *Tg(ins:mCherry)*^{jh2}*;Tg(ptf1a:EGFP)*^{jh1}*nol9*$^{sa1022/+}$*;tp53*$^{zdf1/+}$. All fish were maintained in accordance with regulations from the Institute and the Home Office.

For breeding purposes, adult males and females were placed in breeding tanks with a divider to separate the adults from the eggs. Embryos were collected and incubated in water containing and 2 mg/l methylene blue (Sigma). When stated, the embryos were maintained in water containing 0.18g/l sea salt and 0.002% Phenylthiourea (PTU, Sigma) in 0.18 g/l sea salt was added at 8-24 h.p.f. to inhibit melanocyte formation (Karlsson et al., 2001). All the embryos were raised at 28.5°C and staged according to Kimmel *et al.*, 1995 (Kimmel et al., 1995).

2.1.2 Genotyping of zebrafish embryos, larvae and adults

Genomic DNA was extracted using the Hot Shot method (described below) from whole embryos or fin clips and by Proteinase K digestion from embryos processed for whole-mount *in situ* hybridisation or immunohistochemistry. Genotyping was then performed by competitive allele-specific PCR (KASP) genotyping system (KBioscience).

Zebrafish, at least 3 months old, were anaesthetised in 0.02% 3-amino-benzoic acid ethyl ester (Sigma) before the tip of the tail fin was clipped and placed in individual well of a 96-well plate. They were individually kept until genotyping was completed. Whole zebrafish embryos were fixed at the appropriate stage in 100% methanol at -20°C overnight. Individual embryos were placed in wells of a 96-well plate and allowed to dry. Fin clips or whole embryos were digested in Hot Shot alkaline lysis buffer (25 mM NaOH, 0.2 mM EDTA) (50ul or 25ul respectively) at 95°C for 30 mins, followed by 5 mins on ice. An equal volume of Hot Shot neutralisation buffer (40mM Tris-HCl) was then added. DNA was diluted to a working concentration of 1.25-12.5 ng/µl.

Embryos processed for whole-mount *in situ* hybridisation and immunohistochemistry were placed in wells of 96-well plate and 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, with occasional vortexing to ensure complete disruption. Proteinase K was inactivated at 80°C for 30 mins. 50 µl isopropanol was added and plates were repeatedly inverted. DNA was precipitated by centrifugation at 4200rpm at 4°C for 30 mins. The DNA pellet was washed twice with 100 µl 70% ethanol, allowed to dry and resuspended in 25 µl of water to a working concentration of 1.25-12.5 ng/µl.

Genotyping was carried out using the competitive allele-specific PCR (KASP) genotyping system (KBioscience). 4 µl of DNA was pipetted into a black 96-well PCR plate followed (Bio-Rad) by 4 µl of PCR mix, according to the manufacturer's protocol (KBioscience). Plates were read using PHERAstar plus (BMG labtech) and analysed using the software KlusterCaller (KBioscience). Table 2-1 shows the sequences used for design of KASP assays by KBioscience to genotype *nol9*^{sa1022}, *las1l*^{sa674} and *tp53*^{zdf1}.

Gene	KASPar Sequences
nol9	GGTCATGTAGAAGTGCTGGGCTTCACCATAGAGGAGGGTCAACAGCCTTA[C/A]CCTSTG TTTTCACCACCGACCCACTGCCCGCTCACTATCACGGCCTTAGG
las1l	CTAACCACACCGCCAAAACAACGCAGATTCCGCCCACTGAGGAGCTCCAA[G/T]AGAAGY TGAGCACAGAAACTGTGCAGGAGAGGAACTYGGCTCTACAGGGA
tp53	ATTTTGCCTTATAATAGGAGGGTAATGTGAATCTAACCTGGCA[G/T]GTTTGGTGAAAGAA TCTTCTTCAGCTACATTACGACCTGAGGGGAGCAAAAA

Table 2-1 Sequences of *nol9*, *las1l* and *tp53* that was used to design KASP genotyping assays. Red shows mutation position with wild-type base followed by alternative base.

2.2 RNA extraction and DNAse treatment

RNA extraction was carried out on wild-type embryos at 1 d.p.f. and 2 d.p.f. for cDNA synthesis to generate *in situ* hybridisation RNA probes (Section 2.3.2), at 5 d.p.f. on mutant and wild-type siblings for Differential Expression Transcript Counting Technique (DeTCT; Section 2.6.1) and for cDNA synthesis to generate DIG-labelled probe for Northern blot analysis (Section 2.5.1).

Embryos were homogenised in 10 volumes of TRIzol® (Life Technologies), followed by a 5 minute incubation at room temperature. 0.2 ml chloroform per 1 ml TRIzol® reagent initially used, was added and mixed by vigorous shaking for 15 seconds. The tubes were spun at 12,000rpm at 4°C for 10 mins. RNA was precipitated from the aqueous phase by adding 0.5 ml isopropanol per 1 ml TRIzol® reagent initially used and mixed by repeated inversion. After 10 minute incubation at room temperature, the tubes were spun at 12,000rpm at 4°C for 10 mins. 1 ml 75% ethanol per 1 ml TRIzol® reagent initially used was added to wash the RNA pellet and the tubes were spun at 7500rpm at 4°C for 5 mins and this step was repeated. To dry the RNA pellet, the tubes were incubated at 37°C for 5-10 mins. 17µl RNAse-free water (Sigma) was added and incubated at 55°C for 10 mins. RNA was quantified on a NanoDrop ND-1000 Spetrophotometer (Thermo Scientific) and 1 µl was run on a 1% agarose (Invitrogen) gel in 1x Tris-acetate-EDTA (TAE) buffer (40mM Tris-acetate, 20mM acetic acid and 1mM EDTA).

For RNA used to make cDNA for RNA probes for *in situ* hybridisation (Section 2.3.2), DNase treatment of RNA was carried out using the DNA-free Kit (Life Technologies) according to the manufacturer's instructions.

For RNA used to make DeTCT libraries (Section 2.6.1), DNAse treatment was carried out as follows: RNAse-free water was added to make the volume up to 89 µl and 10 µl DNAse I Buffer (NEB) and 1µl DNase I (NEB) were added followed by a 10 minute

incubation at 37°C. 1 µl of 0.5M EDTA (Sigma) was added and the enzyme was heat inactivated at 75°C for 10 mins. 2 µl glycogen (Roche), 10 µl 3M NAOAc pH 5.2 (Sigma) and 300 µl 100% ethanol were added and the tubes were incubated at either -80°C for 1 hour or -20°C overnight. The RNA pellet was washed twice with freshly made 75% ethanol and dried at 37°C for 5 mins. The RNA was then dissolved in 9 µl RNAse-free water (Sigma) at 55°C for 5 mins.

2.3 RNA and protein expression detection

2.3.1 Embryo fixation

Embryos were fixed at the desired stage according to use. For *in situ* hybridisation, immunohistochemistry with specific antibodies and TUNEL assay, embryos and larvae were fixed in 4% paraformaldehyde (PFA) 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 4°C overnight. The embryos were then dehydrated in series of methanol in 1x PBST (PBS, 0.1% tween-20): 5 min in 25% (v/v) methanol; 5 min in 50% (v/v) methanol; and 5 min in 75% (v/v) methanol and washed and stored in 100% methanol at -20°C overnight or longer. For immunohistochemistry against Cytokeratin, larvae were fixed in 80% MeOH/20% dimethyl sulfoxide (DMSO) at 4°C overnight.

2.3.2 Whole-mount RNA *in situ* hybridisation

The probes for RNA *in situ* hybridisation were generated using a RT-PCR-based approach. The RNA was first reverse transcribed to cDNA, PCR was then performed using gene-specific primers with the antisense primer containing a T7-promoter sequence and finally *in vitro* transcription of the PCR product was carried out.

The cDNA synthesis was carried out using the Transcriptor High Fidelity cDNA synthesis Kit (Roche) according to the manufacturer's instructions. In brief, a 11.4 µl reaction containing 500ng of RNA and 1 µl of OligodT was incubated at 65°C for 10 mins. 4 µl of 5x Transcriptor High fidelity reaction buffer, 0.5 µl RiboLock Ribonuclease Inhibitor, 2 µl dNTPs, 1 µl 0.1M DTT and 1.1 µl Transcriptor High Fidelity Reverse Transcriptase were added and the reaction was incubated at 55°C for 2 hours.

The PCR was performed using the forward and reverse primers in Table 2-2. In a 30 µl reaction containing 5 µl of 1:20 wild-type cDNA generated, 15 µl JumpStart Taq Ready mix (Sigma) and 1 µl 10 µM primers were incubated and PCR was carried out under the following conditions:

1. 94°C for 2 mins;
2. 9 cycles of (94°C for 30 secs, 62°C (-0.5°C/cycle) for 30 secs, 72°C for 1.5 mins);
3. 31 cycles of (94°C for 30 secs, 58°C for 30 secs, 72°C for 1.5 mins) and
4. 72°C for 5 mins.

Gene	Primer Sequences
nol9	Forward: GACAATGAAAGTACACAAGGTTC Reverse: TAATACGACTCACTATAGGGTAACACTGCACGGTTCTTGG
foxa1	Forward: CATGACGAACAGCAGCATGA Reverse: GAAATTAATACGACTCACTATAGGCCGCTGGACTGCTCTCTCTT
gata6	Forward: CTGTCATGCGCAAACTGTCA Reverse: GAAATTAATACGACTCACTATAGG CGAAGTATCCGTTGGCATCA
pdx1	Forward: CCTTCCAGAGACACCCCAAC Reverse: GAAATTAATACGACTCACTATAGGCTGGTTGCCGTTGCATACAT
prox1	Forward: GAGCATCTAAGGGCCAAACG Reverse: GAAATTAATACGACTCACTATAGGGGTCCCTGGCTCTTTCCTCT
fabp2	Forward: GGCTCGGGGTAAAGTTAGGC Reverse: GAAATTAATACGACTCACTATAGG GGGCTGCCAATCATTAAAGC

Table 2-2 Forward and reverse primer sequences to generate probes for RNA *in situ* hybridisation

The *in vitro* transcription was carried out in a 20 µl reaction containing 1 µg of DNA template, 4 µl of 5 x Transcription Buffer (Fermentas), 1 µl RiboLock Ribonuclease Inhibitor (Fermetas), 1 µl of 0.1M DTT (Life Technologies), 2 µl of 10x DIG RNA labelling mix (Roche), 2 µl T7 RNA polymerase (Fermentas) and the tube was incubated at 37°C for 2 hours. 2 µl of DNase I (Fermetas) was added and incubated at 37°C for 15 min. The probes were cleaned up using Lithium Chloride precipitation Solution (Life Technologies) according to manufacturer's instructions. An equal volume of hybridisation buffer was added to the RNA probe and stored at –20°C.

Whole-mount RNA in situ hybridisation was performed using a protocol adapted from (Thisse and Thisse, 2008). The embryos were rehydrated through successive dilutions of methanol in 1x PBST: 5 min in 75% (v/v) methanol; 5 min in 50% (v/v) methanol; and 5 min in 25% (v/v) methanol and subsequently rinsed several times in PBST. The embryos were permeabilised using Proteinase K (Invitrogen; 10 µg/ml) according to the developmental stage: 1 d.p.f. – 5 mins, 2 d.p.f. – 10 mins, 3 to 4 d.p.f. – 30 mins and 5 d.p.f. - 45 mins. Embryos were then rinsed in PBST and fixed in 4% PFA in PBS at room temperature for 20

mins. The embryos were rinsed in PBST again before being placed in hybridisation buffer (50% formamide, 5x saline-sodium citrate buffer (SSC), 0.1% Tween-20, 150 µg/ml heparin, 5 mg/ml Ribonucleic acid from Torula yeast) at 68°C for 5 mins. The buffer was then replaced with fresh prewarmed hybridisation buffer and embryos were incubated at 68°C for 2 hours. The embryos were incubated in 200 µl of 1 µg/µl DIG-labelled RNA probes in hybridisation buffer at 68°C overnight. The embryos were washed with serial dilutions of hybridisation buffer in 2x SSC: 5 min in 75% (v/v) hybridisation buffer, 50% (v/v) hybridisation buffer, 25% (v/v) hybridisation buffer and 5 min in 2x SSC. The embryos were then washed twice in 0.2x SSC for 30 mins. The embryos were blocked in blocking solution (PBST, 2% goat serum, 2 mg/ml BSA) at room temperature for 2 hours on a rocker. The embryos were then incubated in an alkaline phosphatase-conjugated anti-digoxygenin antibody (Roche) diluted 1:2000 in blocking solution at 4°C overnight on a rocker. The embryos were then washed briefly in PBST and six times in PBST for 15 mins and then incubated with AP substrate nitro blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl phosphate toluidine salt (NBT/BCIP; Roche). The staining was stopped when required by rinsing in PBST for 10 mins and fixed in 4% PFA at room temperature for 20 mins. The embryos were then incubated for 15 mins in series of glycerol in PBS: 25% (v/v) glycerol, 50% (v/v) glycerol and subsequently washed in 100% glycerol and stored in 100% glycerol at 4°C.

2.3.3 Immunohistochemistry

Immunohistochemistry was used to study the endocrine pancreas development and cell differentiation and ducts of the exocrine pancreas. For immunohistochemistry against Carboxypeptidase-a and Cytokeratin, the fixed embryos were rehydrated through successive dilutions of methanol in 1x PBST: 5 min in 75% (v/v) methanol; 5 min in 50% (v/v) methanol; and 5 min in 25% (v/v) methanol. The larvae were digested with 0.1% collagenase (Sigma) in PBST for 30 mins. For immunohistochemistry, the larvae were then washed twice for 5 mins, then twice for 30 mins in PBS/0.1% Triton/0.2% BSA. They were then washed in blocking buffer (5% goat serum in PBS/0.1% Triton/0.2% BSA) for 1 hour and incubated in anti-Carboxypeptidase A1 antibody (Sigma) diluted 1:100 and anti-Cytokeratin (Santa Cruz Biotechnology) diluted 1:50 in blocking buffer at 4°C overnight. Larvae were then washed several times in PBS/0.1% Triton/0.2% BSA then once with blocking buffer before being incubated in Alexa-546 goat anti-rabbit (Invitrogen) diluted 1:1000 in blocking buffer at 4°C overnight. The larvae were washed several times before being mounted with Vectashield Mounting Media (Vector laboratories). For immunohistochemistry against Insulin, Glucagon, Somatostatin and Pdx1, embryos and larvae were deyolked before being incubated in blocking buffer (1% DMSO, 1% goat serum, 1% BSA and 1% Triton X-100 in 1X PBS) for at least 1 hour and incubated in primary antibodies in blocking buffer overnight 4°C overnight. The primary antibodies were polyclonal guinea pig anti-Insulin (Dako; 1:200), polyclonal rabbit anti-Somatostatin (Dako; 1:200), monoclonal mouse anti-Glucagon (Sigma; 1:1000) and polyclonal guinea pig anti-zebrafish Pdx1 (generous gift from Dr Chris Wright, Vanderbilt University, TN, USA; 1:200). Embryos/larvae were washed several times in PBST and incubated in blocking buffer for at least 1 hour before being incubated in secondary antibodies in blocking buffer at 4°C overnight. The secondary antibodies were diluted 1:1000 and included Alexa-594 goat anti-guinea pig (Invitrogen), Alexa-546 anti-rabbit (Invitrogen), Alexa-546 anti-mouse (Invitrogen) Alexa-660 anti-mouse (Invitrogen) and Alexa-680 anti-rabbit (Invitrogen). The larvae were washed several times before being mounted with Vectashield Mounting Media (Vector laboratories).

2.3.4 Microscopy

Images of live embryos and embryos subjected to whole mount RNA *in situ* hybridisation were taken using either the Leica DFC 450 CCD camera attached to LM80 dissecting microscope and Leica Application Suite software or a Leica DFC 420 CCD camera

attached to a Leica MZ16 FA dissecting microscope (Leica Microsystems, Germany) and Leica Application Suite software. Images of *Tg(ins:mCherry)*^{jh2}*;Tg(ptf1a:EGFP)*^{jh1}*;las1l*^{sa674}, *Tg(ins:mCherry)*^{jh2}*;Tg(ptf1a:EGFP)*^{jh1}*;nol9*^{sa1022} and embryos subjected to immunohistochemistry and TUNEL assay were taken using either the Leica DFC310 FX attached to Leica M205 FA microscope or the Leica TCS SP5/DM6000 confocal microscope with Leica Application Suite Advanced Fluorescence (v2.0.0 build 1934) software. The volume of the exocrine pancreas was measured from Z-stacks images with a 10 μm slice interval using the Measure Stacks plugin in the ImageJ64 software (National Institutes of Health (NIH), http://imagej.nih.gov/ij/). Photos were cropped and modified using the GIMP software.

2.4 Characterisation of loss of function mutants

2.4.1 Morpholino injections

1- to 2-cell stage embryos were injected with 4 ng of morpholino in 0.25% phenol red (Sigma). The translation blocking morpholino were designed and synthesised by Gene-Tools. The morpholino sequences were as follows: *nol9*: ACCTTGTGTACTTTCATTGTCATCC, std: CCTCTTACCTCAGTTACAATTTATA.

2.4.2 Inhibition of Notch-signalling

To study the secondary islet formation, the larvae were incubated in 100 μM of *N*-[*N*-(3,5-Difluorophenacetyl)-$_L$-alanyl]-*S*-phenylglycine *t*- butyl ester (DAPT, StressMarq) in egg water containing PTU from 3 d.p.f. until 5 d.p.f.

2.4.3 Flow cytometry analysis

Flow cytometry analysis was used to analyse the cell cycle of *nol9*^{sa1022} mutants and wild-type siblings. 80-100 larvae were incubated in 1 ml Accumax (Innovative Cell Technologies) at room temperature for 15 mins. Larvae were homogenized using a 1ml syringe and 21 and 25 gauge needle (Becton Dickinson). The tubes were spun at 1500rpm for 10 mins at 4°C. The pellet was washed with 1x PBS, strained through a 100μm nylon cell strainer (Fisher Scientific) and spun at 3000rpm for 3 mins. The pellet was fixed in 1% PFA at room temperature for 15 mins. The tubes were spun at 3000rpm for 3 mins and the pellet was fixed in cold 70% ethanol at 4°C for 2 hours. The tubes were spun at 3000rpm for 3 mins and resuspended and kept in 1 ml PBS at 4°C. For staining, the pellet was resuspended in 0.5 ml PBS containing 25 μg/ml propidium iodide (Sigma), 0.1 mg/ml RNase Type I, DNase free (Sigma), 0.002% Triton X-100 (Sigma) and 0.1% sodium citrate pH7.4 at 37°C for 40 mins in the dark. The tubes were spun at 3000rpm for 3 mins and the pellet was resuspended in 300 μl PBS. Cell analysis was carried out on BD LSRFortessa (BD Biosciences) and cell cycle measurements were performed using the program FlowJo v7.6.5.

2.4.4 Cell Proliferation

The BrdU assay was used for cell proliferation analysis. 4 d.p.f. larvae were chilled on ice for 15 mins before incubation in 10mM BrdU (Life Technologies) in egg water on ice for 20 min. The larvae were then incubated with pre-warmed egg water for 3 hours at 28°C. The

embryos were then fixed in 4% PFA at 4°C overnight. The larvae were washed 4 x 10 mins in PBST, 3 x 5 mins with ddH2O, 2 x 5 mins with 2N HCL, incubated in 2N HCL for 1 hour and washed 2 x 10 mins with 0.1% PBST and 1 x 10 mins in Tris-HCL pH 9.5. The larvae were incubated in blocking solution (10% FBS (Sigma), 1% DMSO, 0.1% Triton-X-1000 in 1X PBS) for 1 hour and incubated at 4°C overnight with monoclonal rat anti-BrdU (Abcam; 1:200). The larvae were washed several times in PBST and incubated in blocking solution for 1 hour before being incubated at 4°C overnight with anti-rat IgG-Cy3 conjugated (Millipore; 1:400). The larvae were washed several times before being mounted with Vectashield Mounting Media (Vector laboratories).

2.4.5 Cell Death

The TUNEL assay was used to detect apoptosis. The fixed embryos were rehydrated through successive dilutions of methanol in 1x PBST: 5 min in 75% (v/v) methanol; 5 min in 50% (v/v) methanol; and 5 min in 25% (v/v) methanol and the larvae were digested with 0.1% collagenase (Sigma) in PBST for 30 mins. The In Situ Cell Death Detection Kit, TMR red (Roche) was used according to the manufacturer's instructions.

2.4.6 Alcian blue staining

3 d.p.f. embryos and 5 d.p.f. larvae were rehydrated through successive dilutions of methanol in 1x PBST: 5 min in 75% (v/v) methanol; 5 min in 50% (v/v) methanol; and 5 min in 25% (v/v) methanol and subsequently rinsed several times in PBST. The embryos/larvae were stained overnight in solution containing 0.1% Alcian blue (Sigma) dissolved in 0.1N HCl. They were then rinsed twice in water and digested using 0.025% trypsin (Sigma) in a saturated solution of sodium tetraborate for 30 mins. The embryos/larvae were rinsed three times in water and transferred in series of glycerol in water: 25% (v/v) glycerol; 50% (v/v) glycerol and 75% (v/v) glycerol. The embryos and larvae were then imaged.

2.4.7 O-dianisidine staining

3 d.p.f. and 5 d.p.f. zebrafish were incubated for 30 mins in the dark with a solution containing 0.6 mg/ml o-dianisidine (Sigma), 11mM sodium acetate (pH 4.5), 43% ethanol (v/v) and 0.65% hydrogen peroxide. The embryos and larvae were subsequently washed in sterile water and transferred to 80% glycerol/20% water for imaging.

2.4.8 Statistical approaches

Student's *t*-test and Paired Student's *t*-test were used to compare the means of two populations in Statplus (AnalystSoft). A *p*-value cut-off of 0.05 was used to define statistical significance.

2.5 Study of rRNA processing and ribosome biogenesis

2.5.1 Bioanalyser & Northern Blot analysis

RNA from phenotypic and non-phenotypic larvae was analysed on the Agilent Bioanalyser 2100 according to the manufacturer's instructions. The Northern Blot analysis was carried out in collaboration with Dr Tobias Fleischmann. 1000 µg RNA from both phenotypic and non-phenotypic larvae was digested in 20 µl reaction in water: 0.5 µl RNAse H (NEB), 2 µl RNase H Buffer, with or without 2 µl of 100 µm oligo 5' GGTTCACGCCCTGTTGAAG 3' at 37°C for 15 mins. 20 µl of RNA was loaded on a 6% TBE/UREA gel (Life Technologies) using a Low Range RNA Ladder (NEB) and run for 1.5 hours at 180V in 1x TBE buffer. The gel was then stained with Sybr Safe stain (Invitrogen). The gel was blotted to Hybond-Nylon (Amersham) in 0.5x TBE buffer for 2 hours at 25 V.

The probe for Northern blot was generated using a RT-PCR-based approach and using the High Prime DNA Labeling and Detection Starter Kit II (Roche). The RNA was reverse transcribed as in Section 2.3.2 and purified using QIAquick Gel Extraction Kit (Qiagen), PCR was then performed using the primers 5' CGATGAAGAACGCAGCTAGC 3' and 5' CTGGCCTCGGAGATCGAC 3'. In a 50 µl reaction containing 5 µl 10x Buffer (Novagen), 5 µl 2mM dNTPs (Novagen), 3 µl $MgSO_4$ 1.5 µl (Novagen), 10 µM primers 2 µl cDNA generated and 1 µl KOD Hot Start DNA Polymerase (Novagen) were incubated and PCR was carried out under the following conditions

1. 95°C for 2 mins;
2. 25 cycles of (95°C for 20 secs, 56°C for 10 secs, 70°C for 15 secs);
3. Hold at 4°C

The DIG High Prime DNA Labeling and Detection Starter Kit II (Roche) was used according to the manufacturer's instructions for DNA labelling, hybridisation to the membrane, immunological detection and chemiluminescent signal detection. The signals was visualised using an X-ray film (Kodak).

2.5.2 Polysome fractionation

Polysome fractionation was carried out in collaboration with Dr Felix Weis. 50 phenotypic and non-phenotypic larvae were resuspended in lysis buffer (50mM Tris-HCl pH 7.4, 150mM potatssium acetate, 2.5 mM $MgCl_2$, 6 mM 2-Mercaptoethanol, 1 Tb/25ml

protease inhibitors (Roche), 0.1 mg/ml cycloheximide, 10% glycerol, 120 U/ml RNase inhibitors and 0.5% sodium deoxycholate) and sheared through 23G needle. The lysates were centrifuged at 16,100g for 20min at 4°C. The supernatant were loaded on a 500 µl sucrose cushion (35% in polysome profile buffer containing 50mM Tris-HCl pH 7.4, 80mM NaCl, 2.5 mM $MgCl_2$ and 6 mM 2-Mercaptoethanol) and centrifuged for 2 hours at 32,000 rpm at 4°C on a Beckman rotor TLA 120.2. The pellets were resuspended in 200 µl polysome profile buffer and cycloheximide. 100 µl of the solution were then loaded on 4 ml 10-45% sucrose gradient and unloaded using a Brandel gradient fractionator. A UV monitor (UV-1, Pharmacia) was used to detect the polysome profiles at A_{254} and a Labjack U3-LV data acquisition device with a LJTick-InAmp preamplifier was used to read the electronic outputs of the UV-1 monitor.

2.6 Differential Expression Transcript Counting Technique (DeTCT)

2.6.1 DeTCT Library preparation

DeTCT was developed by Dr John Collins and was used to study mRNA expression analyses of *nol9*^{sa1022}, *las1l*^{sa674}, *tti*^{s450} and *set*^{s453} mutants. For *nol9*^{sa1022} and *las1l*^{sa674}, six pairs of adult carriers were incrossed and for each pair, 25 mutant and 25 wild-type siblings were collected at 5 d.p.f. for RNA extraction (Section 2.2). For *tti*^{s450} and *set*^{s453}, the RNA of six biological replicates, i.e. 25 mutants and 25 wild-type siblings from six incrosses of adult carriers, were kindly provided Dr Joan Heath, Walter+Eliza Hall, Institute of Medical Research, Melbourne, Australia. DNAse treatment was carried out on all the RNA samples (Section 2.2).

The cDNA libraries were made as follows: 9µl of total RNA was fragmented by incubation in 10x Fragmentation Buffer (Ambion) at 70°C for 5 min s. The tube was immediately transferred to an ice bath and 1 µl of stop solution (Ambion) was added and the RNA was kept at 4°C. 62.5 µl Streptavidin Magnetic Beads (NEB) was added to 50 µl 1x Wash/binding buffer (0.5M NaCl, 20mM Tris-HCl pH 7.5, 1mM EDTA, Sigma) and applied to a magnet rack for 1 minute. The beads were washed twice in 100 µl 1x Wash/binding buffer before incubation in 100 µl 1x Wash/binding buffer containing 1 µl of 100µM 14bp polyT primer at room temperature for 5 mins on a tube rotator to allow polyT primer to bind to the beads (Figure 2-1 Step 1). The beads were then washed in 100 µl 1x Wash/binding buffer and then incubated in 50 µl 2x Wash/binding buffer (1M NaCl, 40mM Tris-HCL pH 7.5, 2mM EDTA; Sigma) together with 1 µl RNase Inhibitor (NEB) and fragmented RNA at room temperature for 20 mins on tube rotator. The beads were washed twice in 100 µl 1x Wash/binding buffer and once in 100 µl cold no EDTA/low salt buffer (0.15M NaCl, 20mM Tris-HCl pH 7.5; Sigma) before they were resuspended in 18.9 µl RNAse-free water (Sigma). 2.5 µl 10x T4 RNA ligase buffer (NEB), 1µl RNase Inhibitor (NEB), T4 PNK 3' phosphate minus (NEB) and 2.5µl 10mM ATP (NEB) were added and the reaction was incubated for 30 mins at 37°C. At room temperature, 10 µl 80% PEG (Promega) was added. 1.5 µl 10x T4 RNA ligase buffer (NEB), 1.5 µl 10mM ATP (NEB), 1 µl T4 RNA ligase (NEB) and 1 µl 10µM stRSSA4 primer (Table 2-3) were added and the reaction was incubated for 2 hours at 37°C (Figure 2-1 Step 2). 40 µl 2x Wash/binding buffer was added and incubated for 2 mins at room temperature. The beads were then washed twice with 100µl 1x Wash/binding buffer and once with 100µl cold no EDTA/low salt buffer before they were resuspended in 12 µl

RNase-free water (Sigma). The solution was incubated at 80°C for 2 mins and the poly(A) transcripts ligated to the partial Illumina adapter was eluted (Figure 2-1 Step 3).

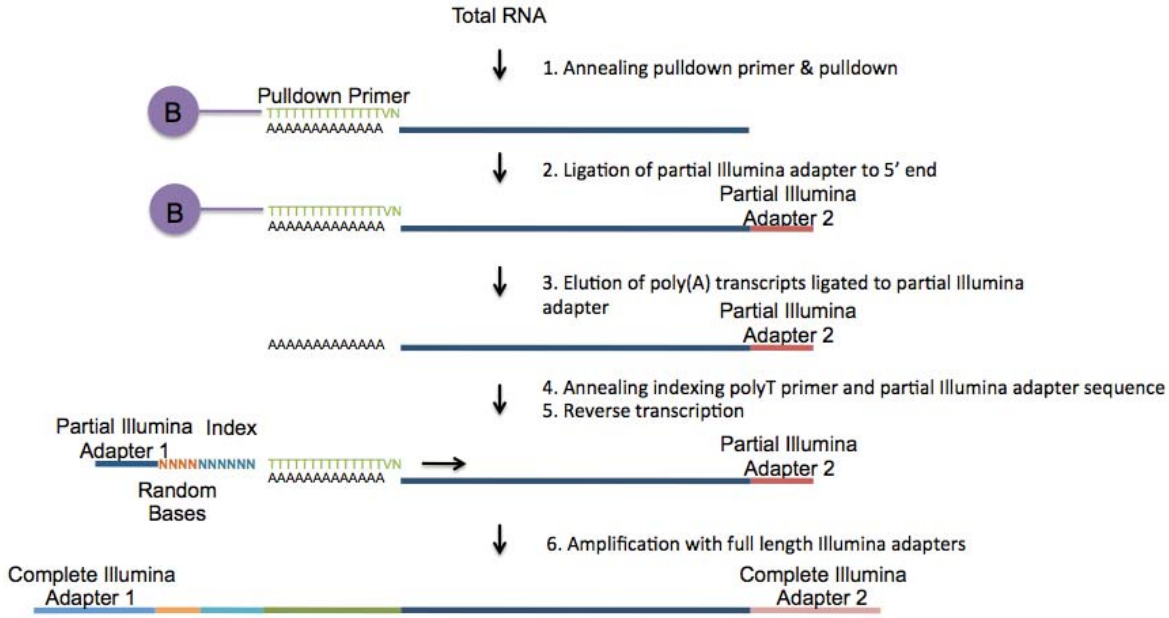

Figure 2-1 The DeTCT library preparation includes RNA extraction, pulldown of poly(A) transcripts, ligation of Illumina adapters and an indexing polyT primer, reverse transcription and amplification. (Dr John Collins, Personal Communication)

1 µl of specific tagged primer (Table 2-4) was added to the appropriate reaction tube, incubated at 70°C for 2 mins and immediately transferred to an ice bath (Figure 2-1 Step 4). For synthesis of first stranded cDNA: 4 µl 1 5xFS buffer (Invitrogen), 2 µl 0.1M DTT (Invitrogen), 1 µl 10mM dNTP (Invitrogen), 1 µl RNase Inhibitor (NEB) and 1 µl SuperScript II (Invitrogen) were added and incubated at 42°C for 1 hour (Figure 2-1 Step 5). The cDNA was cleaned using the QIAquick PCR Purification Kit (Qiagen) according to manufacturer's instructions and eluted in 38 µl EB Buffer (Qiagen). The PCR reaction was carried out in a 96-well PCR plate (Thermo Scientific) as follows: 2.5 µl 10x KOD polymerase buffer (Novagen), 2.5 µl 2mM dNTP (Novagen), 1 µl 25mM $MgSO_4$ (Novagen), 1 µl 10µM stSA.PCR.S.1+2 primer mix (Table 2-3), 0.5 µl KOD polymerase (Novagen) and 17.5 µl template or water. The following program was used on a PTC-225 Peltier Thermal Cycler (MJ Research): 94°C for 2 mins; 15 cycles of 94°C for 15 seconds, 60°C for 30 seconds, 68°C for 3 mins; and 68°C for 5 mins (Figure 2-1 Step 6). The PCR products were cleaned using the QIAquick PCR Purification Kit (Qiagen) according to manufacturer's instructions, eluted in 50 µl EB Buffer (Qiagen) and measured on an Eppendorf BioPhotometer. 5 µl of each sample and the PCR no template control were run on 1.5% agarose (Invitrogen) gel for

45 mins at 70 V in 1x TAE buffer. The DeTCT cDNA library was sequenced on an Illumina HISeq 2000 by the Wellcome Trust Sanger Institute Core Sequencing Facility.

Primer Name	Primer Sequence
stRSSA4	5' Am-CUCGGCAUUCCUGCUGAACCGCUCUUCCGAUCU 3’
stSAPCRS.1:	5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGA 3’
stSAPCRS.2:	5’ CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAAC 3’

Table 2-3 Sequences of primers used in DeTCT library preparation

Sample	Primer Sequence
nol9_mu1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBGAGGCTTTTTTTTTTTTTTVN
nol9_wt1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBAGAAGTTTTTTTTTTTTTTVN
nol9_mu2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBCAGAGTTTTTTTTTTTTTTVN
nol9_wt2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBGCACGTTTTTTTTTTTTTTVN
nol9_mu3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBCGCAATTTTTTTTTTTTTTVN
nol9_wt3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBCAAGATTTTTTTTTTTTTTVN
nol9_mu4	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBGCCGATTTTTTTTTTTTTTVN
nol9_wt4	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBCGGCCTTTTTTTTTTTTTTVN
nol9_mu5	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBAACCGTTTTTTTTTTTTTTVN
nol9_wt5	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBACGGGTTTTTTTTTTTTTTVN
nol9_mu6	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBCCAACTTTTTTTTTTTTTTVN
nol9_wt6	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBAGCGCTTTTTTTTTTTTTTVN
las1l_mu1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBGAGGCTTTTTTTTTTTTTTVN
las1l_wt1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBAGAAGTTTTTTTTTTTTTTVN
las1l_mu2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBCAGAGTTTTTTTTTTTTTTVN
las1l_wt2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBGCACGTTTTTTTTTTTTTTVN
las1l_mu3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBGCCGATTTTTTTTTTTTTTVN
las1l_wt3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBCGGCCTTTTTTTTTTTTTTVN
las1l_mu4	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBAACCGTTTTTTTTTTTTTTVN
las1l_wt4	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBACGGGTTTTTTTTTTTTTTVN
las1l_mu5	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBCCAACTTTTTTTTTTTTTTVN
las1l_wt5	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBAGCGCTTTTTTTTTTTTTTVN
tti_mu1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBGAGGCTTTTTTTTTTTTTTVN
tti_wt1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBAGAAGTTTTTTTTTTTTTTVN
tti_mu2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBCAGAGTTTTTTTTTTTTTTVN
tti_wt2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBGCACGTTTTTTTTTTTTTTVN
tti_mu3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBCGCAATTTTTTTTTTTTTTVN
tti_wt3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBCAAGATTTTTTTTTTTTTTVN
set_mu1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBGCCGATTTTTTTTTTTTTTVN
set_wt1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBCGGCCTTTTTTTTTTTTTTVN
set_mu2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBAACCGTTTTTTTTTTTTTTVN
set_wt2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBACGGGTTTTTTTTTTTTTTVN
set_mu3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBCCAACTTTTTTTTTTTTTTVN
set_wt3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNBAGCGCTTTTTTTTTTTTTTVN

Table 2-4 Sequences of primers used for different samples (Green, Red, Brown Random 4mer, Black tag 6bp, Pink 14bp PolyT).

2.6.2 DeTCT analysis

Dr Ian Sealy carried out the analysis of sequence data from DeTCT libraries of *nol9*^{sa1022}, *las1l*^{sa674}, *tti*^{s450} and *set*^{s453} mutants. The DeTCT analysis pipeline, written by Drs James Morris and Ian Sealy and available from https://github.com/iansealy/DETCT, was used. The sequencing data produces two sets of reads, the read 1 contains the random bases, the index tag, 14 T bases and the remaining is transcript-specific sequence whereas the read 2 is entirely transcript-specific sequence. The DeTCT analysis pipeline consists of the following steps:

1. FASTQ files are extracted from the BAM files;
2. The reads are trimmed: 21 bases are removed from the 3' ends of both read 1 and read 2 and the tag, random bases and polyT sequence are removed from the 5' end of read 1;
3. The reads are aligned to the Zv9 reference genome using the Burrows-Wheeler Aligner (BWA) software (Li and Durbin, 2009);
4. PCR duplicates are flagged using a modified version of the Picard suite's MarkDuplicates tool (available from https://github.com/iansealy/picard-detct);
5. The genome is divided into bins of 100 bases and the number of read 2s aligned in each bin are counted across all libraries;
6. Read peaks are probabilistically identified in the bin data using a Hidden Markov model (HMM). This provides a list of bins with associated read counts and probabilities;
7. Adjacent bins are joined together to make regions;
8. The 3' end associated with each region is identified by extracting the read 1s paired with read 2s within the region. The alignment of read 1 gives the 3' end position and the strand along with a read count;
9. The 3' ends are filtered for artefacts primed from genomic DNA rich in A bases and the 3' ends with the highest read counts are chosen;
10. The number of read 2s in each region for each experimental sample are used to produce count data;
11. The count data are used for differential expression analysis using DESeq (Anders and Huber, 2010). All regions with count sum below the 40th quantile are excluded. DESeq fits two models: one based only on sibling pairings and one based on sibling pairings and experimental condition (i.e. mutant or sibling) and compares which model explains the data best in order to produce a list of differentially expressed regions;

Each region is annotated with Ensembl gene information based on the nearest transcript to the 3' end.

2.6.3 Comparisons between *nol9*^{sa1022}, *las1l*^{sa674}, *tti*^{s450} and *set*^{s453} mutants

The total number of reads for libraries of three pairs of mutant and wild-type siblings of *nol9*^{sa1022}, *las1l*^{sa674}, *tti*^{s450} and *set*^{s453} mutants was normalised to a total read count of 26 M reads using Picard DownsampleSam tool. The steps 4-12 from the DeTCT analysis pipeline (Section 2.6.2) were carried out and the list of differentially expressed regions were filtered to include only the regions that are within 100 bases to the nearest 3' end Ensembl gene. This list was then filtered to include regions that are statistically significant at a DESeq adjusted *p*-value of 0.05 (Benjamini-Hochberg procedure for multiple testing) and the list of regions were sorted for unique genes to produce a list of statistically significant differentially expressed genes. The Ensembl Gene IDs of the statistically significant differentially expressed genes of *nol9*^{sa1022}, *las1l*^{sa674}, *tti*^{s450} and *set*^{s453} mutants were then compared using the tool VENNY (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

2.6.4 Gene ontology enrichment analysis

Dr Ian Sealy carried out the enrichment analysis for Gene Ontology (GO) terms in *nol9*^{sa1022}, *las1l*^{sa674}, *tti*^{s450} and *set*^{s453} mutants using the R topGO package (Alexa et al., 2006). First, the whole list of regions produced by the DESeq-based pipeline was sorted for unique genes and their corresponding highest adjusted *p*-values were considered. The enrichment analysis was performed using the *elim* method and the Kolomogorov-Smirnov like test was used as the test statistic. The statistically significant GO term was defined as having a Ks-value of less than 0.01.

To identify the GO terms that were enriched between *nol9*^{sa1022}, *las1l*^{sa674}, *tti*^{s450} and *set*^{s453} mutants, the statistically significant GO terms (K-S value less than 0.01) of all four mutants were compared using the tool VENNY (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

2.6.5 KEGG pathways enrichment analysis

For enrichment of KEGG pathways in *nol9*^{sa1022}, *tti*^{s450} and *set*^{s453} mutants, the Database for Annotation, Visualization and Integrated Discovery (DAVID) was used (Dennis et al., 2003; Huang da et al., 2009b) (http://david.abcc.ncifcrf.gov). First, the list of differentially expressed regions that was generated by the DESeq-based pipeline was sorted for unique genes to produce a background list of 11170 genes for *nol9*^{sa1022}, 10527 for *tti*^{s450} and 10535 for *set*^{s453} mutants. The list of statistically significant differentially expressed regions (DESeq adjusted *p*-value less than 0.05) was sorted for unique genes to produce a list of 566 for *nol9*^{sa1022}, 730 for *tti*^{s450} and 607 for *set*^{s453} mutants of statistically significant differentially expressed genes. The statistically significant differentially expressed genes were then compared to the respective background list using the DAVID functional annotation tool.