Chapter 2
Materials and Methods

2.1. Reagents

2.1.1. *C. elegans*

2.1.1.1. *C. elegans* strains

The following mutant strains were acquired from the *Caenorhabditis* Genetics Centre (CGC), University of Minnesota, USA (http://www.cbs.umn.edu/CGC/): Bristol N2, *glp-1(or178)*, *lag-2(q420)*, *emb-5(hc61)*, *smg-1(r861)* and *smg-5(r860)*.

2.1.1.2. Nematode Growth Medium (NGM) (Stiernagle, 2006)

NaCl	3g
Peptone	2.5g
Optional[a]: Agar	19g
dd H_2O	to 1L

The solution was autoclaved and cooled to 55°C before addition of:

Cholesterol solution (5 mg/ml in ethanol)	1ml
1M $CaCl_2$	1ml
1M $MgSO_4$	1ml
1M KH_2PO_4, pH6.0	25ml
Fungizone	800µl

in the order as written, with mixing thoroughly after addition of each component.

Solutions were sterile-filtrated through a membrane filter with a pore size of 0.2 µm.

[a] For preparation of agar plates, solution was poured into sterile Petri dishes.

2.1.1.3. M9 Buffer (Stiernagle, 2006)

1M KH_2PO_4	3g
1M Na_2HPO_4	6g
1M NaCl	5g
ddH_2O	to 1L

1ml 1M $MgSO_4$ was added after solution had been autoclaved to sterilize.

2.1.1.4. Freezing buffer (Stiernagle, 2006)

KH_2PO_4	3g
0.05M K_2HPO_4	129ml
0.05M KH_2PO_4	871ml
NaCl	5.85g
Glycerin	30% (v/v)

2.1.1.5. Bleach solution

1M NaOH	250µl
Sodium hypochlorite, available chlorine 10-13%	100µl
Autoclaved H_2O to	1000µl

2.1.2. Bacteria

2.1.2.1. RNAi feeding strains

Bacterial clones used for RNA interference (RNAi) experiments were selected from the Ahringer RNAi feeding library (Kamath *et al*., 2003) and *C. elegans* ORFeome collection (Rual *et al*., 2004).

2.1.2.2. **Luria-Bertani (LB) medium** (Bertani, 1951)

Bacto-tryptone	10g
Bacto-yeast extract	5g
NaCl	10g
Optional[a]: Bacto-Agar	15g
ddH2O	to 1L

pH was adjusted to 7.2 and solution was autoclaved to sterilize.

[a] For preparation of agar plates, solution was poured into sterile Petri dishes.

2.1.2.3. 2 x Tryptone / yeast extract (TY)

Bacto-tryptone	16g
Bacto-yeast extract	10g
NaCl	5g
dd H_2O	to 1L

pH was adjusted to 7.2 and solution was autoclaved to sterilize.

2.1.3. Buffers used for Affymetrix tiling microarray hybridization and processing

(From Affymetrix GeneChip® Whole Transcript (WT) Double-Stranded Target Assay Manual)

2.1.3.1. 12x MES Buffer

MES hydrate	64.61g
MES Sodium Salt	193.3g
Molecular Biology Grade Water	800ml

Mix, adjust volume to 1L and 0.22µm filter. Stored at 4°C in the dark.

2.1.3.2. 2x hybridization buffer

12X MES Stock Buffer	8.3ml
5M NaCl	17.7ml
0.5M EDTA	4.0ml
10% Tween-20	0.1ml
Molecular Biology Grade Water	19.9ml

Stored at 4°C in the dark.

2.1.3.3. Wash Buffer A

20X SSPE (Ambion)	300ml
10% Tween-20	1.0ml
Molecular Biology Grade Water	699ml

0.22µm filtered

2.1.3.4. Wash Buffer B

12X MES Stock Buffer	83.3ml
5M NaCl	5.2ml
10% Tween-20	1.0ml
Molecular Biology Grade Water	910.5ml

0.22µm filtered and stored at 4^{o}C in the dark.

2.1.3.5. 2x Stain Buffer

12X MES Stock Buffer	41.7ml
5M NaCl	92.5ml
10% Tween-20	2.5ml
Molecular Biology Grade Water	113.3ml

0.22µm filtered and stored at 4^{o}C in the dark.

2.1.3.6. Array Holding Buffer

12X MES Stock Buffer	8.3ml
5M NaCl	18.5ml
10% Tween-20	0.1ml
Molecular Biology Grade Water	73.1ml

Stored at 4^{o}C in the dark.

2.1.3.7. Streptavidin Phycoerythrin Stain Cocktail

2x Stain Buffer	300µl
50mg/ml BSA (Invitrogen)	24µl
1mg/ml Streptavidin Phycoerythrin (Molecular Probes)	6µl
Molecular Biology Grade Water	270µl

2.1.3.8. Antibody Stain Cocktail

2x Stain Buffer	300µl
50mg/ml BSA (Invitrogen)	24µl
10mg/ml grade Reagent Goat IgG (Sigma-Aldrich), made up in 150mM NaCl, stored at $4^{o}C$	6µl
0.5mg/ml goat anti-streptavidin biotinylated antibody (Vector Laboratories)	3.6µl
Molecular Biology Grade Water	266.4µl

2.1.4. 10x PCR reaction buffer

100 mM Tris-HCl

500 mM KCl

15 mM $MgCl_2$

pH 8.3 at 25°C

2.2. Protocols

2.2.1. Maintenance of *C. elegans* stocks

C. elegans was maintained on NGM agar plates seeded with OP50 *E. coli* according to standard protocols (Brenner, 1974). For maintenance of large worm populations in liquid culture HB101 *E. coli* grown in 2 x TY was resuspended in NGM. Freshly bleached embryos were then added to HB101 in NGM in conical flasks and shaken at 150r.p.m. at 15^{o}C.

2.2.2. Bleach sterilization of *C. elegans* strains and synchronization

Worms were washed off plates with M9 buffer and pelleted for 1 minute at 1000r.p.m.. Alternatively, worms in liquid culture were pelleted in the same way. The resulting pellet was then resuspended in freshly prepared bleach solution and incubated shaking at room temperature until the worms had broken apart, all of the carcass dissolved and only embryos remained. Embryos were then pelleted for 1 minute at 1000r.p.m. and washed twice with M9 buffer. Embryos were then left shaking for 26h at room temperature in order to obtain a synchronous population growth-arrested at mid-L1 stage (Stiernagle, 2006). Alternatively, if a synchronous population was not required embryos pelleted in M9 buffer were spotted on NGM plates or added to HB101 in NGM for liquid culture.

2.2.3. Freezing and recovery of *C. elegans* stocks

A population of worms containing L1 and L2 stage animals that were approaching starvation were washed off plates in M9 buffer, pelleted by centrifugation at 1000r.p.m.

for 1 minute, and resuspended in an equal volume of M9 buffer and freezing buffer. 1ml of suspension was aliquoted per 1.8ml cryovial. Cryovials were placed into freezing boxes filled with isopropanol to allow a gradual 1°C decrease in temperature per minute when placed at -70°C. Cryovials were stored at -70°C. For thawing, cryovials were placed at room temperature and worms were spotted onto NGM plates seeded with OP50 *E. coli* as soon as all ice had turned to liquid (Stiernagle, 2006).

2.2.4. RNAi by feeding on plates, RNA extraction and visual phenotyping

dsRNA expressing bacteria from glycerol stocks were streaked out onto LB agar plates containing 50mg/ml Amp and incubated overnight at 37^{o}C. The next day the resulting colonies were cultured overnight in 2xTY + 100mg/ml Amp and spotted onto NGM Single Peptone plates containing 50mg/ml Amp and 1mM IPTG, and left overnight to dry. Synchronised L1 stage worms in M9 buffer were then spotted onto plates (or NGM plates sans Amp and IPTG and seeded with OP50 as appropriate) and incubated at the appropriate temperature (20 ^{o}C or 25^{o}C) (Kamath *et al.*, 2003). Worms were washed off plates in M9 buffer at the appropriate timepoint and spun down and washed once in M9 buffer. Worms were then pelleted at 1000r.p.m. for one minute and the pellet resuspended in 4ml Trizol® (Invitrogen) per ml pellet. RNA was then prepared from the Trizol solution according to the manufacturer's protocol, the final pellet resuspended in nuclease-free water. The quantity of RNA was measured with a NanoDrop ND-1000. 100% ethanol was added to give a 70% ethanol solution and stored at -70 ^{o}C. For visual phenotyping, individual animals were transferred to wells of 12 well plates baring the same constituents as above and incubated at 25^{o}C for 24hrs beyond young adult stage.

Adult worms were then removed and the plates returned to the incubator for 24hrs, after which progeny were counted.

2.2.5. DAPI staining

For staining of nuclei with DAPI, whole intact worms were washed off plates in M9 buffer and fixed in cold (-20^{o}C) methanol for 5 min. Fixed worms were washed twice in M9 buffer, incubated 30 min in 100 ng/ml DAPI in M9 and washed two to three times in M9. Worms were then mounted on glass slides and imaged with a Leica SP5 confocal microscope.

2.2.6. Generation of mixed-stage RNA reference sample

Synchronous L1 stage animals were added to *E. coli* strain HB101 in NGM medium in conical flasks shaking at 15^{o}C. HB101 was added into the culture as appropriate such that the animals neither starved nor became anoxic. At the appropriate developmental stage the cultures were placed at 4^{o}C to allow the animals to settle. The animals were isolated and washed twice with M9. RNA was then extracted as in 2.2.4. This gave RNA from synchronous L2, L3, L4, young adult and gravid adult stage populations. The resulting RNA was then mixed and supplemented with RNA extracted from growth-arrested L1 stage animals and asynchronous embryos yielding sufficient RNA for ~1000 microarray hybridizations as detailed in 2.2.7.

2.2.7. RNA labelling and two-colour microarray hybridization

A direct labelling method was used to produce fluorescently labelled cDNA. In all cases the experimental sample (Cy3) was hybridized against a universal reference sample

(Cy5). 20µg of RNA precipitated from 70% ethanol stock by addition of 1/40th volume 3M Sodium Acetate and storage at -70^{o}C for at least 30 minutes. The samples were then spun down at 20800g in an Eppendorf 5415R cooled centrifuge at 4^{o}C. The pellets were then washed once in 70% ethanol and air-dried briefly. The pellets were then resuspended in 14.4µl and 1µl 0.5µg/µl oligo(dT)$_{12-18}$, heated to 70^{o}C for 10 minutes and then placed on ice. The following reagents were then added in order:

- 6.0µl 5 x first strand buffer (Invitrogen)
- 3.0µl 0.1M DDT (Invitrogen)
- 0.6µl dNTP mix (25mM dATP, 25mM dGTP, 25mM dTTP, 10mM dCTP)
- 3.0µl dCTP-Cy3 or dCTP-Cy5 (25mM GE Healthcare)
- 2.0µl Superscript II (Invitrogen)

The mixture was then incubated at 42^{o}C for 2 hours. 1.5µl 1M NaOH was added and incubated at 70^{o}C for 20 minutes to hydrolyse the RNA. 1.5µl HCl was then added to neutralize the solution. The cDNA was then purified from the mixture using QIAGEN PCR Purification columns according to the manufacturers instructions with an additional wash with buffer PE. The eluted cDNA was precipitated in 70% ethanol, 75mM Sodium Acetate at -20^{o}C with 8µg human Cot-1 DNA (Invitrogen), 2µg polyA DNA (Sigma) and 250µg sheared salmon sperm DNA (Ambion) for 30 minutes. The precipitated DNA was then spun down at 20800g for 5 minutes, the pellet washed in 70% ethanol and dried at 70^{o}C for 2 minutes. 10µl nuclease-free water was added to the pellet and heated to 70^{o}C for 5 minutes. 50µl hybridization buffer (50% Dionised Formamide, 5xSSC, 0.1% SDS and 0.1mg/ml BSA) was then added and incubated at 70^{o}C for a further 5 minutes. The

hybridization mix was then allowed to cool to room temperature for 10 minutes in the dark and then centrifuged for 5 minutes at 20800g at room temperature for 5 minutes. 55µl of hybridization mix was spotted on a covered slip of equal width of the microarray slide and sufficient width to cover the printed area. The printed side of the slide was then applied to the cover slip and the slide placed in a saddle in an Advalytix SlideBooster SB800. The SlideBooster had previously been pre-warmed to 42^{o}C with 500µl humidifying buffer (20% Formamide, 2xSSC) added to each reagent reservoir and 30µl coupling buffer (Advalytix) to each saddle. The microarrays were then incubated for 16-24hrs with sonication.

The cover slips were allowed to slide from the arrays in 0.1xSSC, 0.1% SDS. The arrays were washed twice for 15 minutes in 0.1xSSC, 0.1% SDS and then three times for 5 minutes in 0.1xSSC in a slide rack in a pyrex trough in the dark. The arrays were then centrifuged for 1 minute at 1000r.p.m. in slide racks to dry. The microarrays were scanned using a GenePix 4000B scanner at 5µm resolution. All wash buffers were made up with sterile HPLC water.

2.2.8. Affymetrix tiling microarray hybridization

Total RNA was cleaned using Rneasy columns (QIAGEN) according to manufacturers protocol and then Dnase I (Roche) treated with 10U for 30 minutes in 100µl 1x One-Phor-All buffer (Amersham). The RNA was then re-purified using Rneasy columns (QIAGEN).

1µl random hexamers (3µg/µl) were added to 15µg total RNA in 7µl nuclease-free water, and placed in a thermal cycler using the heated lid for the following protocol:

- 70°C for 5 minutes
- 25°C for 5 minutes
- 4°C for 2-10 minutes

The following reagents were then added to the mixture:

- 4µl 5X first strand buffer (Invitrogen)
- 2µl 100 mM DTT (Invitrogen)
- 1µl 10 mM dNTPs (Invitrogen)
- 1µl RNase Inhibitor (Ambion)
- 4µl Superscript II (Invitrogen)

The mixture was then placed in a thermal cycler using the heated lid for the following protocol:

- 25°C 10 minutes
- 42°C 90 minutes
- 70°C 10 minutes
- 4°C 2-10 minutes

The following reagents were then added to the mixture on ice:

- 7.3µl nuclease-free water
- 8µl 5X second strand buffer (Invitrogen)
- 2µl 10 mM dNTPs (Invitrogen)
- 1µl 10 U/ml E. coli DNA Ligase (Invitrogen)
- 1.2µl 10 U/ml E. coli DNA polymerase I (Invitrogen)

- 0.5µl 2 U/ml E. coli RNase H (Invitrogen)

The mixture was then placed in a thermal cycler for the following protocol:

- 16°C 2 hours (without heated lid)
- 75°C 15 minutes (with heated lid)
- 4°C at least 2 minutes

followed by the addition of:

- 10 U/ml RNase H (Epicentre)
- 5 and 20 U/ml RNase A/T1 cocktail (Ambion)

and incubation at 37°C for 20 minutes.

The (ds)cDNA was then purified using QIAGEN PCR Purification columns according to the manufacturers instructions, eluting in nuclease-free water. The eluted RNA was ethanol precipitated by dilution in 100% ethanol to 70% concentration, addition of 1/40th volume 3M Sodium Acetate and incubation at -70°C for at least 30 minutes. The precipitated (ds)cDNA was spun-down at 20800g for 15 minutes, washed once in 70% ethanol, air-dried and resuspended in nuclease-free water. 17µg of (ds)cDNA in 22µl water was digested by the addition of:

- 3µl 10x One-Phor-All buffer (GE Healthcare)
- 5µl Dnase I (Invitrogen) diluted to 0.17U/µl in 1x One-Phor-All buffer

and incubation in a thermal cycler with heater lid using the following protocol:

- 37°C 8 minutes
- 99°C 10 minutes

- 4°C at least 2 minutes

2µg (1.76µl) of (ds)cDNA was assessed on a 1% agarose gel in order to check that the majority of (ds)cDNA was in the desired 50-100bp size-range.

The (ds)cDNA was then labelled by the addition of:

- 17.96µl nuclease-free water
- 14µl 5X TdT buffer (Roche)
- 7µl 25 mM CoCl2 (Roche)
- 2.3µl Affymetrix DNA Labeling Reagent
- 0.5µl Terminal deoxytransferase (8000U; Roche)

followed by incubation at 37°C for 2 hours.

The following was then added to the above mixture:

- 4.17µl Affymetrix Control Oligonucleotide B2
- 2µl 10mg/ml Herring Sperm DNA (Promega)
- 2.5µl 50mg/ml Acetylated BSA (Invitrogen)
- 125µl 2x Hybridization Buffer
- 17.5µl DMSO
- 28.83µl Nuclease-free water

This hybridization cocktail was then heated to 99°C for 5 minutes, cooled to 45°C for 5 minutes, centrifuged at 20800g for one minute and then injected into an Affymetrix

GeneChip® *C. elegans* Tiling 1.0R Array. The array was hybridized for 16 hours in a 45^{o}C Affymetrix hybridization oven at 60 r.p.m..

Hybridized microarrays were washed and scanned according to chapter 5 of the "GeneChip® Whole Transcript (WT) Double-Stranded Target Assay Manual" (https://www.affymetrix.com/support/downloads/manuals/wt_dble_strand_target_assay_manual.pdf).

2.2.9. (ds)cDNA production for Illumina sequencing

Total RNA was cleaned using Rneasy columns (QIAGEN) and then Dnase I (Roche) treated with 10U for 30 minutes in 100ml 1x One-Phor-All buffer (Amersham). RNA was then re-purified using Rneasy columns (QIAGEN). mRNA was purified from total RNA using Oligotex midi kits (QIAGEN) according to the manufacturers protocol. (ds)cDNA was then produced using SuperScript™ Double-Stranded cDNA Synthesis Kit (Invitrogen) and purified using a QIAGEN PCR Purification kit. 1µg of (ds)cDNA was then submitted for sequencing.

2.2.10. Reverse transcription and PCR

Total RNA was cleaned using Rneasy columns (QIAGEN) and then Dnase I (Roche) treated with 10U for 30 minutes in 100µl 1x One-Phor-All buffer (Amersham). RNA was then re-purified using Rneasy columns (QIAGEN). 5µg total RNA was then used to produce first-strand cDNA using SuperScript™ Double-Stranded cDNA Synthesis Kit (Invitrogen) and purified using a QIAGEN PCR Purification kit. 5ng cDNA was used as template for amplification with gene-specific primers in the following PCR mix:

- 3µl 10x PCR reaction buffer
- 3µl 10mM dNTPs
- 2.7µl 1mg/ml BSA
- 0.4µl 5% (v/v) β–Mercaptoethanol
- 0.9µl 10mM primer mix
- 0.6µl Taq polymerase
- 1.5µl template
- 17.9µl nuclease-free water

using the following amplification conditions in a thermal cycler:

- 94°C for 5 minutes
- 30 cycles of:
    - 94°C for 30 seconds
    - 58°C for 30 seconds
    - 72°C for 2 minutes
- 72°C for 5 minutes
- Hold at 16°C

PCR products were then analysed on a 1% agarose ethidium bromide gel.

2.2.11. Two-colour expression microarray data analysis

GenePix Pro 5.0 was used to identify and isolate signal from spots above background and export data. The methodology from this point is described in detail in chapter 3. Briefly, the data were then normalized using a publicly available Perl script available here: http://www.sanger.ac.uk/PostGenomics/S_pombe/software/. Differentially expressed genes between each condition and wild-type were determined by Student's t-test.

Comparative ratios of means of biological replicates were calculated between the relevant conditions via the universal reference sample. Hierarchical clustering was then performed based on a correlation matrix of the differentially expressed genes within all conditions being compared. This was done using GeneSpring

2.2.12. Identifying transcribed regions and visualization of tiling microarray data

Raw spot intensity files (.CEL files) were quantile normalized and scaled in R. The normalized data were processed and then exported as .BAR files using Affymetrix Tiling Analysis Software (TAS) version 1.1 for visualization in Affymetrix Integrated Genome Browser (IGB). A background cut-off was calculated to include the top 5% of all non-genic probes for each condition and interval analysis then performed in TAS to identify transcribed regions above this cut-off. The maxgap and minrun parameters that define the transcribed regions are discussed in chapter 4.

2.2.13. Affymetrix tiling microarray expression data analysis

The raw data for all arrays to be compared were quantile normalized in R. All further data manipulations were performed in Perl. Probe signal was mapped to all genes and exons of the relevant genome release. A background threshold was then calculated for the mean signal of biological replicates in order to include the top 5% of extra-genic probes. Genes were considered expressed if ≥50% of probes were above background in ≥50% of unique exons. Gene intensities of median exonic probes above background within filtered exons were then calculated. Exon intensities used for the splicing analysis were the median probe intensity of probes above background in the exons for which ≥50% of probes were above background.

2.2.14. Illumina sequence data analysis

Sequence reads were then aligned to the genome using Maq (http://maq.sourceforge.net/) to both identify where reads align and the number of reads that overlap a given base pair. The output was then visualized relative to the genome using IGB. Gene intensities based on sequence reads were calculated as the median number of reads spanning a given base of a gene amongst bases for which there is at least one spanning read. Sequence reads spanning exon-exon boundaries were identified as detailed in chapter 4. Briefly, such reads were identified as reads not alignable to the genome using Maq but giving complete alignment across exon-exon boundaries using Maq.