3 MATERIAL AND METHODS

3.1 RNA Preparation from Animal Tissues

Identical sources of total RNA were used for the array hybridization and qPCR using SybrGreen. For the study on individual mice brain tissues [cerebellum, cortex, midbrain] another “batch” of 4 Ts65Dn and 4 euploid littermates was used. All mice tissues were provided by Dr. R.H Reeves from the John Hopkins University School of medecine [Baltimore, USA]. A gel picture with native RNA samples of the individual mice is provided in Appendix (Figure 7-1, p.141). The gel picture of the RNA used in the array hybridization experiments is in Results (Figure 2-1).

Total RNA was extracted from lung, testis, heart, skeletal muscle, kidney, liver, cerebellum, cortex and midbrain of Ts65Dn males and their euploid male littermates using Trizol reagent (Invitrogen), following the manufacturer’s instructions. TRIzol reagent is a solution of guanidine isothiocyanate and phenol, which simplifies the original method published by (Chomczynski and Sacchi, 1987). Briefly, tissue samples were frozen in liquid nitrogen and ground in 1 ml of TRIzol reagent (Gibco) per 100 mg of tissue using a pestle and mortar; they were then incubated 15 min at room temperature to permit the complete dissociation of nucleoprotein complexes. Following homogenization, 0.2 ml of chloroform (Sigma) per 1 ml TRIzol was added to the samples to allow the phase separation. Samples were then centrifuged at 12,000*g* for 15 min at 4°C, and the upper aqueous phase was transferred to a clean tube. The same volume of isopropanol (Merck) was added, and the samples were mixed by vortexing and incubated for 20 min at 4°C to precipitate the RNA from the aqueous phase. After a further centrifugation, the RNA pellet was washed in 75% ethanol, and samples were then suspended in 20 µl nuclease free water. Animals were between 13 and 16 weeks old. Equal amounts of RNAs from four euploid or four trisomic animals were pooled to create the euploid and trisomic sample pools respectively.

DNA contaminations were systematically removed from all total RNA samples. Thus, total RNA was treated with RNase-free Dnase I (Qiagen) following the manufacturer’s guidelines, quantified by UV spectrophotometry (ND-1000, NanoDrop) and its integrity was verified by gel electrophoresis.

3.2 FISH Analysis

Two BACs from the RPCI-23 library, 134F19 and 359P19, were obtained from BACPAC Resources (Oakland, CA). One microgram of BAC DNA was directly labeled with either Spectrum Green or Spectrum Orange in a nick translation reaction (Vysis). 200 ng of labeled probe was then precipitated with a 10-fold excess of mouse Cot-1 DNA (Gibco), resuspended in 10 µl of a solution containing 50% formamide (Roth), 2X SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0), and 10% dextran sulfate (Roth). The probe was diluted 1:10 in DenHyb solution (Insitus Biotechnologies), denatured for 5 minutes at 72 °C, and preannealed at 37°C for 30 minutes. Metaphase spreads were prepared from a colchicine-treated mouse ES cell line containing the Ts65Dn chromosome by previously described methods (Davisson and Akeson, 1987). Slides were prepared as described by Moore *et al.* (Moore *et al.*, 1999) and hybridized with the pre-annealed probe at 37°C overnight. Following hybridization, the slides were washed with 2X SSC at 72°C for 5 minutes, followed by a 1-minute rinse in 2X SSC at room temperature. Chromosomes were counterstained with DAPI II (Vysis) and viewed with a Zeiss Axioskop using a SenSys CCD camera (Photometrics) and Quips Smart Capture imaging software (Applied Imaging).

3.3 Hybridization on cDNA Arrays

Arrays are devices (herein nylon membranes) containing thousands of DNA sequences stuck on at different positions (addresses). Hybridization to complex mixtures of labelled DNA molecules, prepared from cellular RNA, shows the relative expression levels of thousands of genes. This can be used to compare gene expression levels within a sample or look at differences in the expression of specific genes across different samples. A schematic representation of the hybridization workflow used herein is depicted in Appendix (Fig. A-2, p.142).

3.3.1 Clone Selection and Spotting

cDNA clones for mmu21 genes were selected by *in silico* searches in public EST databases or obtained by direct cloning of RT-PCR products (Gitton *et al.*,

2002). 161 EST clones, representing mouse orthologs for 136 genes of human Chr21, have been collected (see Appendix Table 7-1, p.143). cDNAs were PCR-amplified and spotted in duplicate at high-density on nylon filters at the 'Deutsches Ressourcenzentrum für Genomforschung' (RZPD, Berlin, Germany) according to their standard protocols.

An additional 384 Unigene mouse cDNA clones were spotted in duplicate and used for normalization purposes in the analysis, representing a large set of non-triplicated genes. Mapping information was available for 307 Unigene clones showing a homogeneous distribution across the 19 autosomes and the X chromosome: MMU1: 21 clones, MMU2: 24 clones, MMU3: 17 clones, MMU4: 27 clones, MMU5: 18 clones, MMU6: 14 clones, MMU7: 28 clones, MMU8: 15 clones, MMU9: 14 clones, MMU10: 13 clones, MMU11: 23 clones, MMU12: 12 clones, MMU13: 14 clones, MMU14: 10 clones, MMU15: 9 clones, MMU16: 7 clones, MMU17: 17 clones, MMU18: 10 clones, MMU19: 8 clones, MMUX: 6 clones. Accession numbers are provided in Appendix (Table 7-2, p.145).

3.3.2 Probe Preparation

First strand cDNA probes were prepared from 10 µg DNA-free total RNA. RNA was annealed with 21 µM oligo $p(dT)_{15}$ for 10 min at 70°C and chilled on ice. Reverse transcription was carried out in 50 µl of a solution containing [50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 80 units RNAse inhibitor, 0.54 mM dATP, dTTP, dGTP, 0.32 µM dCTP, 0.26 µM alpha-^{33}P-dCTP] with 400 units of Superscript II at 42°C for 1h30. The reaction was stopped by heating to 70°C for 15 min and RNA was degraded in [300 mM NaOH, 0.33% SDS, 17.4 mM EDTA] at 68°C for 30 min followed by a neutralization step in [Tris-HCl pH 8.0 236 mM; HCl 135 mM].

At this step 1 µl of the probe was taken for counting the specific radioactivity of the sample before purification (count A). The probe was purified through a G50 column (Amersham) according to the manufacturers instructions. The volume of the purified probe was evaluated and 1 µl was retained for the radioactivity count of the clean sample (count B). 1/100 of the probe was also taken to check the labeled cDNA quality on an alkaline gel electrophoresis.

For blocking of repetitive sequences, the radiolabeled probe was pre-annealed [99°C for 5 min, 65°C for 20 min] with 17 µg mouse-Cot1 DNA and 11 µg $p(dA)_{40\text{-}60}$ in [0.1% SDS; 5X SSC].

3.3.3 Radioactivity Incorporation

1µl of the probe before purification (count A) and 1µl of the probe after purification (count b) was taken out for measuring the incorporation rate of α-^{33}P-dCTP. Each aliquot was added to ~2.5 ml of measuring solution (liquid scintillation cocktail for aqueous samples) in small measuring vials. The radioactivity was counted in a scintillation liquid counter (1209 Rackbeta, Perkin Elmer). The incorporation rate was given by: I= incorporation rate of α-^{33}P-dCTP= SB/SA*100, where

SA = Specific activity of the 26.10^{-12} mol of α-^{33}P-dCTP = (count A cpm) * 1 µl / (volume of the sample before purification [µl])

SB = Specific activity of the labeled cDNA = (count B cpm) * 1 µl / (volume eluted after G50 column [µl])

The incorporation rate gives a rough estimation of the reverse transcription efficiency and the quality of the cDNA produced, as a direct relation between the incorporation rate and the cDNA quality assessed on an alkaline gel was observed.

3.3.4 Alkaline Gel for cDNA Quality Assessment

In order to check the quality of the labeled cDNA, an aliquot for each sample was loaded on an alkaline gel [1% agarose, 50 mM NaOH, 1 mM EDTA]. The electrophoresis buffer was composed of 50 mM NaOH and 1 mM EDTA. Before loading the sample [1/100 of the sample + 50 mM EDTA] was denatured 5 minutes at 94°C, chilled on ice and supplemented with 1X loading buffer. Labelled λ HindIII marker served as a size standard. The electrophoresis was performed at 13 Volts.hour/cm. The gel was then incubated in 7% TCA at room temperature, dried in a vacuum, exposed over night to a BAS2325 screen, and finally scanned with a BioImage Analyser (BAS1800, Fujifilm).

3.3.5 Labeling of λ HindIII Marker

A 20 µl reaction [1 µg of λ HindIII ladder, 0.5 µl Klenow enzyme, 1X Klenow Buffer, 10 µCi α-^{32}P-dATP] was incubated 15 minutes at room temperature, then stopped with 1µl EDTA and purified on a G50 column. The radioactivity count was usually about 8000 cpm/µl. The amount loaded on the gel depended on the "freshness" of the labeled marker, ranging from 0.2 µl to 2 µl. The half-life of α-^{32}P is ~14,3 days.

3.3.6 Hybridization

Filters were pre-hybridized at 65°C in 20 ml Church buffer (0.25 M Sodium Phosphate buffer pH 7.2, 1 mM EDTA, 1% bovine serum albumine, 7% SDS) supplemented with 200 µg salmon sperm DNA and 5 µg yeast tRNA. Probe was added to pre-hybridised filters and hybridization was carried out in Church buffer at 65°C for 15 h. Two identical filters were hybridized simultaneously with each probe.

3.3.7 Washing and Scanning Filters

Filters were washed in [0.1X SSC; 0.1% SDS] at 65°C for 20 min. Filters were exposed 5 days to BAS2325 screens and scanned with a BioImage Analyser (BAS1800, Fujifilm). An example of a scanned filter is shown in Figure 3-1. Hybridizations were performed 3 times for all tissues except for cerebellum and heart (2 times) and skeletal muscle (1 time) because of the limited amount of RNA available.

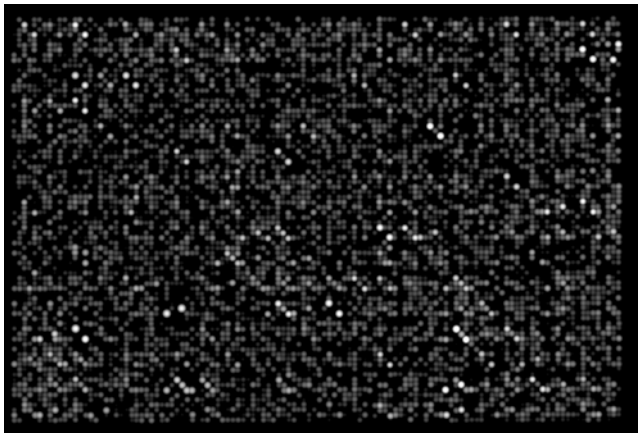

Figure 3-1: Nylon array hybridized with a radioactive probe.

3.4 Array Data Analysis

Data analysis incorporated the following steps: Image analysis, data normalization, judging gene expression, judging differential gene expression between Ts65Dn and control samples, clustering of expression profiles.

3.4.1 Image Analysis

Image analysis was carried out by a semi-automated procedure. A grid was placed manually (Visual grid software, GPC Biotech) on each filter in order to determine the centre of each spot. Afterwards, the pixel intensities were quantified within a pre-defined area around the centre using a Gaussian spot shape (Steinfath *et al.*, 2001).

3.4.2 Data Normalization

For each of the nine tissues under analysis the whole batch of experimental replicates with Ts65Dn and control samples was normalized simultaneously. Normalization should eliminate multiplicative technical bias between the different experiments. Therefore a three-step procedure was implemented:

-In the first step *local background* was subtracted for all spots in each experiment.

-In the second step a *reference experiment* was selected from the batch. In order to treat all experiments equally, the average value was computed for each cDNA across the batch as a virtual signal and defined the set of all virtual signals as the reference experiment. The median value of this reference experiment derived from the sample of 384 non-triplicated mouse Unigene cDNAs was used as the reference median.

-In the third step, for each single experiment a multiplicative factor was calculated and normalization was performed according to this factor: Let x_{ij} be the signal of the ith cDNA in the jth experiment. Let med_{ref} be the reference median and let med_j be the median value of the non-triplicated mouse Unigene cDNAs in the jth experiment, then the signal x_{ij} was replaced by $x_{ij} * med_{ref}/med_j$.

3.4.3 Judging Gene Expression

In order to measure whether a given gene was significantly expressed in a given tissue, its cDNA's signal was compared to a signal distribution derived from negative controls. In our array design, we distributed approx. 3,840 empty spot positions on the array. After quantification a small, non-zero intensity is assigned to each of these empty spots reflecting the amount of background signal on the array. Since these positions are spread uniformly over the array, the distribution of these signals reflects the distribution for signal noise and is an indicator whether signals are at the background level or reflect reliable expression levels. For each cDNA the relative proportion of empty positions on the array smaller than the actual observed intensity (background-tag) was counted. Background-tags from replicated experiments for the same cDNA were averaged. Thus, high values (close to one) indicate that the cDNA is expressed in the tissue tested whereas low values reflect noise. cDNAs were considered "expressed" when their average background tag was above 0.9, a threshold consistent with the limit of visual detection of the spots.

3.4.4 Judging Differential Expression in Ts65Dn and Control Samples

For each cDNA statistical tests were performed, based on the replicate signals in experiments with Ts65Dn and control samples. Three standard tests were used in parallel, Student's T-test, Welch test and Wilcoxon's rank-sum test. To evaluate differential expression of the genes, p-values of Wilcoxon's rank-sum test were preferably used as a reference since this distribution-free test does not require a specific parametric signal assumption such as Gaussian distribution of the two samples like Student's t-test and Welch test. Furthermore, the Wilcoxon test statistic is very robust against outlier values since it is based on the ranks of the signals rather than on the signals themselves. A recursive function was implemented for the calculation of the exact p-values of this test. Fold changes for trisomic versus controls were considered significant when the p-value was <0.05.

Ratios between mean intensities of Ts65Dn and control spots from replicated experiments were averaged, and the statistical error of this mean was calculated to fall within the bounds $\frac{\overline{X}}{\overline{Y}} \pm \frac{1}{\overline{Y}^2}\sqrt{\overline{X}^2 S_y^2 + \overline{Y}^2 S_x^2}$, where $\overline{X}$ and $\overline{Y}$ were the mean

ratios of each replicated experiment, and S_x and S_y were the respective standard errors of the means (SEM) calculated as $S_x = \sqrt{\frac{1}{(N-1)N}\sum_{i=1}^{N}(X_i - \overline{X})^2}$ and $S_y = \sqrt{\frac{1}{(M-1)M}\sum_{i=1}^{M}(Y_i - \overline{Y})^2}$.

To calculate the reproducibility of a signal across a number of replicated experiments, a coefficient of variation (CV) was calculated for each clone, i.e. the ratio of the mean, m, and the standard deviation, s, of these repeated signals. The CV measures the proportion of error related to the signal. If this ratio is close to 0, the reproducibility is nearly perfect, if it is close to 1 or higher, the reproducibility is poor.

3.4.5 Clustering of Expression Profiles accross Tissues

For each clone, the logarithm (base 2) of the ratio between the intensity in the specific tissue and the average of intensities of this clone across all tissues was calculated. Thus, spot intensities below the average of intensities across all tissues are represented by a color gradient spanning from light to dark green. Conversely, spot intensities above the average of intensities across all tissues are represented by a color gradient spanning from dark to light red. The tool J-Express (www.molmine.com) was used to construct dendrograms with clustering parameters according to the average-linkage hierarchical clustering method with Pearson correlation as a pair wise similarity measure. In order to judge whether a given cluster has numerical evidence a re-sampling procedure was used: A numerical quality value was computed for the selected group (here: average correlation of the group members with the group center), then N (N = 1,000,000) random groups of data points of the same size were selected and the number of quality values, K, higher than or equal to the observed quality value was denoted. The p value for the selected group was computed as the ratio K/N.

3.5 Quantitative Real Time PCR

Real Time PCR allows monitoring the progress of the PCR as it occurs (i.e., in Real Time). Thus it represents a powerful tool for the detection and quantification of mRNA. Data is therefore collected throughout the PCR progress, rather than at the end. In other words, in Real Time PCR, reactions are characterized by the point in time during cycling when amplification of a target is first detected rather than the amount of target accumulated after a fixed number of cycles. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

Two types of chemistries were used herein to detect PCR products:

>SYBR Green I Dye Chemistry: the SYBR Green I dye chemistry uses SYBR Green I dye, a highly specific, double stranded DNA binding dye, to detect PCR product as it accumulates during PCR cycles.

>TaqMan Chemistry: the TaqMan chemistry uses a fluorogenic-labeled probe and the 5´nuclease activity of Taq DNA polymerase to enable the detection of a specific product as it accumulates during PCR cycle. The TaqMan probe contains a reporter dye (FAM) at its 5' end, and a quencher dye (TAMRA) at its 3' end.

The most important difference between the TaqMan and SYBR Green I dye chemistries is that the second one will detect all double-stranded DNA, including non-specific reaction products. A well-optimized reaction is therefore essential for accurate results. The principle of both chemistries is recapitulated in Appendix (Figure 7-3, p.151).

3.5.1 Design of Primers for SybrGreen Assays

To validate the array data by an independent method, 39 genes (29 triplicated and 10 duplicated) have been analyzed by quantitative Real Time PCR using SybrGreen chemistry. For each gene tested by SybrGreen qPCR, a primer pair was designed using the Primer Express software (PE Applied Biosystems) with one of the primers spanning an intron-exon boundary. Sizes of amplicons ranged from 52 to 111 bp. Primer sequences are provided in Appendix (Table 7-3, p.152).

3.5.2 Selection of TaqMan Assays

To study the gene expression variation in single mice, 50 genes (33 triplicated and 17 duplicated) have been analyzed by quantitative Real Time PCR using TaqMan chemistry. TaqMan® Gene Expression Assays for each gene were selected via Applied Biosystems web page (http://www.appliedbiosystems.com/). These Assays-on-Demand (AoD) were provided as a pre-formulated Assay (20x mix) containing:

- 2 unlabeled PCR primers (900nM each final concentration)
- 1 FAM™ dye-labeled TaqMan® MGB probe (250nM final concentration)

TaqMan® Gene Expression Assays use universal cycling conditions, which eliminate the need to optimize conditions individually. All assays met the amplification efficiency criteria of 100%±10% (ABI, ApplicationNote 127AP05-02) and were comparable to each other. A list of all used assays is in Appendix (Table 7-4, p.153).

3.5.3 Sample Preparation

Reverse transcription was performed in 20 µl reactions: 1 µg of DNase-treated total RNA, 0.5 mM each dATP, dCTP, dTTP and dGTP, and 250 ng Random Hexamers (Roche) were heated at 65°C for 5 min and quickly chilled on ice. The reaction was then supplemented with 1X first-strand buffer [50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$], 0.01 M DTT, 200 U SuperScript II reverse transcriptase (Invitrogen) and 40 U recombinant RNasin Ribonuclease Inhibitor (Promega), incubated at 25°C for 10 min, and then at 42°C for 1 h, followed by heating at 70°C for 15 min. Generated cDNAs were tested by PCR for integrity and absence of genomic DNA contamination. For the integrity check we used gene specific primers (Atp5a seq) for the mitochondrial ATPase coupling factor 5 subunit (*Atp5a*) gene (Appendix Table 7-3, p.152). The absence of genomic DNA was tested with intron specific primers (Hprt (intronic) for the hypoxanthine guanine phosphoribosyl transferase (*Hprt*) gene (Appendix Table 7-3, p.152).

3.5.4 Quantitative RT-PCR Using SybrGreen

Real Time PCR reactions were performed using the ABI Prism 7900HT Sequence Detection System with 25µl reaction composed of 1/40 volume of the cDNAs, 300 nM primers and 1X SYBR Green PCR Master Mix, containing SYBR Green I Dye, AmpliTaq Gold DNA Polymerase, dNTPs with dUTP, passive reference and optimized buffer components (Applied Biosystems). Cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles at 94°C for 15 s and 60°C for 1 min. A final melting curve[17] was recorded by cooling the reaction mixture to 60 °C for 15 s and then slowly heating the sample to 95 °C with a ramp rate of 0.2 °C/s. Fluorescence was measured continuously during the slow temperature ramp to monitor the dissociation of the SYBR Green. Specificity of PCR amplification was verified by analysis of the melting curve and subsequent electrophoresis on 4% NuSieve:agarose (3:1) gel. Negative controls produced negligible signal detection (38-40 Ct). Three identical reactions were run on 96-well plates for each gene, and each plate was duplicated. Data were normalized using a geometric mean of three housekeeping genes present in each PCR plate: hypoxanthine guanine phosphoribosyl transferase *(Hprt)*, phosphomannomutase 2 (*Pmm2*) and hydroxymethylbilane synthase (*Hmbs*).

3.5.5 Validation Experiment

The following experiment was performed to validate the use of the comparative Ct calculation method (see Chapter 2.5.7). The amount of target, normalized to an endogenous reference and relative to a calibrator, is given by $2^{-\Delta\Delta Ct}$, where $\Delta\Delta$Ct is the difference in ΔCt for Ts65Dn and control sample and ΔCt is the difference in threshold cycles for target and reference.

The $\Delta\Delta$Ct validation required approx. equal efficiencies of target and reference amplification. Therefore, standard curve assays were obtained for all

[17] To prove that only the desired PCR product has been amplified, a melting curve analysis should be performed after PCR. In melting curve analysis the reaction mixture is slowly heated to 95°C, which causes melting of double-stranded DNA and a corresponding decrease of SYBR Green I fluorescence. The instrument continuously monitors this fluorescence decrease and displays it as melting peaks. Each melting peak represents the characteristic melting temperature of a particular DNA product (where the DNA is 50% double-stranded and 50% single-stranded). If PCR generated only one amplicon, melting curve analysis will show only one melting peak. If primer-dimers or other non-specific products are present, they will be shown as additional melting peaks.

target genes and references by measuring the transcripts levels obtained with specific primer sets on adult mouse brain cDNA sample diluted at two fold intervals. For each dilution, transcript levels were plotted against the Log value of the input cDNA concentration. qPCR efficiencies (*E*) were calculated from the slopes, where one cycle in the exponential phase yielded the efficiency: $E=10^{[-1/slope]}-1$. Amplification of transcripts analyzed here showed efficiency values close to 1 with a high linearity (Pearson correlation coefficient > 0.98).

3.5.6 Quantitative RT-PCR Using TaqMan Probes

Real Time PCR reactions were performed using the ABI Prism 7900HT Sequence Detection System with 25µl reaction composed of 1/40 volume of the cDNAs, 300 nM of each primers and probe and 1X TaqMan Master Mix, containing AmpliTaq Gold® DNA Polymerase, AmpErase® UNG, dNTPs with dUTP, Passive Reference 1, and optimized buffer components (Applied Biosystems). Cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 94°C for 15 s and 60°C for 1 min. We verified that no correlation could be found between threshold cycle (Ct) and expression ratio (Ts/Eu) indicating that there was no systematic biases within our Real Time PCR results. Nonetheless, It should be noticed that when the Ct value increases above ~32, the Standard error also increases, indicating a loss of precision of the replicate measurements. For normalization purposes 18 non-mmu21 control genes were tested on samples cDNA. We identified the most stable genes across samples using the geNorm method (Steinfath *et al.*, 2001). Thus two genes (*Hprt* and *Hmbs*) were selected and data were normalized to their geometric mean. All assays were performed in triplicates. To minimize intra assay variation, sample's cDNA was premixed with the PCR mastermix and distributed equally into each reaction. For a given target gene all tissue-samples were run on the same reaction plate. This increases the accuracy of inter individual comparison, as the mRNA of interest is amplified under the same PCR conditions in all tissue-samples. To validate the reproducibility of our system, one experiment including two cDNA samples was duplicated. The correlation between the two independent experiments was over 99%.

3.5.7 Analysis of Quantitative Real Time PCR

Amplification plots and predicted threshold cycle (Ct) values (fractional cycle number at which the amount of amplified target reaches a fixed threshold) from the exponential phase of PCR were obtained with the Sequence Dectection Software (SDS version 2.0, PE Applied Biosystems). Further calculation and graphics were carried out in Excel 2000. A common threshold value was chosen for all genes and the baseline was set manually for individual genes.

The relative expression calculation method relies in the principle on the comparative Ct method (User Bulletin #2, P/N 4303859B, www.appliedbiosystems.com). Ct values were first normalized to a geometric mean of the two normalization genes ($\Delta Ct=Ct_{target}-Ct_{reference}$) and converted to a relative expression quantity (NE) by the formula $NE=100*2^{-\Delta Ct}$. The NE standard deviation was calculated according to following formula: $\sigma_{NE} = NE * Ln2 * \sqrt{\sigma_{Ct_{target}}^{2} + \sigma_{Ct_{reference}}^{2}}$

For electronic pool calculation the NE value for each individual of a given group (Ts65Dn or euploid) were averaged.

The Ts/Eu ratio (R) was obtained from the quotient of NE values. The standard deviation of the ratio is given by:

$$\sigma_R = \sqrt{\left(\frac{\sigma_{NE_{Ts65Dn}}}{NE_{Euploid}}\right)^2 + \left(\frac{NE_{Ts65Dn} * \sigma_{NE_{Euploid}}}{NE_{Euploid}^{2}}\right)^2}$$

An example of the calculation method is given in Appendix (Figure 2-1, p.155)

To calculate the coefficient of variation of a group following formula was used:

$CV = \frac{\sigma}{\overline{NE}} \times 10$, where $\overline{NE}$ is the mean expression over the four samples and σ is the standard deviation of the mean and is given by the formula;

$\sigma = \sqrt{\frac{n\sum x^2 - (\sum x)^2}{n(n-1)}}$, where n is the number of observations and x the values to average (here NE).

The Technical Variance (TechVar) and the Biological Variance (BioVar) were calculated according to following formulas:

$$TechVar = \sum_{i=1}^{I} \sum_{j=1}^{J} (NE_{ij} - \overline{NE}_{i}.)^2$$

$$BioVar = J \sum_{i=1}^{I} (\overline{NE}_{i.} - \overline{NE})^2$$

where

i= the sample number (four per group).

j= the technical replicate number (three per experiment).

NE_{ij} = NE of each experiement.

$\overline{NE}_{i.}$ = Mean NE over replicates.

$\overline{NE}$ = Mean NE over the samples of a group (euploid or Ts65Dn)

The total Variance (TotVar) is defined by the formulas:

$$TotVar = \sum_{i=1}^{I} \sum_{j=1}^{J} (NE_{ij} - \overline{NE})^2$$

$$TotVar = TechVar + BioVar$$

3.6 DNA Preparation

3.6.1 Cosmid DNA Isolation

Following cosmids were provided by Alan Coulson (Sanger Institute): C47E12, C05D10, C24H12, C33C12, F28B3, ZC373. Glycerol stocks of 8 single colonies for each clone were made upon arrival. Single colonies of cosmids were then grown in 8 ml LB medium with either 75 µg/ml Ampicilline (for C47E12, C05D10, C24H12, C33C12, ZC373) or 50 µg/ml Kanamycin (for F28B3) depending on the resistance of the clone. DNA was isolated using Qiagen Plasmid Mini Purification kit. Quantity and Quality of DNA was assessed by UV spectrophotometry.

3.6.2 YAC DNA Isolation

Following YACs (Yeast Artificial Chromosomes) were provided by Alan Coulson (Sanger Institute): Y116A8, Y71G12, Y105E8, and Y74C10. Glycerol stocks of 8 single colonies for each clone were made upon arrival. Single colonies of YACs were then grown in 15 ml CSM-URA medium. Total DNA from yeast was then isolated following QIAGEN Genomic DNA extraction kit (yeast section) guidelines. The isolated DNAs were eluted in 70 µl TE buffer. This DNA extraction kit is based on the Anion-Exchange Resin method, that allows isolation of high yields of pure genomic DNA ranging in size from 20-150 kb and avoids the use of toxic and hazardous reagents.

3.6.3 Genomic DNA Isolation

Isolation of *C. elegans* genomic DNA from the wild strain N2 was performed according to Sulton and Hodgkin (Sulston and Hodgkin, 1988). Nematodes were washed off an overgrown plate with 1 ml TE in a 1,5 ml tube (Eppendorf). They were allowed to settle for 5-10 minutes by gravity. The volume was reduced to 100 µl leaving the nematode pellet undisturbed. Worms were washed twice in TE, the volume adjusted to 50 µl and frozen at –80°C for 10 minutes. After thawing at room temperature, 50 µl of 2x lysis buffer was added. After incubation at 65°C for 1 hour, proteinase K was inactivated by incubation at 95°C for 15 minutes. The lysate was spun for 5 minutes at 14000 rpm and the supernatant was transferred into a fresh tube. After addition of 5 µl RNAse (5 mg/ml in H_2O), tubes were incubated for 20 minutes at 37°C. Isolated genomic DNA was stored at –80°C. For subsequent PCRs, generally 1-2 µl/reaction was used.

Lysis Buffer: 20 mM Tris pH 8.3, 100 mM KCl, 10 mM EDTA, 0,9 % Tween, 0,9 % NP-40, 0,2 mg/ml Proteinase K (20 mg/ml stock in ddH_2O).

3.6.4 Plasmid DNA Isolation

A single colony was picked from Ampicillin (Amp) or Kanamycine (Kan) supplemented agar plate and was transferred to 3 ml of LB (0.05 µg/ml Amp or 0.03 µg/ml Kan) in a 15-ml tube. The sample was incubated at 37°C for about 16 hours

under 224 rpm rotation. The bacterial pellets were collected by centrifugation at 5000 rpm for 3 min. The Qiagen Plasmid Mini Kit was used to isolate the plasmid DNA according to the manufacturers instructions. The principle of this isolation is based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to a Anion-Exchange resin under appropriate salt and pH conditions. RNA, proteins, dyes, and low molecular weight impurities are removed by a medium-salt wash. Plasmid DNA is eluted in a high-salt buffer, and then concentrated and desalted by isopropanol precipitation. Finally the isolated plasmid DNA was eluted in 30 µl of nuclease free water and stored at -20°C.

3.6.5 DNA Quantification and Quality Control

DNA was quantified by spectrophotometric analysis (ND1000, Naodrop) following the rule that 1 OD at 260 nm equals 50 µg DNA per ml. The A_{260}/A_{280} ratio between 1.9 and 2.1 is for pure DNA. DNA quality and size were also checked by agarose gel electrophoresis. Normally a 0.8% agarose gel was used for analyzing genomic DNA and plasmids or long PCR products (>6 kb), 1% or 1.2% agarose gel were used to analyze PCR fragments (or DNA fragments obtained by restriction enzyme digestion). 4% agarose gels were used to check small PCR products (<150 bp). 0.5 µg of λHind III, 1 kb, 100 bp or 25bp DNA markers (Fermentas) were loaded in one slot of the gel as size standard. Before loading, 1X of DNA loading buffer was added to the DNA sample. Gels (~10cm) were run at 50 Volts for 30 minutes.

3.6.6 DNA Analysis by Restriction Enzymes Digestion

Digestions by restriction endonucleases were made in 20 µl volume composed of 1X restriction buffer (New England Biolabs (NEB)), 10 U of each enzyme (2 enzymes maximum per reaction sharing a compatible NEB buffer) and 1µg of template DNA. The reaction was incubated at 37°C for 1-2 hours. The resulting bands were separated on 1% low melt nuclease free agarose gels. If necessary, a specific single band was excised from the gel using a clean sharp scalpel and treated following the QIAquick Gel Extraction Kit instructions (Qiagen).

The purified DNA was eluted with 30 µl free water and immediately processed or stored at -20°C.

3.6.7 DNA Sequencing

DNA sequencing was carried out using ABI PRISM™ BigDye Terminator Cycle sequencing ready reaction Kit directly from purified PCR product or plasmid DNA. For each sequence reaction, 2 µl Terminator Ready Reaction Mix (P/N 4337454, Applied Biosystems), 1 µl primer (10 µM), About 300-400 ng of plasmid DNA (10% of size) or 50-100 ng PCR fragments (10 ng per 100 bp), and sterile H_2O were mixed to final reaction volume of 10 µl in 8-tube strips. For sequencing plasmid DNA, the mixture was heated at 96°C for 5 min, followed by 30 cycles at 96°C for 20 sec, 50-60°C (depending on primer) for 10 sec and 60°C for 4 min. For sequencing PCR templates, the reaction was heated at 96°C for 3 min, followed by 25 cycles at 96°C for 10 sec, 50-60°C (depending on primer) for 5 sec and 60°C for 4 min. The sequence reaction was then precipitated using 25 µl 100% ethanol and mixed by vortexing. The sample was incubated at room temperature for 10 min and then centrifuged at room temperature at 2700 g for 60 min, ethanol solution was removed and the sample was washed twice with 200 µl 70% ethanol. The pellet was dried for 2 to 5 min and then stored at -20°C. Sequences were read on ABI 377 automatic sequencers.

3.7 PCR and Cleanup

3.7.1 PCR Protocols

Standard PCR: A standard PCR protocol was used for amplification of specific fragments from cDNA, and plasmid DNA. The 50 µl reaction was composed of 1X PCR buffer (Roche), 800 mM $MgCl_2$ (Promega), 800 µM dNTP (200 µM each), 250 nM of each primer, 3 Units of Taq polymerase (Promega or in house) and 40 to 200 ng template DNA. The PCR reaction was carried out using a standard PCR program.

Long range PCR: For amplification of long DNA fragments for, two systems were used: the eLONGASE PCR sytem (Invitrogen) and the TaqPlus Precision system (Stratagene). Reactions were set-up according to the manufacturers instructions. The reaction mix was similar to a the standard PCR described above except that the enzymes and buffer used were those supplied by the system. When a fragment was difficult to amplify, the reaction was supplemented with 5% DMSO.

Colony PCR: The colony PCR protocol was used to amplify products from bacterial colonies on agar plates. Single colonies were re-suspended in 20 µl of sterile H_2O, filled up with the amplification mixture (same mixture and conditions as for the standard PCR).

3.7.2 PCR Programs

All PCRs were run on a PTC200 machine (MJ Research).

Standard PCR: 94°C for 2 min; 30 cycles [94°C for 30 sec (denaturation), 50-60°C for 0.5-2 min (annealing); 72°C for 1 min 30 (extension)], 5 min at 72 °C (final extension).

Long range PCR: 94°C for 1-2 min; 30 cycles [94°C for 1 min , 45-60°C for 1 min; 72°C for 1 min per kb of target], 10 min at 72 °C.

Colony PCR: The cycling parameters were the same as the Standard PCR except that the initial denaturation step was performed at 95°C for 10 min.

3.7.3 PCR Product Cleanup

PCR products ranging from 100 bp to 10 kb were purified from primers, nucleotides, polymerases and salts using QIAquick PCR purification kit according to the manufacturer's instructions. In order to purify single band from more than one-band PCR products, PCR products were separated on 1% low melt agarose gels. The band of interest was excised from the gel using a clean sharp scalpel and treated following the QIAquick Gel Extraction Kit instructions. The purified DNA was eluted with 30 µl free water and stored at -20°C.

3.8 *C. elegans* Nomenclature

C. elegans nomenclature was originally described by Horvitz (Horvitz *et al.*, 1979) and is summarized at: http://biosci.umn.edu/CGC/Nomenclature/nomeguid.htm.

Gene names are in italics, and consist of three letters, a hyphen and an Arabic number, e.g. *rrf-3*. Mutation names follow the gene name; they consist of one or two letter and an Arabic number, and are also in italic, e.g. *rrf-3(pk1426)*. The letters identify the isolating laboratory. The wild-type allele of a gene is defined as that present in the Bristol N2 strain. Wild-type alleles can be designated by a plus sign in parenthesis immediately after the gene, for instance *rrf-3*(+).

Strains are given non-italicised names consisting of two uppercase letters followed by a number, e.g. CB4856 for the Hawaiian wild type strain. The strain letter prefixes refer to the laboratory of origin and are distinct from the mutation letter prefixes.

Phenotypes are indicated by a non-italicised three letter abbreviation, which mostly corresponds to the gene name; the first letter is capitalised, e.g. *Unc*, for Uncoordinated. The protein product is identical to the gene name, but is written in non-italic capitals, e.g. RRF-3.Gene fusions incorporated in transgenes that consist of a *C. elegans* gene fused to a reporter such as GFP are indicated by the gene name followed by two colons and the reporter, all italicised, e.g. *daf-9::GFP*.

3.9 Nematode Strains

Wild type strain *(N2):* This is the wild-type Bristol strain, originally isolated from an English mushroom farm in the 1950's. Almost all *C. elegans* is based on derivatives of this original strain.

VH525 strain: *[nre-1(hd20); hdIs16(glr-1::YFP, unc-47::YFP,unc-129::YFP, rol-6(su1006))]*. This strain was use for RNAi experiments as nre-1 is a mutation that causes neurally expressed genes to be more sensitive to RNAi.

rrf-3(pk1426) strain: This strain was use for RNAi experiments. The mutation in rrf-3 gene results in a loss of function of a putative RNA-directed RNA polymerase (RdRP) of *C. elegans*, RRF-3, causing a substantial enhancement of

sensitivity to RNAi in diverse tissues (Simmer *et al.*, 2002). This is particularly striking in the nervous system; neurons that are generally refractory to RNAi in a wild type genetic background can respond effectively to interference in an *rrf-3* mutant background.

lin-15(n765) *strain:* This strain carries a chromosomal *lin-15*(*n765*) mutation, which leads to a multivulva (*Muv*) phenotype that can only be scored in adults. These worms are often used for microinjections, as *lin-15* is the most commonly used rescuing marker. *n765* is a temperature sensitive allele, the *lin-15(n765)* animals are raised at 15°C for injection. After microinjection the worms are raised at 20°C.

3.10 Culture Conditions

Nematode strains were cultured on NGM agar plates seeded with OP50 , a uracil-requiring strain of *E. coli* as a food source, as described by Brenner and Sulston (Brenner, 1974; Sulston and Hodgkin, 1988). NGM plates are seeded with OP50, which were grown at 37°C for 1 day, to create a bacterial lawn. Worms can be added to the lawn and cultivated. In a few days, the worms will eat up all the food and begin to starve. A starved stock plate can be kept for 8 weeks or more at 15°C, without loosing viability in the worm population (dauer stage). The main limitation is dessication: the agar medium eventually dries out completely and the worm dies. Sealing the plate with parafilm will delay this. Worms were cultured at 20 °C unless indicated otherwise. A List of nematode strains used and generated in this work can be found in (Appendix Table 7-5, p.156)

Preparation of NGM-agar: 6 g NaCl, 5 g Peptone, 33,7 g Agar (Difco), 1,925 l dH_2O, 50 ml of 1 M KPO_4, 2 ml of 5 mg/ml Cholesterol in 95% EtOH. After 40 minutes autoclaving the medium is supplemented with: 4 ml of sterile $CaCl_2$ (0.5 M) and 2 ml of sterile $MgSO_4$ (1 M).

$CaCl_2$ (1 M): 29.4 g $CaCl_2$ dehydrate, 200 ml dH_20.

$MgSO_4$ (1 M): 49.29 g $MgSO_4$ Heptahydrate, 200 ml dH_2O.

3.11 Freezing Worms

C. elegans can be stored at -80°C or in liquid nitrogen. They remain viable indefinitely in this stage. Thus, the strains are cleaned up by alkaline hypochlorite if necessary. The clean strain is cloned onto 3* 6 cm agar plates. Worms are grown until food is gone and they have just starved out. They should be let to starve for one day until eggs hatch to L1, but before dauer larvae have formed (important!). Then 1ml of sterile M9 is pipette onto each plate. An equal volume of sterile freezing solution (with $MgS0_4$ added) is added. Pipette up and down several times to mix and wash worms. The washed worms are dispensed in 0.5 ml each into 7 sterile, labeled cryovials. The vials are put in styrofoam rack and covered for slow freezing in the –80°C freezer (1 degree/minute). The next day, 3 tubes are placed in –80°C freezer box, 3 tubes in liquid nitrogen, and 1 tube is thwan for testing on 6 cm plate.

Freezing solution: For 1 liter, 6 g NaCl, 50 ml Potassium phosphate buffer 1M pH6, 200 ml Glycerol, qsp 1 liter with water. Autoclave. Supplement with 3 ml of $MgSO_4$ 0.1M.

Potassium buffer 1M, pH6: 120 g KH_2PO_4, 21 g K_2HPO_4, qsp 1liter with dH_2O. Autoclave.

3.12 Cloning

All plasmids made for this study are listed in Appendix (Table 7-6, p.156). This table gives an overview of the final plasmid size, primers, restriction sites, PCR templates and inserts size obtained during the different steps. Two types of construct were generated, plasmids for gene expression localization in *C. elegans* (GFP fusion), and plasmids for RNAi feeding experiments. The cloning strategies are described in the Results chapter. All primers sequences used for the generation of GFP fusion constructs are listed in Appendix (Table 7-7, p.157) and the primers used to generate construct for RNAi feeding experiments are listed in Appendix (Table 7-8, p.158).

Following reagents were used:

Restriction enzymes: New England Biolabs (NEB).

Polymerases: Taq Plus Precision (Stratagene), eLONGASE (Invitrogen), HouseTaq (MPIMG).

Cloning: TOPO XL PCR cloning kit (Invitrogen), Rapid DNA ligation kit (Roche), Shrimp alkaline phosphatase (USB), QIAquick PCR purification kit (QIAGEN), QIAquick Gel extraction kit (QIAGEN).

Transformation: For transformation the Invitrogen TOP10 electro-competent cells [F-*mcr*A∆(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*X74*rec*A1*ara*D139 ∆(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R)*end*A1 *nup*G] were used.

For the RNAi feeding experiment the plasmids were transformed into HT115(DE3) RNase III-deficient *E. coli* strain by chemocompetence. The HT115 genotype is: F-*mcr*A, *mcr*B, IN(*rrn*D-*rrn*E)*1, λ̃, rnc*14*::Tn*10 (DE3 lysogen: lacUV5 promoter-T7 polymerase). The RNase III gene is disrupted by a Tn10 transposon carrying a tetracycline-resistance marker.

3.13 Preparation of Chemically Competent Cells

E. coli HT115 cells were cultured overnight in 4 ml LB medium. 400 ml LB medium were inoculated with the starter culture and grown at 37°C until a OD_{600} of 0.5 was reached. The cells were then centrifuged at 3,000g for 10 min at 4°C and the pellet was resuspended in 8 ml TSS. 0.8 ml of 87% glycerol was added, 410 µl aliquots were prepared and stored at -80°C.

TSS: 85% LB-broth, 10% (w/v) PEG-8000, 5% DMSO, 50 mM MgCl2

LB medium: for 1 liter, 10 g Bacto-Tryptone, 5 g Yeast extract, 5 g NaCl, 1ml NaOH 1M, qsp 1 liter with dH2O. Autoclave.

3.14 Preparation of *C. elegans* Total RNA

Worms were grown on 10 cm NGM-agar plates until close before starvation (this gives ~0.5 ml packed worms, 0.5 mg total RNA). Worms were harvested in M9 medium, and washed 3 times in M9 (by centrifugation at 2,000 rpm for 5 seconds in 15 ml tubes). The supernatant was removed and 1 ml quartz sand (washed with HCl and water, baked at 400°C overnight) and 3 ml Tri reagent

(phenol/isothiocyanate; MRC) were added and vortexed for 10 minutes. After 5 minutes at room temperature (RT), 0.6 ml chloroform was added, vortexed and left at RT for additional 15 min. The mixture was spun down for 15 seconds at 4°C and maximum speed. The aqueous phase was transferred to a fresh tube and supplemented with 1.5 ml isopropanol. After 10 min at RT, and a brief spin (10 sec, 4°C, max speed), the pellet was washed with 3 ml 75% EtOH, vortexed and spun down for 10 sec. The RNA pellet was air dried and dissolved in 100 µl nuclease free water.

3.15 Glycerol Stocks

Glycerol stocks of generated bacterial clones were prepared by picking single colonies from their selective LB agar plates and grown in 100 µl LB medium (supplemented with the adequate antibiotic) for 4-5 hours at 37°C. Sterile glycerol was then added to the mini-culture to 25 % final concentration. After snap-freezing the glycerol stocks were kept at –80°C.

LB agar plate: for 1 liter, 10 g Bacto-Tryptone, 5 g Yeast extract, 5 g NaCl, 15 g Agar, 1ml NaOH 1M, qsp 1 liter with dH2O. Autoclave. Swirl, let cool down and pour plates.

3.16 Microinjections

To make transgenic *C. elegans* we used the microinjection technique as described by Mello and colleagues (Mello *et al.*, 1991). Briefly, the DNA construct (plasmid) with the gene of interest was mixed with a co-injection marker and injected into the distal syncytial gonad (Figure 3-2). The injected DNA is taken up into the mature oocyte's nucleus. The DNA exists as an extra-chromosomal array (i.e. not integrated in the chromosome), which segregates randomly and can be lost, that is why a marker is needed to follow which animals have the array. F1 progeny that shows the co-injection marker phenotype are picked and transgenic lines are established by keeping those animals that segregate the array in their F2 generation. Usually 1 in 10 F1 progeny with the array will gives a transgenic line. All GFP constructs (in vector L3781) were injected at a concentration of 10 ng/µl

together with the *lin-15*(+) [75 ng/µl] marker (Clark *et al.*, 1994) into *lin-15(n765)* animals. A list of all strains obtained by injections is found in the Appendix Table 7-5 (p.156).

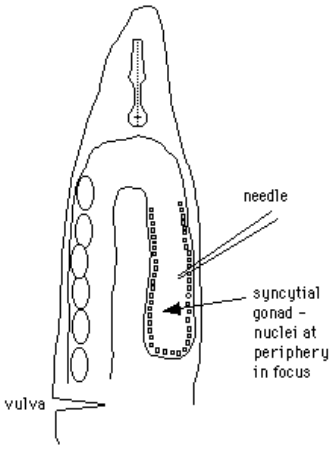

Figure 3-2: Microinjection into the Hermaphrodite Syncytial Gonad

In summary, DNA was injected at 10 ng/µl final concentration, along with the marker DNA (final concentration, 90 ng/µl) in the *lin-15* mutants. For microinjection needles were pulled using capillaries (Harvard GC100TF-15), at a heat setting of 30/64.4 (Narshige PC-10). The needle quality was checked under binocular. 10 µl of injection cocktail (DNA, Marker, qsp 10 µl with M9 buffer) was prepared and spun for 5 sec to remove particules. Needles were loaded with a small amount of injection cocktail avoiding air bubbles. The loaded needle was placed in the microinjection apparatus at 10° angle. One day old adults were placed on plate without food and let run for ~5 min before loading. 50 µl of paraffin oil was placed on dried 2% agarose pads. Worms (~5) were placed onto the pad. Once the focus was made with the 40x objective, the needle was broken gently on the agar surface. The needle was then inserted into gonad syncitium at 30° angle and the mixture injected until it fill like a ballon. Once injected the worms were covered with 25 µl M9 supplemented with 20 mM glucose for 5-10 min and then transferred to OP50 plate and left at 20°C. F1 were scored after 3 days and non multivulva adult worms were further cloned. F2 lines were than analyzed.

3.17 Microscopy

Nematodes were scored by Leica MZ8 dissection microscope. Slides were prepared as described by Sulston and Hodgkin (Sulston and Hodgkin, 1988). A drop of 1 mM levamisole in M9 medium was placed on an agar pad and the nematodes to score were picked into the drop. Levamisole is used to anaesthetize the animals. For GFP analysis, nematodes were scored by Nomarsky DIC microscopy using a Zeiss Axioscope 2 plus microscope. Photographs were taken with a Sony progressive 3 CCD camera and with a Hamamatsu orca ER digital CDD camera. Adobe PhotoShop was used to adjust brightness and contrast.

M9 medium (for 1 liter): 3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml MgSO4 1 M qsp 1liter with dH_2O.

5% Agar for microscope slides (for agar pad): 12.5 g agar, 250 ml dH_2O. Swirl and pour in 3 ml/ glass test tubes.

3.18 RNAi

Nematodes were fed with *E. coli* strains expressing siRNAs directed against selected target mRNAs. RNAi constructs for 7 of the 9 candidate genes (C33C12.9, Y74C10AL.2, ZC373.1, Y116A8C.36, C24H12.5, F28B3.1, and C47E12.7) were obtained by PCR amplfication of the target sequence and cloning of the purified PCR fragment in a vector containing RNA polymerase promoters (L4440, See Fire Lab Vector Kit Documentation at http://www.addgene.org/labs/Fire/Andrew/Vec99.pdf). For all bacteria-mediated RNAi experiments L4440 plasmids were used with HT115 (DE3) *E. coli* that has an inducible T7 RNA polymerase. Initially, the topo-plasmids containing the inserted fragment of interest were digested with BglII restriction enzyme, cloned into the multiple cloning site of the L4440 feeding vector (Fire kit) and transformed into *E. coli* strain HT115. A list of generated plasmids is in Appendix Table 7-8 (p.158). Additional constructs were selected from the RNAi library of the Ahringer lab , available from MRC gene service (Kamath and Ahringer, 2003) (Appendix Table 7-9, p.158). In both cases the *E. coli* strain HT115 contained the L4440 expression vector with an insert of the target gene. Selected strains were grown on Tet/Amp

plates (100 µg/ml Ampicillin, 12,5 µg/ml Tetracycline) overnight at 37°C. RNAi experiments were performed as described in Kamath *et al.* (Kamath *et al.*, 2001).

For standard screens, bacteria (HT115) were grown over night at 37°C in 5 ml LB medium with 50 µg/ml ampicillin. After a 10-minute spin at 3000 rpm (4°C), the culture volume was reduced to 1 ml; the pellet was resuspended and seeded onto Amp/IPTG NG agar plates (50 µg/ml ampicillin, 1 mM IPTG). The induction of dsRNA production was achieved at room temperature over night.

To achieve a stronger induction of dsRNA, an alternative protocol was used, in which the induction didn't occur directly on the plates, but in liquid culture. HT115 were grown at 37°C in 25 ml LB medium, containing 100 µg/ml ampicillin and 15 µg/ml tetracycline, to an OD_{595}= 0,4. To induce siRNA production, 10 µl IPTG (1 M) was added to each culture and bacteria were grown for additional 4 hours. The culture were then supplemented with additional 100 µg/ml Amp, 15 µg/ml Tet and 20 µl IPTG (0,5 M). Following a 5 minutes spin at 200 rpm, the culture volume was reduced to 7.5 ml, the pellet resuspended and bacteria were seeded onto Tet/Amp NG agar plates (100 µg/ml Amp, 15 µg/ml Tet).

For the RNAi experiments two L4 larvae of the wild type strain (N2) and the RNAi hypersensitive strains *rrf-3(pk1426)* and the VH525 strain as well as from selected GFP strains were transferred onto RNAi plates and grown at 25°C. Bacteria (HT115) containing empty vector L4440 served as a negative control, and bacteria containing *unc-22* gene fragment (from RNAi library, Ahringer lab (Kamath and Ahringer, 2003)) served as positive controls. *Unc-22* gene[18] was selected as a positive control for the RNAi experiments, because it has clear null phenotype that can be recognized by quick and simple inspection of the worms; they show Uncoordinated movements.

[18] The *unc-22* gene encodes a serine-threonine protein kinase that may regulate contraction. The enzyme is involved in myosin regulation may be involved in regulating final stages of sarcomere assembly. Mutants are uncoordinated and exhibit uncontrolled twitching of body-wall muscle cells, muscle cells have disordered myofilament lattices.