Electronic Supplementary Material (ESI) for Molecular BioSystems.

EXPERIMENTAL SETUP

In this work we analysed data gathered from 30 recombinant inbred lines (RILs) [1]. They were produced by the cross of two strains of *C. elegans*, the widely used reference strain N2, isolated in Bristol, U.K., and the strain CB4856 sampled in Hawaii (HW) [2]. RILs are usually produced by picking a number of individuals in the F2 generation of the initial cross and letting them self for several generations until the resulting strains are isogenic. This way each line has a random combination of the two parental genomes in the homozygous state, representing a powerful tool for performing a genome-wide dissection of complex biological traits [3].

Each line was genotyped by Rockman and Kruglyak in a previous work at 1,455 SNP markers [4]. Out of 208 lines genotyped in the laboratory before, 30 lines have been selected for this project and used for small RNA sequencing. Mutations in most miRNA genes show only a modest effect on phenotypes and appear to be functionally redundant [5,6]. This suggests that the majority of miRNAs present a feedstock of evolution. Small RNA abundance has been measured using small RNA sequencing technology of Illumina in all of the 30 RILs as well as in both strains of N2 and HW.

miRNA mapping from N2 to HW

To produce the set of miRNAs in HW the library of annotated miRNAs in N2 from miRBase [7] was mapped to the genome assembly of HW with BLAST [8]. First, release 17 (v17) of the miRBase was mapped on the HW genome assembly sequenced using the Illumina GenomeAnalyzer IIx. Next, new miRBase release 18 (v18) was applied to a different HW genome assembly, with a considerably higher sequencing depth. The raw sequencing reads for the second assembly were kindly provided to us by Matthew V. Rockman from the Department of Biology and Center for Genomics and Systems Biology at the New York University. The assembly of both of the sequenced genomes of HW was done at the Berlin Institute of Medical Systems Biology (BIMSB). The set of known miRNAs in N2 was extended

by 34 miRNAs annotated and kindly provided for this work by Rina Ahmed earlier (unpublished data). Results of miRNA mapping are shown in Table SM1. miRNAs were considered as mapped, if the position of the mapped hairpin on the target genome overlapped with the position of the mapped mature and/or the star sequence.

Out off 266 miRNAs in N2 overall, 253 were successfully mapped onto HW strain. 212 (84%) of them have identical nucleotide sequence in both strains. Out off 40 miRNAs with non-identical sequence only 7 miRNAs demonstrate an identity below 90%: *mir-261*, *mir-1833*, *mir-2218b*, *mir-4922-1*, *mir-4930*, *mir-5547* and *mir-IV_118138*. The full list of miRNAs in HW with explicit annotation of any sequence differences are reported in Table SM1.

Table SM1 - Overview of all miRNAs with sequence differences between N2 and HW.

miRNA_name	Mutation	Identity
mir-40	1 mutation	98.9
mir-60	1 mutation	98.9
mir-86	1 mutation	99.0
mir-231	1 mutation	99.0
mir-234	1 mutation	99.0
mir-242	2 mutations	97.9
mir-261	deletion in the middle	89.2
mir-262	1 mutation	99.1
mir-267	1 mutation	98.9
mir-272	1 mutation	98.9
mir-273	2 mutation	97.9
mir-355	1 mutation	99.1
mir-791	1 mutation	98.8
mir-794	1 mutation	98.8
mir-796	4 mutations	95.5
mir-1817	2 mutations	98.1
mir-1821	1 mutation	99.0
mir-1822	1 mutation	98.9
mir-1833	8-mutations/internal insert	72.2
mir-2215	1 mutation	98.5
mir-2209b	1 mutation	98.4
mir-2218b	internal insert	88.0
mir-2219	1 mutation	98.7
mir-4806	2 mutation	96.7
mir-4812	1 mutation	98.7
mir-4922-1	4 mutations/2 internal inserts	83.1
mir-4929	5p-gap/internal-gap	95.5
mir-4930	4 mutations/inserts/losses	83.9
mir-4933	1 mutation/3p-gap	90.8

mir-5547	1 mutation/3p-gap	78.2
mir-5595	1 mutation	98.5
mir-I_285	2 mutations	96.7
mir-I_245	1 mutation/1bp-gap	97.5
mir-V_24974	2 mutation	96.4
mir-IV_11813	7 mutations/inserts	83.1
mir-X_28864	1 mutation	98.3
mir-X_27916	1 mutation	98.3
mir-I_2736	1 mutation	98.5
mir-IV_3136	1 mutation	98.4
N2 novel -3	1 mutation	98.6

miRNA predictions with miRDeep2

Novel miRNAs in both HW and N2 were predicted via the application miRDeep2 [9]. Using reference genome and small RNA deep sequencing reads this software can predict novel miRNAs based on the consistency with the miRNA biogenesis. For the N2 strain, the version WS200 [10] was used as a reference genome. The miRDeep2 cutoff score of 2 used for the prediction of novel miRNAs was selected to guarantee a signal-to-noise ratio >= 10. Equal threshold was used in the miRDeep2 paper for all nematodes [9], as well as by other publications [11,12]. 10 new miRNAs were predicted in HW and 6 in N2. From miRNAs predicted in this way, only those meeting all of the following criteria were considered to be truly novel:

- miRNAs should not map onto a known rRNA/tRNA
- miRNAs whose seed regions are unique in N2
- miRNAs whose seed regions are unique among related nematodes:
    - *C. briggsae*,
    - *C. remanei*,
    - *P. pacificus*

Applying such criteria resulted in 7 novel miRNAs in HW and 3 in N2. For consistency reasons they were also mapped to respectively other strain as described above. As can be seen on Table SM2, one of

HW novel miRNAs did not map on N2 genome. Vice versa one of the N2 novel miRNAs did not map on the HW assembly.

Table SM2 - Results of miRNA mapping from N2 to HW for distinct groups of miRNAs. For miRBase annotations release 18 (v18) was used. Predictions of novel miRNAs are reported. HW novel were predicted in HW and mapped onto N2.

Strain	miRbase miRNAs	Novel by R. Ahmed	N2 novel	HW novel	total
N2	223	34	3	6	266
HW	213	31	2	7	253

Expression NGS data analysis

The analysis of the genetic data was done using our Spire software. In particular, the mapping of Illumina small RNA reads to the two reference miRNA libraries of N2 and HW was performed using microRazorS program release 1.0 [13]. Only the best forward hit per read was considered to reduce the number of false positive hits (-m 1 and -f). For the full description of the pipeline please refer to the methods in the publication and the online manual. We were able to identify a list of candidate eQTLs, as reported on Table SM3 using several different eQTL mapping algorithms, including univariate as well as multivariate approaches. The top 5 results were predicted by both interval mapping (em) and multiple imputations (imp) univarite methods, as well as the Random Forest approach. Seventeen eQTLs were identified by Random Forest alone, of which ten were supported by at least three or more runs. Further 7 eQTLs were predicted by univariate methods only (em and/or imp). An output of a univariate method (interval mapping in this case) of *lin-4* can be seen on Figure SM1 and Figure SM2. Random Forest output for the miRNA *mir-4936* can be seen on Figure SM3.

Table SM3 - Full list of eQTL predictions on the *C. elegans* dataset. Each prediction that is supported by the standard interval mapping (em), multiple imputations (imp) and in at least 3 out of 5 Random Forest runs was plotted on the Fig. 2 in the main paper. Other results are also present here.

Mirna Name	Mirna marker	Mirna Chr	Mirna pos	QTL Marker	QTL position	QTL Chr	QTL Support Interval markers	QTL support interval positions	Supported by methods	p-value	Cis or trans
mir-799	124	X	8600630	128	8665271.0	X	[124, 132]	[8394252.0, 8923304.0]	Imp	0.0369	cis
									Em	0.0355	
									RF (3/5)	< 0.05	
lin-4	553	2	5902266.5	1390	16623881.0	5	[1390, 1390]	[16623881.0, 16623881.0]	Em	0.0036	trans
									Imp	0.0058	
									RF (5/5)	< 0.05	
mir-4936	743	3	3249194.5	276	1563141.0	1	[271, 280]	[1265969.0, 1823874.0]	Em	0.0459	trans
									Imp	0.0454	
									RF (5/5)	< 0.05	
mir-78	1107	4	15165654.5	270	1130938.0	1	[269, 270]	[1079165.0, 1130938.0]	Em	0.0193	trans
									Imp	0.019	
									RF (5/5)	< 0.05	
mir-242	944	4	4274287.5	944	4265784.0	4	[944, 945]	[4265784.0, 4281915.0]	Em	0.0257	cis
									Imp	0.0216	
									RF (4/5)	< 0.05	
mir-787	171	X	11294680.5	460	14267212.0	1	[457, 462]	[14155576.0, 14325175.0]	RF (3/5)	< 0.05	trans
mir-4932	387	1	9512352.5	1238	6259748.0	5	[1238, 1239]	[6259748.0, 6336140.0]	RF (5/5)	< 0.05	trans
mir-49	145	X	9989264	266	884391.0	1	[263, 268]	[637477.0, 990151.0]	RF (5/5)	< 0.05	trans
mir-357	1262	5	8580573.5	1078	13532205.0	4	[1078, 1078]	[13532205.0, 13532205.0]	RF (5/5)	< 0.05	trans
mir-793	203	X	13857930.5	422	11722283.0	1	[419, 424]	[11600954.0, 11805353.0]	RF (4/5)	< 0.05	trans
mir-IV_11813	969	4	6294591	974	6544139.0	4	[969, 978]	[6278860.0, 6790549.0]	RF (4/5)	< 0.05	cis
mir-5547	649	2	12238570.5	649	12280277.0	2	[649, 649]	[12280277.0, 12280277.0]	RF (5/5)	< 0.05	trans

mir-240	117	X	7882615	276	1563141.0	1	[271, 280]	[1265969.0, 1823874.0]	RF (3/5)	< 0.05	trans
mir-355	636	2	11833506.5	456	14113165.0	1	[456, 456]	[14113165.0, 14113165.0]	RF (4/5)	< 0.05	trans
mir-2219	1184	5	2924616.5	1188	2919446.0	5	[1184, 1191]	[2675409.0, 3111900.0]	RF (4/5)	< 0.05	cis
mir-64	726	3	2172890.5	222	14961400.0	X	[221, 224]	[14888419.0, 15074614.0]	RF (1/5)	< 0.05	trans
mir-74	31	X	2369085	270	1130938.0	1	[269, 270]	[1079165.0, 1130938.0]	RF (1/5)	< 0.05	trans
mir-II_5556	501	2	2136866.5	502	2182381.0	2	[501, 502]	[2121018.0, 2182381.0]	RF (1/5)	< 0.05	cis
mir-1833	1102	4	15012343	1095	14423623.0	4	[1091, 1099]	[14263782.0, 14664093.0]	RF (1/5)	< 0.05	trans
mir-2212	239	X	16207776.5	266	884391.0	1	[263, 268]	[637477.0, 990151.0]	RF (1/5)	< 0.05	trans
mir-1829a	231	X	15588262.5	234	15563593.0	X	[231, 238]	[15500580.0, 15842349.0]	RF (1/5)	< 0.05	cis
mir-1833	1102	4	15012343	1100	14843111.0	4	[1100, 1101]	[14843111.0, 14857053.0]	RF (1/5)	< 0.05	trans
mir-1829c	225	X	15236313.5	281	2068168.0	1	[263, 288]	[637477.0, 2538742.0]	Imp	0.0185	trans
									em	0.0117	
mir-240	117	X	7882615	271	1265969.0	1	[263, 281]	[637477.0, 2068168.0]	Imp	0.0167	trans
									em	0.0221	
mir-1833	1102	4	15012343	1091	14263782.0	4	[1088, 1102]	[14112559.0, 15008938.0]	Imp	0.00285	cis
									em	0.00085	
mir-67	776	3	5931348	482	762240.0	2	[477, 497]	[426624.0, 1750144.0]	Imp	0.0359	trans
									em	0.0346	
mir-49	145	X	9989264	269	1079165.0	1	[255, 271]	[39041.0, 1265969.0]	Imp	0.0334	trans
									em	0.0484	
mir-257	1399	5	17140688.5	288	2538742.0	1	[281, 288]	[2068168.0, 2538742.0]	em	0.00795	trans
									imp	0.0234	
mir-II_4504	565	2	6943916.5	482	762240.0	2	[477, 482]	[426624.0, 762240.0]	em	0.2347	trans

A eQTL hotspot analysis was performed using the list of significant eQTLs. The significance of the results was calculated assuming a background Poisson distribution. The hotspot at the SNP position 1265969 (Marker 271) at the beginning of the chromosome 1 showed a p-value of 0.02. The expression at this locus correlates with the expression of 4 miRNAs: *mir-1829c*, *mir-4936*, *mir-240*, *mir-49*.

For a protein-protein interaction (PPI) network we used the experimentally validated targets of the top 15 miRNAs with an identified eQTL from our results. We used the DIANA-TarBase database [14], resulting in 149 protein targets overall. We observed 99 interactions within this group, with the biggest subgroup containing 47 proteins. We analysed the GO terms within biological processes, as well as

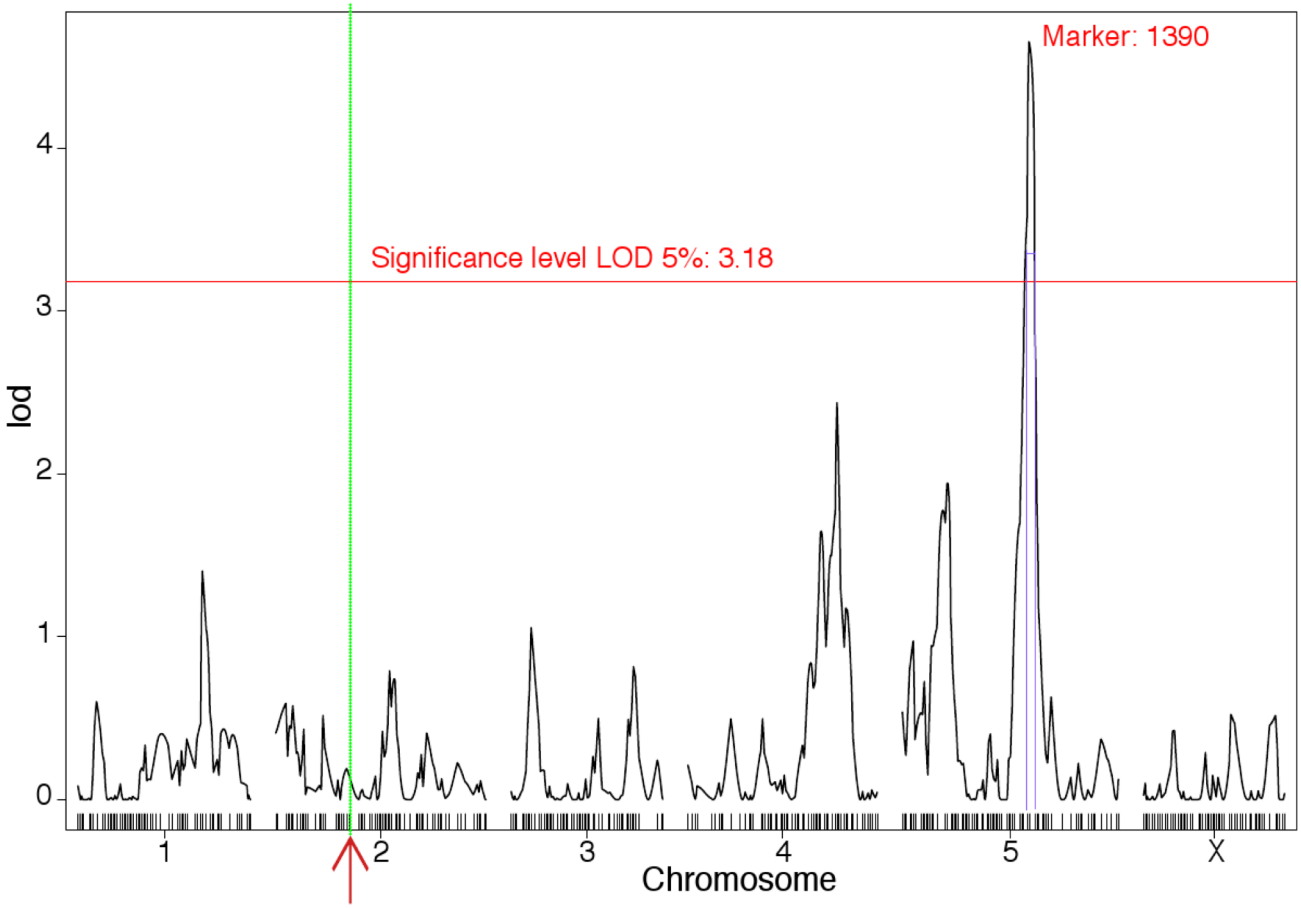

Figure SM1 - An example result of interval mapping (em) method used for eQTL mapping on the miRNA *cel-lin-4*. The green line indicated by red arrow is the genomic position of the miRNA. The blue lines indicate the confidence interval of the eQTL at the marker 1390.

KEGG pathways for potential enrichment. In particular, we used the predefined constraint, higher-order set of GO terms GOslim in our work. The p-values were adjusted for multiple testing with FDR. The results are summarized in the tables SM4 and SM5.

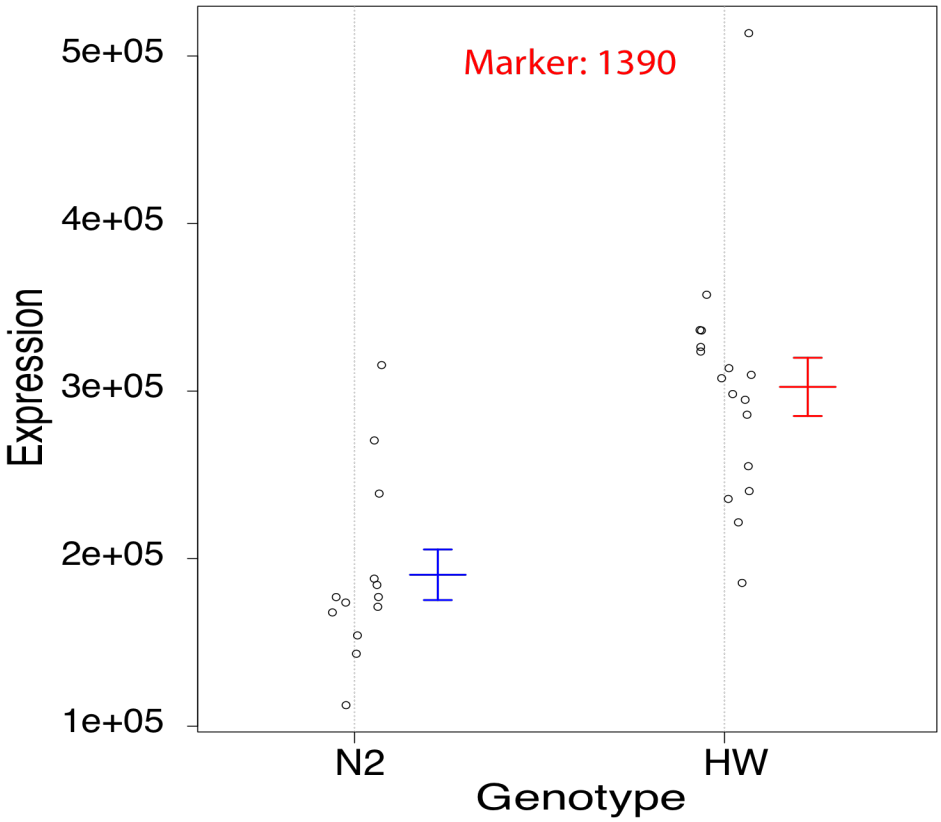

Figure SM2 - An effect plot of the results on the Figure SM1. Each point represents one RIL line out of the whole dataset. Next to

Table SM4 - Significantly enriched GO Terms amongst the protein targets of the top 15 miRNAs in our results.

GO term	GO ID	Number of Genes	P-values (FDR corrected)
Anatomical structure development	GO:0048856	54	0.00862

Table SM5 - Significantly enriched KEGG pathways amongst the protein targets of the top 15 miRNAs in our results.

KEGG term	ID	Number of Genes	P-values (FDR corrected)
Citrate cycle (TCA cycle)	00020	5	0.000353
Carbon metabolism	01200	6	0.00246
Lysine degradation	00310	3	0.0465
Metabolic pathways	01100	13	0.0317

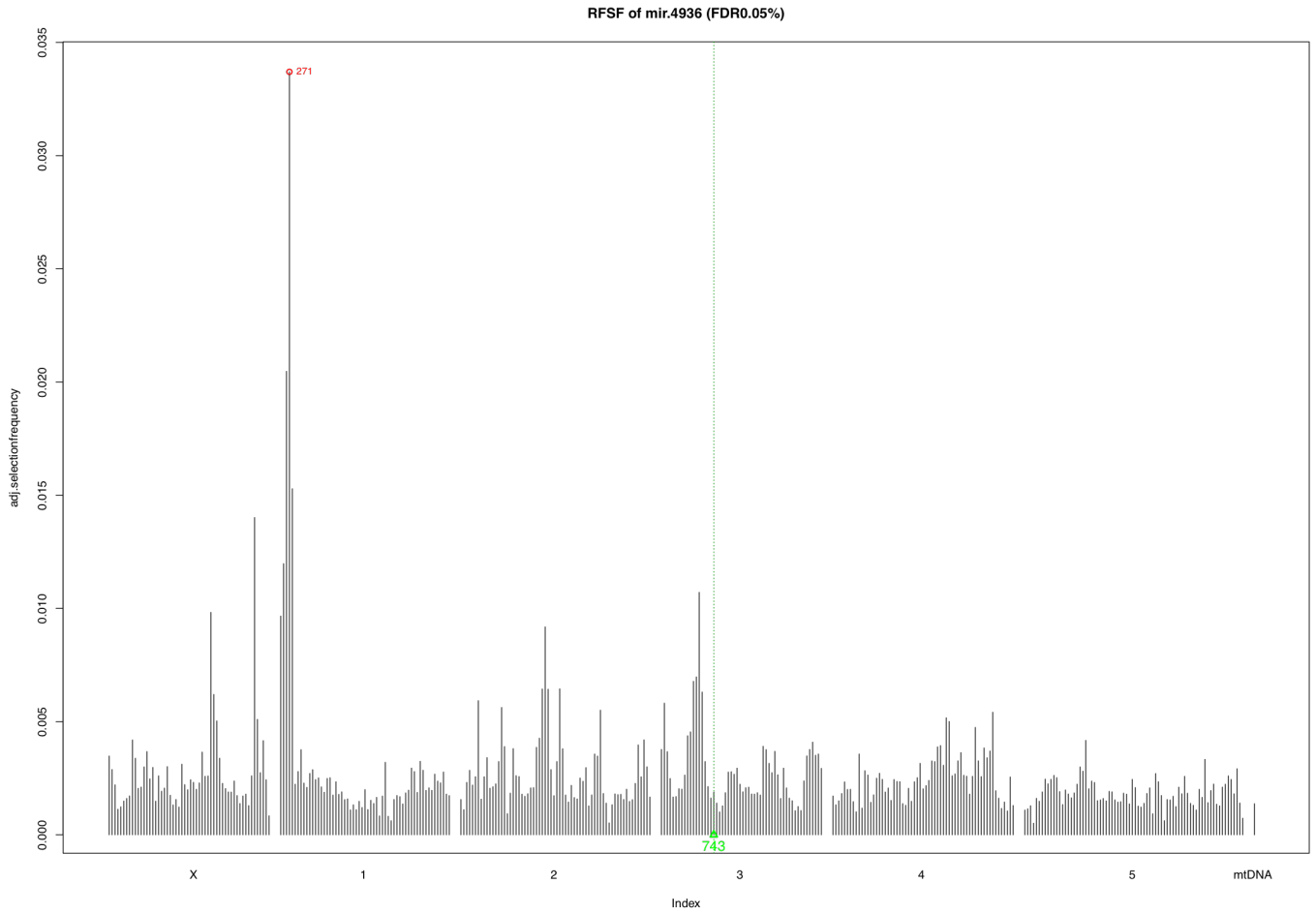

Figure SM3 - An example result of random forest (RF) method used for eQTL mapping on the miRNA cel-mir-4936. The green line and the green number indicate the genomic position of the miRNA. The identified eQTL is at the Marker 271 on Chr 1 (at the hotspot).

VALIDATION

For a follow-up validation of the eQTL results with a qPCR we selected the top 15 miRNAs and the hotspot mRNA gene **ghr-1**. We further picked the 16 RILs, produced by crossing the N2 and the HW worms as described above, with the strongest predicted eQTL at the SNP markers next to the 16 genes of interest.

For the most miRNAs in our selection the sequence identity between the two *C. elegans* strains is either not present or in the region, that is of no importance for the miRNA primer design procedure. The only exception presents the recently validated novel miRNA *mir-IV_11813* (*mir-8201*) [11], for which two different qPCR primers for the miRNA mature sequence have been designed. The first variant "5'-

TCTGGATCGATAATGTAAACCT-3' " is the consensus sequence produced by the reads that map to the *mir-IV_11813* sequence in the RILs with the HW genotype for the gene of interest. On the other hand the second sequence variant "5'- TCTTAGGATCGATAATGTAATTATATAGAATCT-3' " is the result of the *mir-IV_11813* mature N2 sequence mapped to the HW genome. Please note, that for the eQTL mapping we used the miRNA hairpin sequence. The HW sequence was thus defined through mapping of the N2 miRNA sequence to HW genome. Both *mir-IV_11813* mature sequences have been used for the qPCR validation of the predicted eQTL effect (See Figure SM4 panel a). Using the second sequence variant for the primer design, we were able to show the predicted difference of expression for *mir-IV_11813* at its eQTL, depending on the genotype at the locus (Figure SM4 panel b). Together with the unclear signal for the first sequence variant (Figure SM4 panel c) it indicates a high sequence variance of *mir-IV_11813* between *C. elegans* strains and thus its low conservation. This suggests an evolutionary very young miRNA.

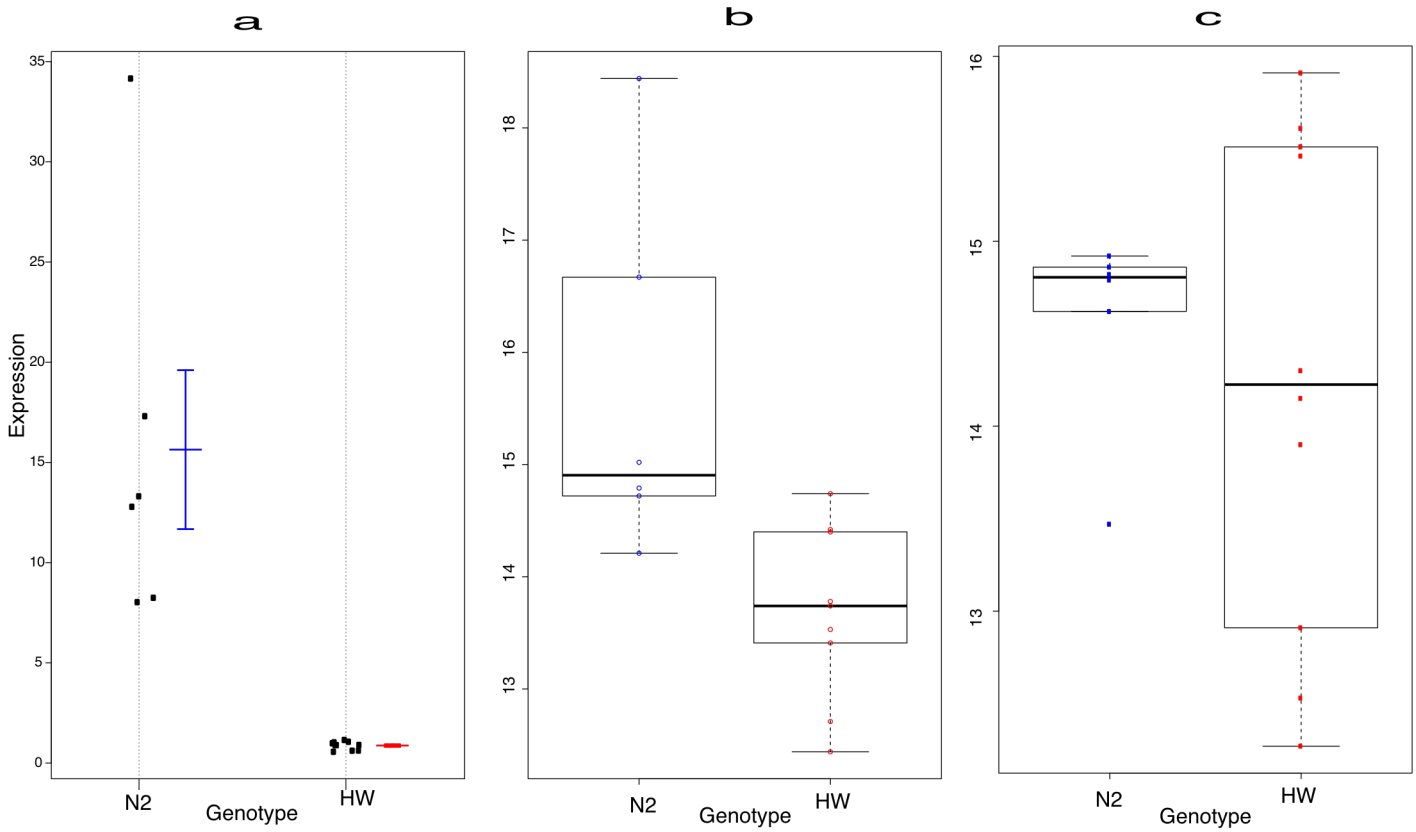

Figure SM4 - Comparison of different expression levels of the miRNA cel-mir-IV_11813 (cel-mir-8201). In each plot, every dot represents one of 16 preselected RILs. These are grouped by genotype for each given RIL at the SNP marker of the genomic position of the eQTL (Marker 969, Chr: 4, Position: 6544139). For each genotype, the phenotypic mean is plotted, with error bars at ±1SE. a) Predicted distribution before qPCR. b) and c) Distribution of expression values after qPCR. b) HW genotype sequence was used for the primer construction. c) N2 genotype sequence was used for the primer construction.

Nematodes cultivation

Nematodes were maintained and propagated at 20°C on solid Nematode Growth Medium (NGM) seeded with OP50 *E. coli* (Caenorhabditis Genetics Center, University of Minnesota, USA) for food. To prepare age-synchronized animals, nematodes were transferred to fresh NGM plates after reaching maturity at 3 days of age and allowed to lay eggs overnight. Isolated hatchlings from the synchronized eggs (day 1) were cultured on fresh NMG plates at 20°C.

RNA extraction and quality control

RNA was extracted from ~ 30.000 synchronized worms at L4 larval stage (day 4). To this end, nematodes were collected and resuspended in 60% sucrose solution (ref). After centrifugation at 3000 rpm x 5 min, worms were collected, washed two times with M9 buffer (REF) and placed into 700 µl QIAzol Lysis Reagent (Qiagen). Nematodes were homogenized immediately using Ultra-Turrax homogeniser (IKA) until the sample was uniformly homogeneous (usually 2 min). Total RNA was then purified according to the RNeasy Mini kit instruction reported in the animal tissues Handbook (miRNeasy Mini Handbook, Qiagen). RNA concentration and purity were then evaluated by determining the absorbance ratio at 260 nm/280 nm (A260/A280) on NanoDrop-ND 1000 Spectrophotometer (Nanodrop Technologies, ThermoScientific), whereas the RNA integrity was evaluated according to the RNA integrity number (RIN) by using RNA 6000 Nano kit on Bioanalyzer 2100 instrument (Agilent Technologies, Inc. Santa Clara, USA). Only samples with RIN>7 and A260/A280 ratio between 1.8-2 were included in the analysis.

cDNA synthesis

Reverse transcriptions were performed using the miScript Reverse Transcription (RT) Kit (Qiagen) according to the manufacturer's instructions, starting from 1µg of total RNA. cDNAs products were diluted 1:50 and 1:5 for PCR detection of miRNAs and mRNA respectively, and stored at -20°C.

Primers design

We tested 15 miRNAs, plus 1 mRNA gene (GRH-1) (Table 1). Because the sensitivity and specificity of the qPCR assay are influenced by primer design, miRBase and WORMBase databases were used to analyse the sequences of miRNAs and mRNA respectively. To test miRNAs, we used miScript primer

assay from Qiagen. Some of the miRNA primer sequences were designed manually and checked by the OligoCalc software (http://www.basic.northwestern.edu/biotools/oligocalc.html) [15]. To test GRH-1 expression level, the primers were designed using Primer3 software [16] (Table SM6 and SM7). NCBI Blast tool was used to check the primer specificity. The qPCR efficiency (E) and correlation coefficients were calculated by Rotorgene Q software (Qiagen). For each primer a standard curve (5 points) was made in triplicate from 10-fold serial dilution of pooled cDNAs. For each primer set the qPCR efficiency ranged from 97% to 110%, however all correlation coefficients were higher than 0.99, confirming the reaction linearity of all primer sets.

Table SM6 - miRNA qPCR primer designs.

miRNA	Primer design	mirBASE accession
miR-799	MS00020174 [1]	MIMAT0004234
lin-4	MS00018900 [1]	MIMAT0000002
mir-242	MS00019474 [1]	MIMAT0000298
mir-78	MS00020055 [1]	MIMAT0000050
mir-357	MS00028700 [1]	MIMAT0000699
mir-49_1	MS00019859 [1]	MIMAT0000020
mir-355	MS00019726 [1]	MIMAT0000697
[3]***mir-2219_N2***	MS00028679 [1]	MIMAT0011459
mir-793	MS00020132 [1]	MIMAT0004228
mir-240	MS00019460 [1]	MIMAT0000296
mir-787	MS00020083 [1]	MIMAT0004222
[3]***mir-2219_HW***	designed manually	-
mir-4932	designed manually	MIMAT0020138
mir-4936	designed manually	MIMAT0020142
[2]***mir-IV_11813 _N2***	designed manually	-
[2]***mir-IV_11813_HW***	designed manually	-

[1] miscript primer assay Qiagen.
[2] *mir-IV_11813* is a novel miRNA (not present in the miRBase database), therefore, because the sequence is highly variable between the HW and N2 strains we tested two primers, likewise
[3]*mir-2219* has a difference of one nucleotide in the middle of the sequence. All other miRNAs have the same sequence in the two strains.

Table SM7 - mRNA qPCR primer design.

Gene	Primer design	wormBase accession
GRH-1	Primer3 software	WBGene00001707

qPCR with SYBR Green

qPCR reactions were performed in triplicate on a Rotor-Gene Q instrument (Qiagen, Valencia, CA, USA) and using the miScript SYBR® Green PCR Kit (Qiagen). For detection of miRNAs expression, the PCR volume reaction was 20 µl containing: 10 µl of 2x QuantiTect SYBR Green PCR Master Mix, 2 µl of 10x miScript Universal Primer, 2 µl of 10x miScript Primer Assay (Qiagen) or specific primer, 2 µl of diluted cDNA. For detection of mRNA expression, the PCR volume mix was 20 µl containing: 10 µl of 2x QuantiTect SYBR Green PCR Master Mix (Qiagen), 2 µl of 10x forward and reverse Specific Primer, 2 µl of diluted cDNA.

Amplification conditions were: initial incubation step at 95°C for 15 minutes, followed by 40 amplification cycles at 94°C for 15 seconds, 55°C for 30 seconds, and 70°C for 30 seconds. Afterwards a melting curve analysis (60°C to 95°C) was used to verify the specificity of each product. In addition, each PCR reaction included a no-reverse transcriptase enzyme and a no-template control to check for potential genomic DNA contaminations.

Data analysis

Data analysis was performed using the normalization with reference gene as endogenous control. It is the most common method used in qPCR relative expression [17]. Therefore the selection of an appropriate endogenous control is a critical step in the experimental design. To achieve reliable relative expression results it is important that the qPCR E of both the target and the endogenous control is approximately equal and the linear relationship (slope) of differences between the Ct (ΔCt) versus the log of template amount is < 0.1.

For analysing miRNAs expression, we have tested three small nucleolar RNAs (sno234, U18, and U6), as possible endogenous controls, whilst we have analysed two candidate reference genes (TPA and PMP-3) for mRNA normalization (Table SM8 and SM9).

Table SM8 - Candidate reference genes for microRNA normalization.

Gene name	GenBank accession
sno234	DQ789547.1
U6	X07829.1
U18	Z75111.1

Table SM9 - Candidate reference genes for mRNA normalization.

Gene Name	Gene Bank accession
TBA-1	NM_001264284.1
PMP-3	NM_001269679

The expression stability of the selected candidate reference genes was analysed by the comprehensive tool RefFinder [18] that integrates four software packages: geNorm [19], Normfinder [20], BestKeeper [21]. To select the most reliable reference gene a comprehensive approach was developed on the 4 programs above reported [18]. As results, the most stable reference genes were sno234 and pmp3 for analysing miRNAs and mRNA respectively (Table SM10 and SM11).

Table SM10 - Comprehensive ranking order for miRNA reference genes.

Method (reference genes miRNAs)	1	2	3
Delta CT	Sno234	U6	U18
BestKeeper	Sno234	U18	U6
Normfinder	Sno234	U6	U18
Genorm	U6	Sno234	U18
Recommended comprehensive ranking	Sno234	U6	U18

Table SM11 - Comprehensive ranking order for mRNAs reference genes.

Method (reference genes mRNA)	1	2
Delta CT	PMP3	TPA
BestKeeper	TBA1	PMP3
Normfinder	PMP3	TPA
Genorm	TBA1	PMP3
Recommended comprehensive ranking	PMP3	TPA

Cycle threshold (CT) scores > 34 were considered to be undetectable [22] and excluded from the final data analysis. The relative expression levels of targets were calculated by the ΔCt method and the Ct

mean of the three replicates was used for data analysis [23] with relative standard deviation (+/- SD). Higher levels of expression correspond to smaller ΔCt values.

Table SM12 - qPCR result for tested miRNAs.

mRNA *vs* PMP3	Mean ΔCt	SD
mir-799	12.39	+/- 2.50
lin-4	1.81	+/- 1.22
mir-242	8.24	+/- 1.03
mir-4936	8.23	+/- 0.89
mir-78	8.14	+/- 0.91
mir-357	6.45	+/- 1.23
mir-49	4.11	+/- 0.91
mir-4932	8.97	+/- 1.39
mir-355	10.89	+/- 1.05
mir2219-N2*	7.76	+/- 1.78
mir2219-HW	7.58	+/- 1.51
mir-240	5.95	+/- 1.50
mir-787	8.14	+/- 0.86
mir-IV_11813_HW	14.18	+/- 1.06
mir-IV_11813_N2	14.47	+/- 1.07

Table SM13 - qPCR result for the tested gene grh-1.

mRNA *vs* Sno234	Mean ΔCt	SD
GRH1	0.055	+/- 1.23

qPCR results Analysis

Table SM14 shows the expression of the miRNAs quantified by using Rotor Gene Real-Time PCR System (Qiagen) with Sybr Green miRNA assays (Qiagen) in accordance with the manufacturer's instructions.

Table SM14 – Full list of expressions (dCT values) obtained by qPCR for each of the measured miRNAs across the 16 preselected RILs.

QX/ miRNA	9	22	52	55	40	71	17	233	31	42	58	61	147	157	194	237
in-4	2,44	0,96	2,56	0,32	2,43	2,20	3,74	3,69	0,02	2,08	2,69	0,85	0,32	0,78	0,90	3,04
mir-799	14,73	10,56	11,76	9,36	17,03	11,64	12,0	14,8	8,99	14,4	14,8	12,0	8,08	12,9	10,7	14,3
mir-78	8,35	7,44	8,11	8,83	7,91	8,72	9,13	7,64	7,96	8,83	8,49	10,0	7,52	8,16	6,57	6,48
mir-242	8,01	8,05	8,20	7,04	8,88	9,80	9,85	9,15	6,97	8,92	9,13	8,77	7,25	8,09	6,94	6,78
mir-357	6,26	5,77	6,80	5,78	6,43	7,05	7,33	6,48	6,67	6,27	6,84	9,48	4,79	7,83	4,21	5,29
mir-2219	8,52	5,80	7,84	8,35	7,80	8,05	7,99	7,40	8,38	9,05	8,99	9,10	9,10	9,94	4,76	3,12
mir-355	10,86	10,77	10,36	9,02	12,07	12,22	12,3	10,7	11,4	11,4	11,3	11,8	9,14	9,28	10,5	10,9
mir-49	4,47	3,35	4,18	3,47	5,15	5,09	5,06	5,22	3,50	4,66	5,54	3,12	3,01	3,89	3,21	2,90
mir-793	6,28	6,09	7,26	4,82	7,37	6,85	6,61	6,85	6,83	8,00	7,97	7,70	6,54	6,42	7,12	8,94
mir-240	6,30	3,98	6,95	5,29	7,06	7,90	5,91	6,38	3,34	7,04	8,06	7,76	3,59	5,33	5,43	4,83
mir-4936	7,82	8,87	8,30	7,40	8,04	7,48	7,65	7,43	8,48	8,06	8,50	7,20	7,32	7,71	8,38	8,29
mir-787	8,41	7,42	8,07	7,10	9,35	8,35	7,50	8,51	8,74	9,53	8,94	8,15	6,98	6,90	ND	ND
mir-2219	7,97	5,83	7,73	8,15	7,42	7,75	8,04	7,69	9,07	8,37	8,18	8,36	8,60	8,95	6,35	2,89
mir-4932	10,06	8,65	9,89	9,48	9,55	9,04	9,82	9,15	8,67	9,57	10,4	9,34	9,54	9,12	5,63	5,61
mir-IV_11831 N2	13,90	12,53	14,30	15,51	14,15	14,82	15,61	15,46	14,79	14,62	14,92	13,47	15,91	14,86	12,91	12,27
mir-IV_11831 HW	13,41	12,44	13,74	13,78	13,53	14,21	14,40	14,42	18,44	16,67	14,79	14,72	14,74	15,02	ND	12,71

Proceeding the dCt analysis, we used the ddCt (ΔΔCt) method, which was described in previous works [23,24]. It is defined as:

$$ddCT=(CT(target,untreated)-CT(ref,untreated))-(CT(target,treated)-CT(ref,treated)),$$

where

CT(*target*,*untreated*) = CT value of gene of interest in untreated sample

CT(*ref*,*untreated*) = CT value of control gene in untreated sample

CT(*target*,*treated*) = CT value of gene of interest in treated sample

CT(*ref*,*treated*) = CT value of control gene in treated sample

Since we were interested in the absolute differential expression, we calculated both the scenarios (the first with N2 genotype as the *treated* group and HW as *untreated* group and viceversa), considering in each case the best ratio between two. The results are summarized in the Table SM15.

The absolute amount of material that we obtain through PCR for each sample for each primer pair is inversely proportional to 2^(*CT)* [23].

Under that presumption the ratio of the target gene (miRNA) in the treated sample (HW) relative to the untreated sample (N2) is 2^(ΔΔ*CT)*.

Table SM15 - ddCT results of qPCR experiment with 15 different miRNA mature sequences. *mir-IV_11831* has been tested using two diverse sequences, where N2 indicates the first sequence variant and HW the second one (see text for further details).

miRNA Name	ddCT ((HW-N2)	Ratio (HW-N2)	ddCT (N2-HW)	Ratio (N2-HW)	Best ratio
mir-799	3,64	12,4	-3,64	0,08	12,4
lin-4	1,61	3,06	-1,61	0,33	3,06
mir-IV_11831HW	-1,96	0,26	1,96	3,88	3,88
mir-4932	-1,08	0,47	1,08	2,12	2,12
mir-787	0,93	1,91	-0,93	0,52	1,91
mir-4936	0,89	1,86	-0,89	0,54	1,86
mir-242	-0,89	0,54	0,89	1,85	1,85
mir-793	0,87	1,82	-0,87	0,55	1,82
mir-357	-0,69	0,62	0,69	1,61	1,61
mir-78	-0,37	0,77	0,37	1,29	1,29
mir-IV_11831N2	-0.33	0.79	0.33	1.25	1.25
mir-240	0,28	1,21	-0,28	0,82	1,21
mir-49	0,22	1,16	-0,22	0,86	1,16
mir-355	0,20	1,15	-0,20	0,87	1,15
mir-2219	-0,05	0,97	0,05	1,03	1,03

Follow-up validation of the hotspot

For the further investigation of the identified hotspot and the transcription factor **grh-1** as a candidate for the regulator we compared pairs of RILs as described in the main paper. Table SM16 summarizes the distribution of genotypes for the hotspot candidate gene **grh-1** and the corresponding expression as normalized dCT value.

Table SM16 – Full list of expressions (dCT values) obtained by qPCR the transcription factor **grh-1**. Last row shows the corresponding genotypes of **grh-1**. The dCT values were produced and normalized as described above.

QX/ miRNA	9	22	52	55	40	71	17	233	31	42	58	61	147	157	194	237
Grh-1 expressio n	-0,19	0,74	1,11	0,42	0,52	0,28	-0,22	0,58	-1,99	-0,68	0,02	-1,37	1,62	0,47	-0,72	0,30
Grh-1 genotype	N2	N2	HW	N2	N2	N2	N2	N2	N2	HW	N2	N2	N2	HW	HW	N2

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