List of Supplemental Materials

Figure S1 Genetic experiments supporting those in Figure 4.

Figure S2 Genetic interaction of *mir-35-41(nDf50)* with *sup-26(lf)* and *nhl-2(lf)* with respect to lethality. Provides context for genetic interactions observed in Figures 4 and 5.

Figure S3 Additional characterization of *sup-26(GFP_null)* and *sup-26::flag* alleles, supporting Figures 4 and 5.

Figure S4 Controls for HITS-CLIP data presented in Figure 6.

Figure S5 Additional characterization of *nhl-2* reporter alleles, supporting Figure 7.

Figure S6 Genetic test of interaction between *mir-35* family mutant lethality and sex chromosome karyotype. Provides context for the genetic interaction of the *mir-35* family and the sex determination pathway delineated in Figures 4-5.

Table S1 List of genes differentially regulated in *mir-35-41(nDf50)* mutant embryos, as shown in Figure 1.

Table S2 List of predicted *mir-35* family target genes screened by RNAi for interaction with the *mir-35-41(nDf50); her-1(gf)* synTra phenotype.

Table S3 Genomic coordinates of significant peaks of SUP-26::FLAG binding identified by HITS-CLIP.

Supplemental Figure Legends

Supplemental Materials and Methods Detailed CRISPR and HITS-CLIP protocols, names and genotypes of strains used, sequences of oligonucleotides used in the study.

Supplemental Figure Legends

Figure S1 *sup-26(lf)* or *nhl-2(lf)* suppresses the *her-1(gf)* Egl phenotype. Quantification of sex determination phenotypes; darker colored bars indicate more severe masculinization.

Figure S2. Neither *sup-26* nor *nhl-2* loss of function suppresses *mir-35(nDf50)* lethality. (A) Quantification of embryonic and early larval lethality at 20°C in indicated genotypes. (B) Quantification of nature of highly-penetrant embryonic and early larval lethality at 25°C in indicated genotypes. As in Alvarez-Saavedra and Horvitz 2010, arrested embryos were categorized according to terminal morphology. Embryos that appeared as a ball of cells with no distinguishable morphological features were classified as “amorphous mass.” Two-fold embryos and poorly-elongated embryos possibly of later developmental stage are difficult to distinguish and thus grouped together.

Figure S3. *sup-26(GFP_null)* allele is feminizing and displays a male-specific phenotype. (A) Schematic of *sup-26* rescuing transgenes generated by MosSCI. Blue regions represent Gateway cloning recombination sites. (B) Top: Quantification of sex determination phenotypes showing suppression of *her-1(gf)* masculinization by *sup-26(GFP_null)* and rescue by *sup-26* rescuing transgenes. Bottom: Quantification of sex determination phenotypes showing suppression of *her-1(gf);mir-35-41(nDf50*/+*) masculinization by *sup-26(GFP_null)*. (+* denotes mIn1 balancer chromosome.) Data for control genotypes are also shown in Figure 3C. (C) Mating assays showing impaired mating by *sup-26(GFP_null);him-8(e1489)* males and rescue of the mating phenotype by *sup-26* rescuing transgenes. Different numbers of *him-8(e1489)* males in top panel demonstrates the quantitative nature the of the assay. All assays in bottom panel contained 6 males. (A-C) *Sup-26(GFP_null)* denotes *sup-26(ma268null)*. *Sup-26(rescue_1)* and *sup-26(rescue_2)* denote *maIs398* and *maIs399*, respectively. (D) Anti-FLAG immunofluorescence in *sup-26::flag* strain in dissected hermaphrodite germline. In addition to germ cells, SUP-26::FLAG is expressed in the somatic gonad in the distal tip cell (DTC) and the hermaphrodite spermatheca, marked by an asterisk (containing an embryo, the eggshell of which is not permeabilized by the staining procedure).(E) Deletions of *mir-35* family seed match in *sup-26::flag* 3’ UTR are not sufficient to alter sex determination phenotype in a *her-1(n695)* background.

Figure S4. SUP-26::FLAG CLIP signal is FLAG-specific and dependent on UV-crosslinking. (A) Quantification of sex determination phenotypes in *her-1(n695gf)* with indicated *sup-26* alleles. The *sup-26(ma265flag)* allele does not dramatically alter the phenotype. (B) Schematic of HITS-CLIP procedure. (C) Left: SUP-26::FLAG CLIP signal depends upon the presence of the FLAG tag and on UV crosslinking. This indicates that only RNA targets that are crosslinked to SUP-26::FLAG are cloned and sequenced. Right: Optimization of micrococcal nuclease concentration by SDS-PAGE (middle) and corresponding RNA gel (right). (D) Lack of gene expression changes in *sup-26(GFP_null)* hermaphrodite embryos versus wild type. (E) Example CLIP traces of SUP-26::FLAG binding in 3’UTR regions.

Figure S5. GFP::NHL-2 is expressed in the maternal germline and enriched in P granules via a post-transcriptional mechanism. (B) Epifluorescence image of GFP::NHL-2 showing fluorescence in oocytes (*nhl-2(ma371)*). (C) Epifluorescence image of a *Pnhl-2::GFP::let-858*

allele at the *nhl-2* genomic locus, showing that transcription under the control of *nhl-2* genomic regulatory elements does not confer P lineage enrichment of GFP (*nhl-2(ma372)*).

Figure S6. *mir-35-41(nDf50)* does not alter the percent of male self progeny from *him-8(e1489)* hermaphrodites. The *him-8(e1489)* allele causes frequent X chromosome nondisjunction, resulting in ~40% males in broods derived from self-fertile hermaphrodites. If XX-specific lethality were to occur, the proportion of males observed in the brood would increase. However, *mir-35-41(nDf50)* does not significantly alter the proportion of males in a *him-8(e1489)* background. Strains were grown on HT115 containing the empty RNAi vector (L4440). p-value > 0.05 (Two-tailed *t*-test with Welch's correction for unequal sample size.) Animals that died as embryos or larvae were excluded from the quantification.

Figure S1

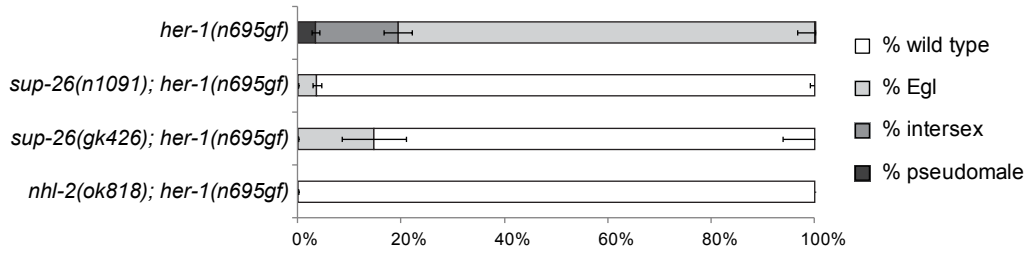

her-1(n695gf)
sup-26(n1091); her-1(n695gf)
sup-26(gk426); her-1(n695gf)
nhl-2(ok818); her-1(n695gf)
0%
20%
40%
60%
80%
100%
% wild type
% Egl
% intersex
% pseudomale

Figure S2

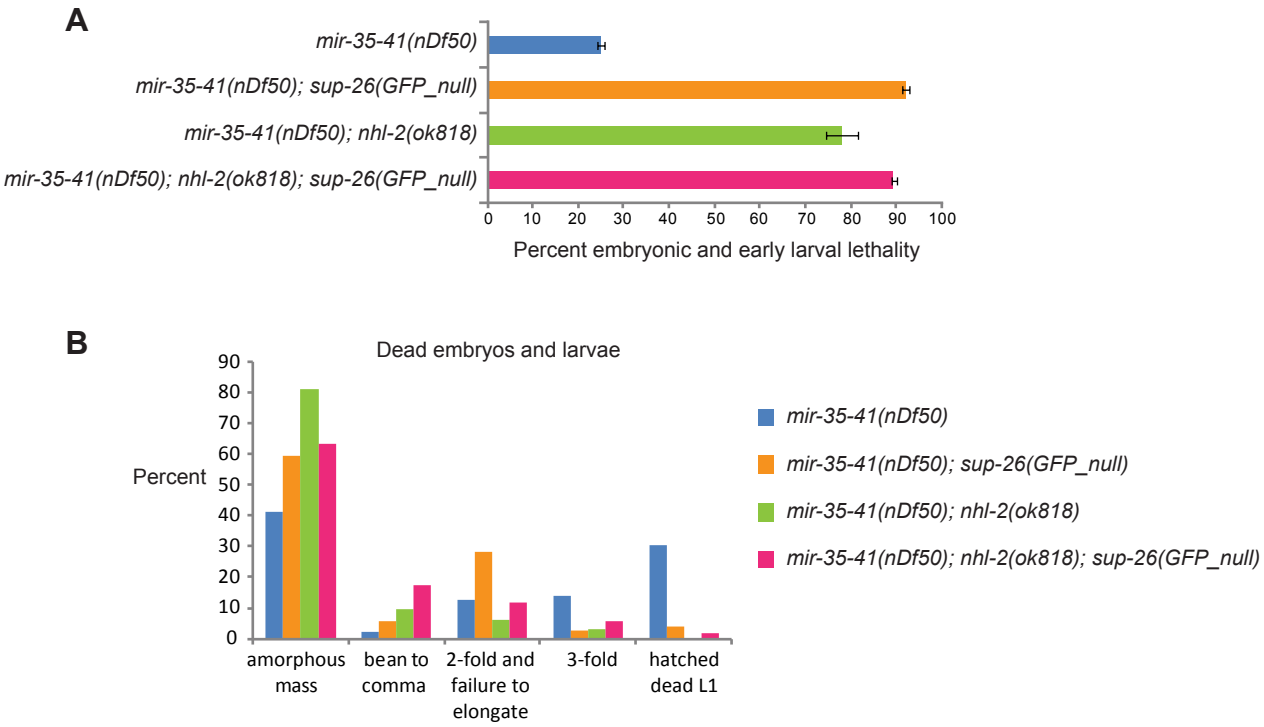

A
mir-35-41(nDf50)
mir-35-41(nDf50); sup-26(GFP_null)
mir-35-41(nDf50); nhl-2(ok818)
mir-35-41(nDf50); nhl-2(ok818); sup-26(GFP_null)
0 10 20 30 40 50 60 70 80 90 100
Percent embryonic and early larval lethality
B
Dead embryos and larvae
Percent
0 10 20 30 40 50 60 70 80 90
amorphous mass
bean to comma
2-fold and failure to elongate
3-fold
hatched dead L1
mir-35-41(nDf50)
mir-35-41(nDf50); sup-26(GFP_null)
mir-35-41(nDf50); nhl-2(ok818)
mir-35-41(nDf50); nhl-2(ok818); sup-26(GFP_null)

Figure S3

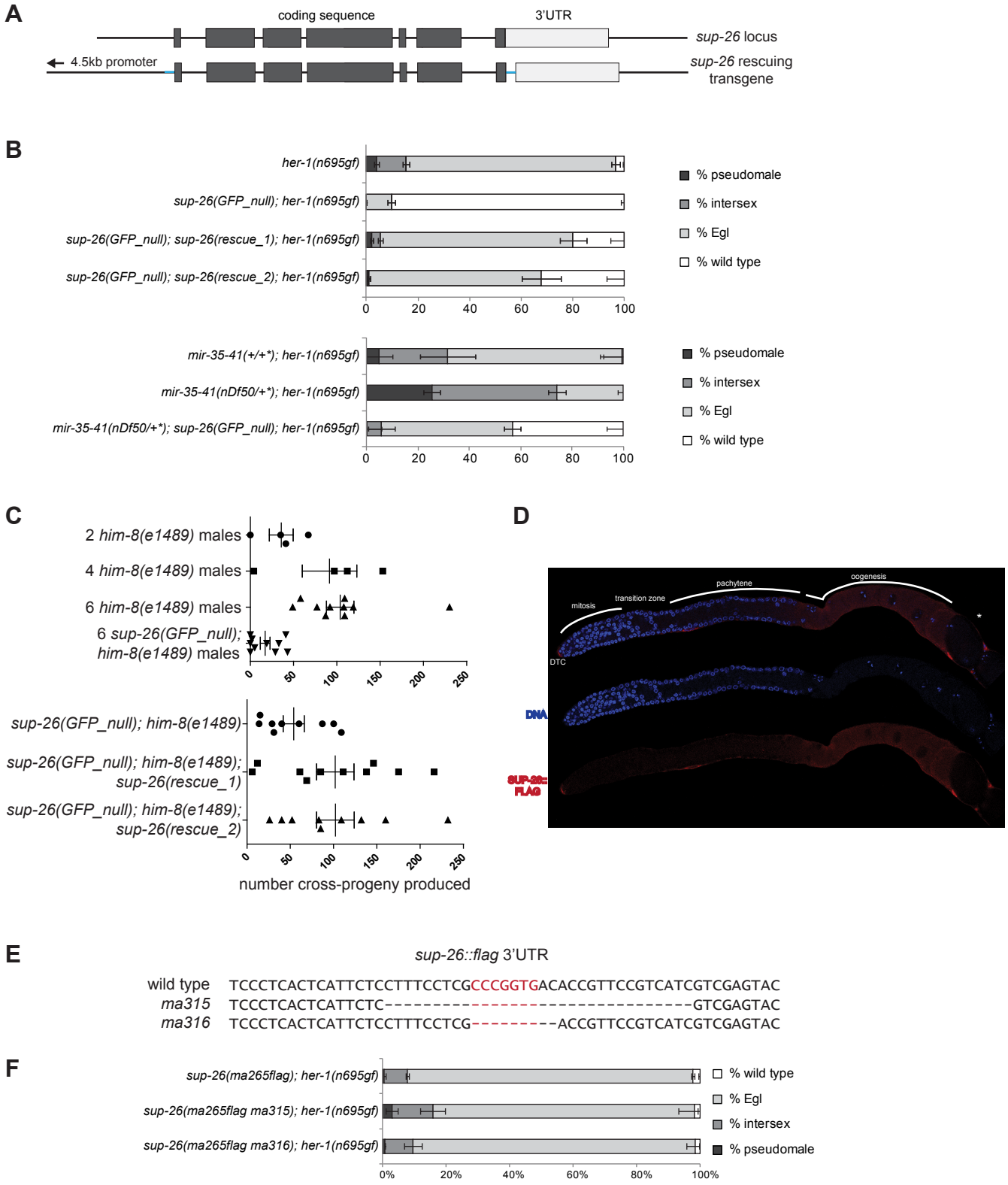

A
coding sequence
3'UTR
sup-26 locus
4.5kb promoter
sup-26 rescuing transgene
B
her-1(n695gf)
sup-26(GFP_null); her-1(n695gf)
sup-26(GFP_null); sup-26(rescue_1); her-1(n695gf)
sup-26(GFP_null); sup-26(rescue_2); her-1(n695gf)
% pseudomale
% intersex
% Egl
% wild type
0 20 40 60 80 100
mir-35-41(+/+*); her-1(n695gf)
mir-35-41(nDf50/+*); her-1(n695gf)
mir-35-41(nDf50/+*); sup-26(GFP_null); her-1(n695gf)
C
2 him-8(e1489) males
4 him-8(e1489) males
6 him-8(e1489) males
6 sup-26(GFP_null); him-8(e1489) males
sup-26(GFP_null); him-8(e1489)
sup-26(GFP_null); him-8(e1489); sup-26(rescue_1)
sup-26(GFP_null); him-8(e1489); sup-26(rescue_2)
0 50 100 150 200 250
number cross-progeny produced
D
mitosis
transition zone
pachytene
oogenesis
DTC
DNA
SUP-26::FLAG
E
sup-26::flag 3'UTR
wild type TCCCTCACTCATTCTCCTTTCCTCGCCCGGTGACACCGTTCCGTCATCGTCGAGTAC
ma315 TCCCTCACTCATTCTC----------GTCGAGTAC
ma316 TCCCTCACTCATTCTCCTTTCCTCG---------ACCGTTCCGTCATCGTCGAGTAC
F
sup-26(ma265flag); her-1(n695gf)
sup-26(ma265flag ma315); her-1(n695gf)
sup-26(ma265flag ma316); her-1(n695gf)
% wild type
% Egl
% intersex
% pseudomale
0% 20% 40% 60% 80% 100%

Figure S4

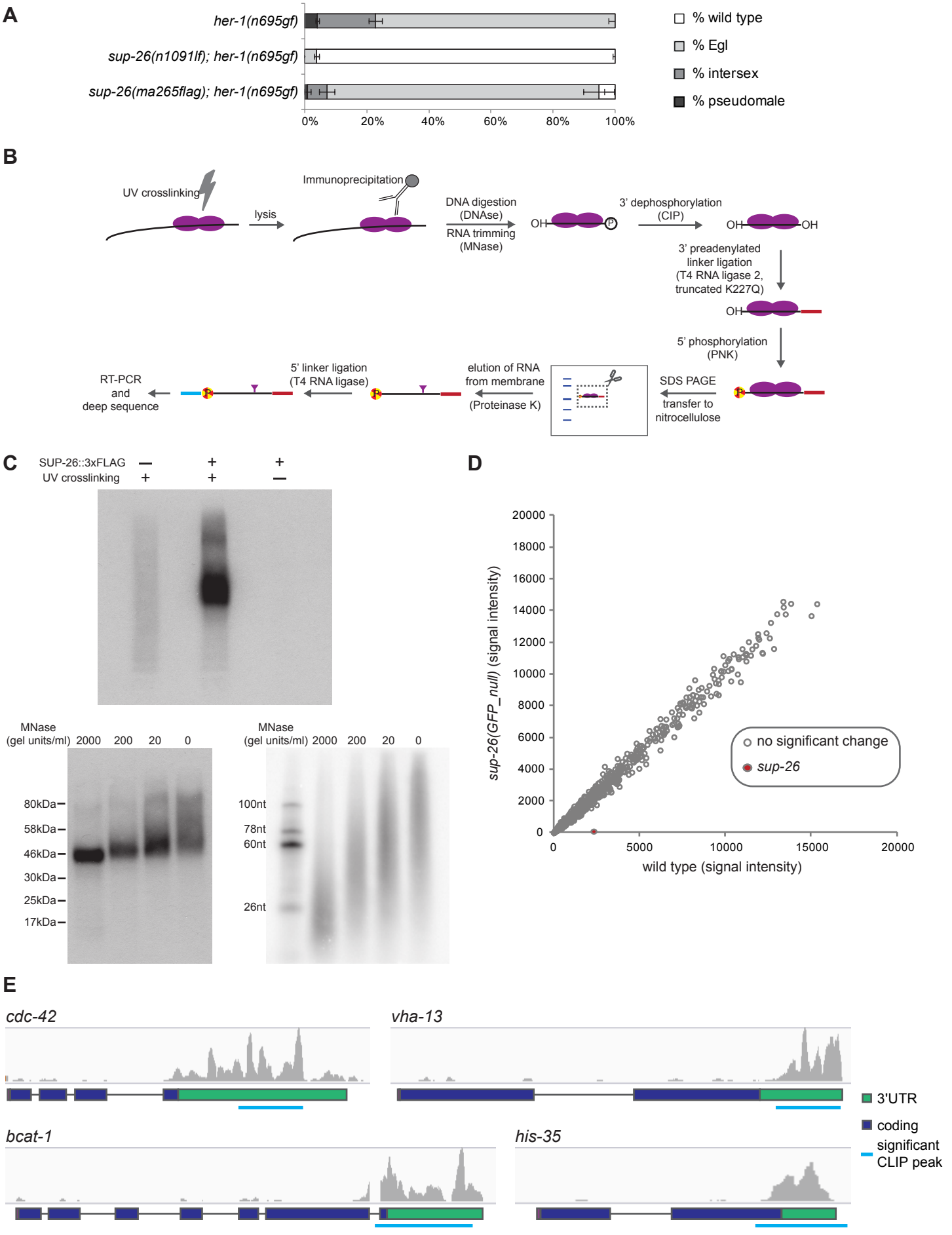

A
her-1(n695gf)
sup-26(n1091lf); her-1(n695gf)
sup-26(ma265flag); her-1(n695gf)
0%
20%
40%
60%
80%
100%
% wild type
% Egl
% intersex
% pseudomale
B
UV crosslinking
lysis
Immunoprecipitation
DNA digestion (DNAse)
RNA trimming (MNase)
3' dephosphorylation (CIP)
3' preadenylated linker ligation (T4 RNA ligase 2, truncated K227Q)
5' phosphorylation (PNK)
SDS PAGE
transfer to nitrocellulose
elution of RNA from membrane (Proteinase K)
5' linker ligation (T4 RNA ligase)
RT-PCR and deep sequence
C
SUP-26::3xFLAG
UV crosslinking
MNase (gel units/ml) 2000 200 20 0
80kDa
58kDa
46kDa
30kDa
25kDa
17kDa
100nt
78nt
60nt
26nt
D
sup-26(GFP_null) (signal intensity)
wild type (signal intensity)
no significant change
sup-26
E
cdc-42
vha-13
bcat-1
his-35
3'UTR
coding
significant CLIP peak

Figure S5

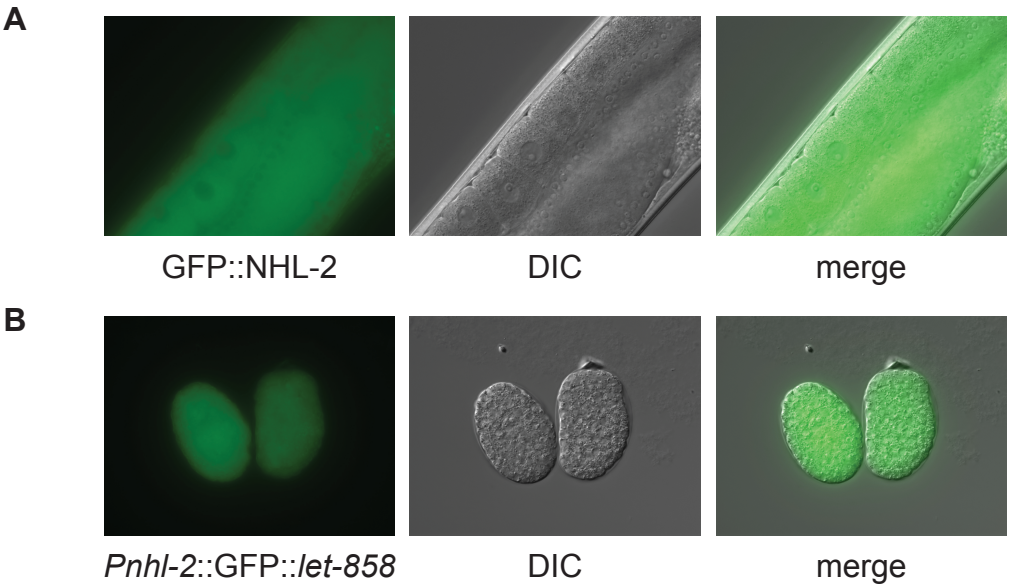
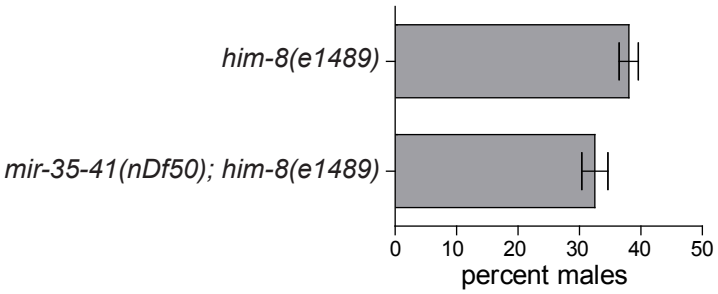


Figure S6


him-8(e1489)
mir-35-41(nDf50); him-8(e1489)
0
10
20
30
40
50
percent males

Supplemental Experimental Procedures

Q-PCR primers

	forward	reverse
her-1	TTGCTCGCTTCTGGATAATG	CAGACTCCTCGGAAAGTTCG
W01F3.2	GCTCTCGAACGGTGGTAAAT	GTAAATGTGCGCATGTCCTT
ZK970.7	CCAGAAGCAAAGAAAGCTGA	GCTGGGAGGGTGGTAAGATA
B0024.4	GTCCAAGAGACGATGCTGAA	CGACAGGAAAGAACATTTGC
C53B7.3	CTAACTCTGGATCCACTTGCC	GTATTGGGCGACACAAACTC
cnc-4	TGGAGCAAAGTCCGTTGTAA	ATTCCGTAGCCACCGTACAT
ttr-21	TGGAACCATATGGGATTCTAAA	TGGAGCAAAGTCCGTTGTAA
ttr-26	AGACTGGATCAGACGGAACA	GCAGAGCCCGTTATAATTGC
C45B2.2	TTGAGAGACAAGGAGTTTGGG	GTCACGCTCGTAGTTCTTGC
cpi-1	CGCTGTTCTTGACGGAATAA	AACAACCTTAATTGGAACCATGT
ttr-15	TTTGGGAGAAAGATACTCTCGAC	CCTTCTTCACGTTGCAGTTG
B0348.2	CCTACGCTCACTGAATGCAC	CAGTAGCAGATTGGCCAGAA
F16B4.4	ACCTGCTGGAGGATATGGAC	GTCGAAACCTCCTTGGTTGT
K09D9.1	ATTTCGGAGAAGGACAATGC	CAACCTCCCATCGTTTCTTT
nhl-2	GAAGGCAGGAGAGGTTCTTG	AACGCACCGAGAAAGTCTAAA

Generation of transgenic strains by CRISPR/Cas9 genome editing

Plasmids encoding Cas9 and single guide RNAs (sgRNAs) targeting the site of interest and sites of visible “co-CRISPR” markers were injected into young adult germlines. F1 animals were cloned if they bore a co-CRISPR or coinjection event, or if they were wild type but had many affected siblings. F2 animals were genotyped by PCR for the desired heritable genome editing event.

Single guide RNAs targeting the 3’ end of the coding sequence of *sup-26* were generated in the U6-guide RNA scaffold described in Friedland et al., 2013. The protospacer sequences used, with underlined PAM, were TCCATTTATTGTGGATTCATCGG, GCCGATGAATCCACAATAAATGG, and TGAATCCACAATAAATGGACAGG. To generate *sup-26(GFP_null)* and *sup-26::flag*, these guides were injected with an equimolar concentration of a guide targeting *unc-22* as well as *eft-3::Cas9* and pRF4 (Mello et al. 1991; Friedland et al. 2013; Kim et al. 2014). For *sup-26(GFP_null)*, a vector previously used to generate a single-copy *sup-26* transcriptional reporter was used as a donor for homologous recombination (McJunkin and Ambros 2014). For the *sup-26::flag* donor, Gibson assembly PCR was used to introduce the following underlined sequence in frame prior to the stop codon, with 678bp and 887bp of homology upstream and downstream, respectively: GAATCCACAAGACTACAAAGACCATGACGGTGATTATAAAGATCATGATATCGATTACAAG GATGACGATGACAAGTAAATGGACA in pBluSKP.

To tag *nhl-2* at its endogenous locus, we used a donor containing the self-excising drug selection cassette according to the published protocol (Dickinson et al. 2015). To generate *nhl-*

2(ma371), the donor was generated by digesting pDD282 with SpeI and ClaI, and using the HiFi assembly cloning kit (New England Biolabs) to fuse the backbone to PCR fragments generated using the following primers to amplify fragments of the *nhl-2* locus from genomic DNA::
donor arm upstream of NHL-2 CDS
ACGTTGTAAAACGACGGCCAGTCGCCGGCATGAAGAAAATGGCATCCAAAGTGC
TCCAGTGAACAATTCTTCTCCTTTACTCATCTTCACGGAATGAACGTTCGACAC
donor arm containing beginning of NHL-2 CDS
CGTGATTACAAGGATGACGATGACAAGAGAAAAAAAGCGGCAGATCGGCCG
TCACACAGGAAACAGCTATGACCATGTTATCATCGGATTACGCCAGGAGGT
The sgRNA and Cas9 vector was generated by performing quickchange mutagenesis using pJW1219 as a template and a universal primer, AAGACATCTCGCAATAGGAGGTG, along with each of the following primers:
CTACCATTCACCGCCGAAGTGTTTAAGAGCTATGCTGGAAACA
CGACTTCGGCGGTGAATGGTGTTTAAGAGCTATGCTGGAAACA
CGGCCGACTTCGGCGGTGAAGTTTAAGAGCTATGCTGGAAACA
One correct clone for each guide RNA was injected along with the donor plasmid, and an integrated line was isolated by hygromycin selection. Animals from this line were heat shocked to yield a line in which the drug selection cassette is excised, fusing GFP and three FLAG tags in frame with the NHL-2 coding sequence at its N terminus (VT3554 *nhl-2(ma371)*).

To generate *nhl-2(ma372)*, quickchange mutagenesis was used to delete Cre recombinase from the donor plasmid used to generate *ma371* using the primers
ATTTTTGCTTTCGTCGTAAATCTACAC and
CTGAATCTCAAATATTTTATTAGAAAACACCAAC. The resulting vector was used as a donor plasmid, and injected alongside the three guides mentioned directly above. An integrated line was isolated by drug selection (VT3564 *nhl-2(ma372)*). In this strain, *nhl-2* upstream sequences and start codon are followed by the GFP coding sequence and the *let-858* 3'UTR. This is followed by a loxP site, a *sqt-1(e1350)* dominant Rolr cassette, a hygromycin resistance cassette, another loxP site, then the remainder of the *nhl-2* coding sequence (which is theoretically inactivated by the upstream transcriptional units). This strain does not contain the Cre recombinase gene. The result is an *nhl-2* null allele containing an *nhl-2* transcriptional reporter (*Pnhl-2::GFP::let-858)* (Dickinson et al. 2015).

For deletions of *mir-35* family seed matches in *sup-26* and *nhl-2*, an sgRNA vector adapted from Arribere et al., 2014 was used in which the Cas9-binding stem loop of the sgRNA was extended as in Chen et al., 2013 (pRB1017_enhanced). In conjunction, a vector in which the *eft-3::Cas9* cassette was subcloned in the pRB1017 backbone (pOI_90) was used to enhance the formation of extrachromosomal arrays via homology between injected plasmids. Both the *unc-22* sgRNA and a *dpy-10* sgRNA co-CRISPR marker were cloned into pRB1017_enhanced, and used as co-CRISPR markers (Arribere et al., 2014; Kim et al., 2014). For *sup-26*, the protospacer sequences used, with underlined PAM, were CGATGACGGAACGGTGTCAC<u>CGG</u>, CGGAACGGTGTCACCGGGCG<u>AGG</u>, and GATGACGGAACGGTGTCACC<u>GGG</u>. For *nhl-2*, the sequences were ATCCGCCTTTTTGTTGTCCC<u>CGG</u>, CAACACCGGGGACAACAAAA<u>AGG</u>, and CACCGGGGACAACAAAAAGG<u>CGG</u>.

To quantify the efficiency of CRISPR events around the *mir-35* seed match in *nhl-2*, an F1 was included in the analysis only if its progeny indicated that it harbored at least one co-CRISPR

event in each targeted co-CRISPR locus. For example, if the segregated F2s were consistent with the F1 having the genotype *dpy-10(mut/mut);unc-22(mut/mut)*, *dpy-10(mut/mut)*;*unc-22(mut/+)* or *dpy-10(mut/+)*;*unc-22(mut/+)*, the F1 was sequenced and included in the analysis. If the F2 phenotypes indicated that the F1 was *dpy-10(mut/+);unc-22(+/+)*, *dpy-10(mut/mut)*;*unc(+/+)* or *dpy-10(+/+)*;*unc-22(mut/mut)*, then the F1 was excluded from the analysis.

Quantification of fluorescent micrographs Epifluorescence images were obtained with a Zeiss Imager.Z1 using the 63x objective. Images were exported as jpeg and quantified in Fiji (ImageJ) by drawing a region of interest around each embryo of interest. The average fluorescence intensity per embryo is plotted. Background off-embryo signal was negligible.

HITS-CLIP library preparation

CLIP libraries were prepared as in Zisoulis et al., 2011, with cloning steps modified from Gu et al., 2011. Recently-starved plates were irradiated with 3kJ/m^2 in a Stratagene UV Stratalinker 1800. Worms were washed 3 times in M9 buffer, and pellets were stored at -80°C until use. Biological replicates were collected on different days. Pellets were thawed in cold homogenization buffer [100mM NaCl, 25mM HEPES pH=7.5, 250µM EDTA, 2mM DTT, 25U/ml RNAse inhibitor (ThermoFisher RiboLock), protease inhibitors (Complete Mini, Roche)] at 4°C. Samples were sonicated on ice at 30W using 20s pulses with 60s rest intervals (Branson SLPe). Sonication continued until pellet was nearly completely homogenized (such that only small volume pellet, ~50µl, was visible after the sample settles). Samples were centrifuged at 16000rcf at 4°C for 15min, and supernatant was moved to a fresh tube. Protein was quantified, and concentration was adjusted to 3mg/ml in homogenization buffer.

Trimming, Immunoprecipitation, 3' Ligation: For all wash steps, wash buffers were cold (4°C) and 500µl was used per wash per sample. For each sample, 50µl of Anti-FLAG M2 magnetic bead slurry (Sigma-Aldrich) was washed 3 times in homogenization buffer. (Anti-FLAG M2 beads were only pipetted with beveled tips.) For each sample, 1ml of 3mg/ml lysate was added to the washed beads and rotated overnight at 4°C. Beads were washed twice with wash buffer (20mM Tris-HCl pH=7.4, 137mM NaCl, 0.10% SDS, 0.50% sodium deoxycholate, 0.50% NP-40), twice with high-salt wash buffer (100mM Tris-HCl pH=7.4, 685mM NaCl, 0.10% SDS, 0.50% sodium deoxycholate, 0.50% NP-40), and twice with PNK buffer (50mM Tris-HCl pH=7.4, 10mM $MgCl_2$, 0.50% NP-40), all at 4°C. A 100µl reaction containing 10U TurboDNAse in 1x reaction buffer (ThermoFisher) supplemented with 0.4U/µl RNAse inhibitor, was added to washed beads and incubated at 37°C for 15min with intermittent shaking (1200rpm for 1min, then 1200rpm for 15s every 3min). Beads were washed twice with PNK buffer. Micrococcal nuclease diluted in 500µl MNase buffer (50mM Tris-HCl pH=7.9, 5mM $CaCl_2$) was added to beads, followed by 10min incubation at 4°C with rotation. (Micrococcal nuclease concentration must be empirically determined for each protein. See Figure S6B. We used 20gel units/ml because this concentration yielded RNA fragment sizes that were of short but mappable length, ≥25nt excluding adaptors.) Beads were washed twice with PNK+EGTA buffer (50mM Tris-HCl pH=7.4, 20mM EGTA, 0.50% NP-40), twice with wash buffer, and twice with PNK buffer, all at 4°C. An 80µl reaction containing 30U CIP in 1x reaction buffer (New England Biolabs) was added to beads, followed by incubation at 37°C for 10min with shaking at 1200rpm for 15s every

3min. Beads were washed twice with PNK+EGTA buffer, twice with PNK buffer, and once with 0.1mg/ml BSA. The 3' adapter ligation was performed using 4.5µl homemade T4 Rnl2 truncated K227Q (concentration unknown) in a 45µl reaction containing 15% DMSO, 15% PEG 8000, 50mM Tris-HCl pH=7.5, 10mM $MgCl_2$, 10mM DTT, 100µg/ml BSA, and 66.6µM of the 3' adapter. Ligation mix was incubated with beads for 4h at room temperature, then at 16°C overnight with shaking at 1300rpm for 15s every 5min. Beads were washed thrice with PNK buffer at 4°C.

5' Labeling, SDS-PAGE: End labeling was performed in an 80µl reaction containing 40U T4 PNK in 1x reaction buffer (New England Biolabs) and 300µCi ^{32}P-γ-ATP. Reactions were incubated at 37°C for 10min with intermittent shaking, then supplemented with 10µl of 1mM nonradioactive ATP and incubated for 5 additional min. Beads were washed 3 times with PNK+EGTA buffer and resuspended in 30µl PNK+EGTA buffer with 10µl 4x NuPAGE LDS sample buffer (ThermoFisher). Samples were incubated at 70°C for 10min, then loaded onto a 10% Bis-Tris gel in MOPS running buffer supplemented with 500µl NuPAGE antioxidant (ThermoFisher). Gel was run at 180V, then transferred to nitrocellulose in Bis-Tris transfer buffer at 40V for 4h at 4°C. The membrane was rinsed in PBS and exposed to film or phosphor screen. The film was used as a mask to excise bands/smears containing protein-RNA complexes. Proteinase K (Roche/Sigma-Aldrich) was diluted to 4mg/ml in Proteinase K buffer (100mM Tris-HCl pH=7.5, 50mM NaCl, 10mM EDTA) and incubated at 37°C for 20min before being added to membrane slices. Of the proteinase K mix, 200µl was added to each membrane slice, and these were incubated at 37°C for 20min with shaking at 1200rpm. Then 200µl of Proteinase K buffer supplemented with 7M urea was added to each slice, and they were incubated for 20 additional min at 37°C with shaking at 1000rpm. Each sample was incubated with 530µl Acid Phenol:Chloroform (5:1, pH=4.5) at 37°C for 20min at 1000rpm; then the aqueous phase was extracted and precipitated with 50µl 3M sodium acetate pH=5.2, 7.5µg GlycoBlue (ThermoFisher) and 1000µl of ethanol:isopropanol (1:1) in a siliconized tube overnight at -80°C. Pellet was spun down at 16000rcf for 20min at 4°C, then washed twice with 300µl cold 75% ethanol.

5' ligation, reverse transcription, PCR: Pellet was resuspended in 3µl water, then incorporated into a 10µl reaction containing 5U T4 RNA ligase 1 in 1x reaction buffer (New England Biolabs) supplemented with 1mM ATP, 1U/µl RNAse inhibitor, 0.1µg/µl BSA, 10% DMSO, and 10µM barcoded 5' adapter. Reactions were incubated for 6h at 15°C, followed by overnight at 4°C. Each reaction was diluted with 300µl water, then extracted with 400µl Acid Phenol:Chloroform (5:1, pH=4.5). RNA was precipitated with 50µl 3M sodium acetate pH=5.2, 7.5µg GlycoBlue (ThermoFisher), and 1000µl of ethanol:isopropanol (1:1) in a siliconized tube overnight at -80°C. Pellet was spun down at 16000rcf for 15min at 4°C, then washed twice with 300µl cold 75% ethanol and resuspended in 9.5µl water. Reverse transcription was performed using Superscript III (ThermoFisher), according to the manufacturer's specifications, with 0.5µM of the RT oligo. Libraries were amplified from cDNA using AccuPrime SuperMix I (ThermoFisher), with the first 10 PCR cycles annealing at 55°C, followed by 14 or more cycles annealing at 65°C. Libraries were sequenced on an Illumina NextSeq500, using the Small RNA sequencing primer.

All oligonucleotides were ordered from IDT with the indicated modifications.

3' Adapter: AppCTGTAGGCACCATCAAT/ddC/

5' Adapters (DNA/RNA hybrid oligo):
barcode A: GTTCArGrArGrUrUrCrUrArCrArGrUrCrCrGrArCrGrArUrCrNrNrNrTrGrArC
barcode B: GTTCArGrArGrUrUrCrUrArCrArGrUrCrCrGrArCrGrArUrCrNrNrNrCrArGrT
barcode C: GTTCArGrArGrUrUrCrUrArCrArGrUrCrCrGrArCrGrArUrCrNrNrNrGrCrTrG
barcode D: GTTCArGrArGrUrUrCrUrArCrArGrUrCrCrGrArCrGrArUrCrNrNrNrArTrCrA
RT primer (CMo11518): ATTGATGGTGCCTACAG
PCR primers:
ATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGACGATC
CAAGCAGAAGACGGCATACGAGATATTGATGGTGCCTACAG

CLIP data analysis

Basespace: The full 3' adapter and PCR primer sequence was trimmed with 0.9 stringency (CTGTAGGCACCATCAATATCTCGTATGCCGTCTTCTGCTTG). 3' ends were then trimmed for PolyA/T tails (minimum length 1), and reads shorter than 27bp after trimming were discarded.

Galaxy: The trimmed FASTQ file was uploaded to Galaxy, and filtered by quality (quality cutoff = 20, percent of read above cutoff = 90). The FASTQ was converted to FASTA, and sequences were collapsed into unique reads. The 3bp randomer was trimmed from the 5' end, and the file was converted back to FASTQ. Reads were separated according to barcode, resulting in 8.1M, 2.1M, 3.2M, and 2.5M unique high-quality reads for replicates A, B, C and D, respectively. The UMass Biocore Seqmapping 1.2 pipeline was then used for mapping using Bowtie2 and Tophat2. First, Bowtie2 was used to align the reads to index files containing rRNA, miRNA, tRNA and snRNA and rmsk sequences allowing 2 mismatches (parameters -N 2 -v). Reads that aligned to the common RNA index files were separated into an annotated read count file. The remaining reads were aligned to the genome using Tophat2 (parameters --library-type fr-secondstrand -g 1). As a control for input RNA abundance, an RNAseq dataset generated using poly(A) selection from a similar sample (Accession number GSM1290044) was processed in the same way, except Tophat2, which was adjusted for the unstranded nature of the library (parameters -g 1).

Piranha: Plus and minus strands from alignment file were separated into two files for each alignment using samtools view. Files were converted from BAM to BED using bamtools convert. BED files were used as input for Piranha version 1.2.0, with binning of 100nt or 200nt (-z 100 or -z 200), and using the RNAseq sample (Accession number GSM1290044) as the covariate. Peaks that were called using 100nt or 200nt binning were combined, and plus and minus strand files were rejoined into a single BED.

Annotation: Bedtools intersect was used to identify overlap between CLIP peaks and Ensembl 3'UTR annotations. Peaks that did not intersect with Ensembl 3'UTRs were intersected with Modencode, Targetscan, and Wormbase 3'UTR annotations, and other Ensembl gene features, and curated by hand (Mangone et al. 2010; Jan et al. 2011). Gene ontology analysis was performed using DAVID (Dennis et al. 2003).

***mir-35* family microRNA conservation** Small RNA deep sequencing efforts in *C. briggsae, C. remaneii,* and *C. brenneri* previously identified the *mir-35* family members in *C. elegans*-related species (Shi et al. 2013). Albritton et al., 2014 recently determined which genomic contigs in these species are sex-linked or autosomal via genomic copy number analysis, thereby allowing the X/autosome assignment of *mir-35* family members.

Male mating efficiency assays The protocol from Hodgkin, 1983 was used, with slight modification. Six L4 males and 6 L4 *fog-2(q71)* females were placed together on a 3cm NGM plate with a spot of HB101 food ~1.5cm in diameter. After 24h, the twelve adults were removed, and progeny were counted on the following day.

List of strains

CB5362 *tra-2(ar221)* II; *xol-1(y9)* X
MT1446 *her-1(n695gf)* V
TY1807 *xol-1(y9)* X
VT2935 *mir-35-41(nDf50)* II; *nEx1187* [*mir-35 mir-45*(genomic) + *sur-5::GFP*]
VT3042 *mir-35-41(nDf50)* II; *her-1(n695gf)* V; *nEx1187* [*mir-35 mir-45*(genomic) + *sur-5::GFP*]
VT3315 *mir-35-41(nDf50)/* mIn1 [*dpy-10(e128) mIs14*] II; *sup-26(ma268GFP_null)* III
VT3362 *mir-35-41(nDf50)* II; *him-8(e1489)* IV; *nEx1187* [*mir-35 mir-45*(genomic) + *sur-5::GFP*]
VT3343 *mir-35-41(nDf50)/* mIn1 [*dpy-10(e128) mIs14*] II; *her-1(n695gf)* V
VT3077 *mir-35-41(nDf50)/* mIn1 [*dpy-10(e128) mIs14*] II; *sup-26(n1091)* III; *her-1(n695gf)* V
VT3245 *mir-35-41(nDf50)* II; *sup-26(n1091)/* qC1 [*dpy-19(e1259) glp-1(q339) qIs26*] III; *her-1(n695gf)* V
VT3081 *mir-35-41(nDf50)/* mIn1 [*dpy-10(e128) mIs14*] II; *sup-26(gk426)* III; *her-1(n695gf)* V
VT3318 *mir-35-41(nDf50)/* mIn1 [*dpy-10(e128) mIs14*] II; *sup-26(ma268GFP_null)* III; *her-1(n695gf)* V
VT3363 *mir-35-41(nDf50)/* mIn1 [*dpy-10(e128) mIs14*] II; *nhl-2(ok818)* III; *her-1(n695gf)* V
VT3088 *sup-26(n1091)* III; *her-1(n695gf)* V
VT3089 *sup-26(gk426)* III; *her-1(n695gf)* V
VT3364 *nhl-2(ok818)* III; *her-1(n695gf)* V
VT3121 *sup-26(ma265flag)* III
VT3125 *sup-26(ma265flag)* III*; her-1(n695gf)* V
VT3366 *sup-26(ma265flag ma315)* III*; her-1(n695gf)* V
VT3418 *mir-35-41(nDf50)/* mIn1 [*dpy-10(e128) mIs14*] II; *nhl-2(ok818)*
VT3422 *mir-35-41(nDf50)* II; *nhl-2(ok818) sup-26(ma268GFP_null)* III; *nEx1187* [*mir-35 mir-45*(genomic) + *sur-5::GFP*]
VT3437 *sup-26(ma265flag ma316)* III*; her-1(n695gf)* V
VT3423 *nhl-2(ma368) lon-1(e168)* III
VT3152 *sup-26(ma268GFP_null)* III
VT3154 *sup-26(ma268GFP_null)* III; *her-1(n695gf)* V
VT3243 *maIs398* [*sup-26(rescue_1) + cb unc-119 (+)*] II; *sup-26(ma268GFP_null)* III; *her-1(n695gf)* V
VT3244 *maIs399* [*sup-26(rescue_2) + cb unc-119 (+)*]/mIn1 [*dpy-10(e128) mIs14*] II; *sup-26(ma268GFP_null)* III; *her-1(n695gf)* V
VT3253 *maIs398* [*sup-26(rescue_1) + cb unc-119 (+)*] II*; sup-26(ma268GFP_null)* III; *him-8(e1489)* V
VT3254 *maIs399* [*sup-26(rescue_2) + cb unc-119 (+)*] II*; sup-26(ma268GFP_null)* III; *him-8(e1489)* V
VT3554 *nhl-2(ma371)*
VT3564 *nhl-2(ma372)*
VT3630 *sup-26(ma268GFP_null ma391)*
VT3631 *sup-26(ma268GFP_null ma392)*

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