Supplemental: Effects of ADARs on small RNA processing pathways in *C. elegans*

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Supplemental Methods

Northern blots

Total RNA (75 µg), prepared as previously described in the methods in the main text, was enriched for small RNAs using the mirVana isolation kit (Ambion), and 2 µg was run on a 15% denaturing gel and transferred onto a Hybond-NX membrane (Amersham Biosciences). RNA was cross-linked to the membrane using 1M 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), for 1 hour at 50 °C. The membrane was washed in 1x TBE and stored dry at 25 °C.

For northern analysis, membranes were pre-incubated with ULTRAhyb-Oligo buffer (Ambion) for ~1 hour at 42 °C and probed overnight at 42 °C with a DNA oligonucleotide end labeled using T4 PNK (New England Biolabs). Blots were washed 4 x 20 minutes with 2x SSC at 42 °C, autoradiographed and then quantified using ImageQuant (Molecular Dynamics). The U6 snRNA was used for normalization of each lane, and 3-4 biological replicate experiments were performed for each tested RNA.

RT-PCR

For quantitative RT-PCR (qRT-PCR), total RNA, prepared as previously described in the methods in the main text, was DNased using TURBO DNase (Ambion). Reverse transcription (RT) was performed on 5 µg of DNased RNA using Thermoscript (Invitrogen) in a 20 µL reaction. After RT, the sample was diluted to 100 µL with water. qPCR was then performed on 2 µL of cDNA, on a LightCycler 2.0, using a Light Cycler DNA Master^{PLUS} Syber Green kit (Roche), according to manufacturer's guidelines. For primary miRNAs, an RT primer specific to each pri-miRNA was designed to bind within the pre-miRNA loop and destabilize the stem-loop structure. The forward PCR primer was outside of the pre-miRNA sequence, allowing only the pri-miRNA sequence to amplify. RT was performed on *gpd-3*, C18C4.7 and F29F11.6 using an oligo-dT primer, with gene specific primers used for PCR. All PCR

products were ~100 bp in size. Standard curves were performed for each primer pair, using a serial dilution of the PCR product, to determine the absolute concentration of each sample. The *gpd-3* gene was used for normalization between samples, as it does not change expression throughout development [1]. 3-5 biological replicates were performed for each tested RNA species.

Gene expression microarrays

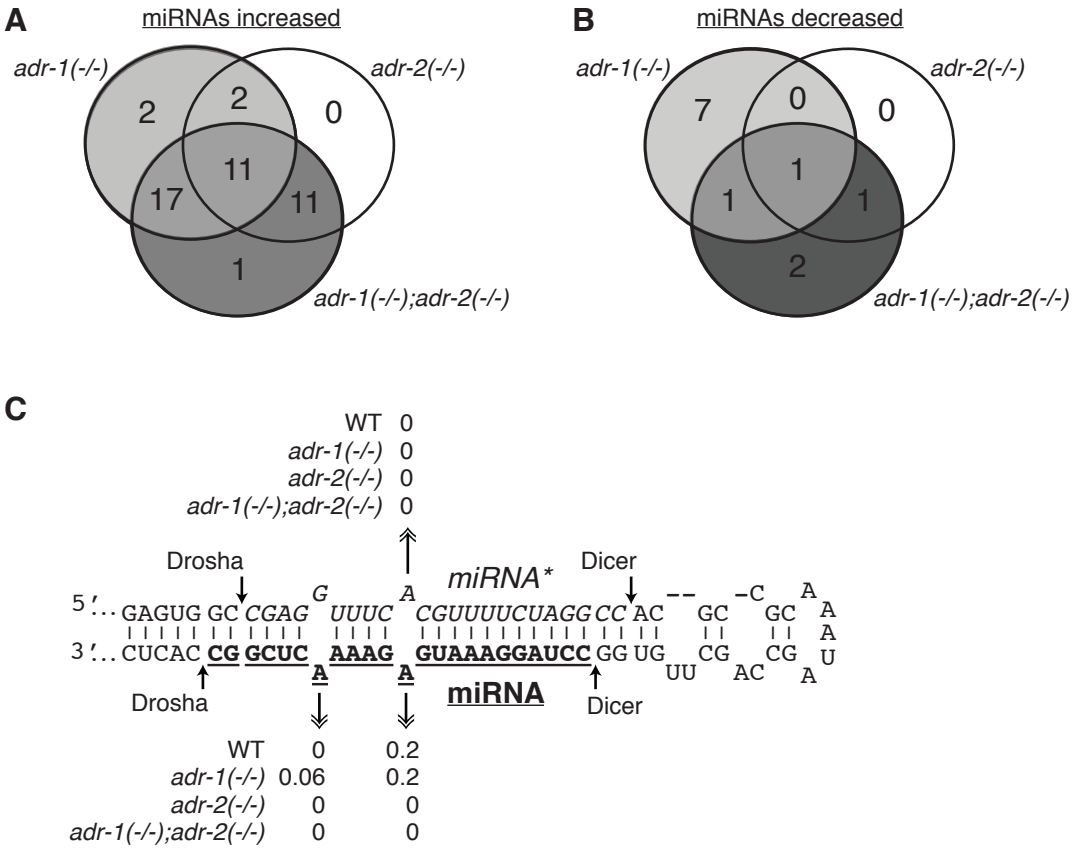
Total RNA (1 µg), as previously prepared, was used to make cRNA with a Two-Color Quick Amp Labeling kit (Agilent Technologies, Ag). cRNA from WT worms was labeled with Cy3 and cRNA from *adr-1(-/-);adr-2(-/-)* was labeled with Cy5. cRNA was then fragmented and hybridized to a *C. elegans* v2 Gene Expression array (Ag) in Hi-RPM hybridization buffer at 65 °C for 17 hours, in a SureHyb hybridization chamber (Ag). Arrays were then washed with Gene Expression wash buffer (Ag), dipped in acetonitrile and air-dried. Arrays were scanned on a G2505C Microarray Scanner (Ag) at 5 µm resolution. Images were loaded into the Feature Extraction software v10.5 (Ag) to align grid positions, measure array and background signals and to initially LOWESS normalize the data. Intensity data were further normalized between arrays in R, using the quantile normalization procedure in the preprocess Core library [2] and batch effects were reduced in R using ComBat [3]. Results were analyzed using GeneSifter (Geospiza) to determine which genes in *adr-1(-/-);adr-2(-/-)* were changed in a significant manner compared to WT, using a t-test with a Benjamini and Hochberg correction. Four biological replicate arrays were performed.

DNA Oligonucleotide sequences

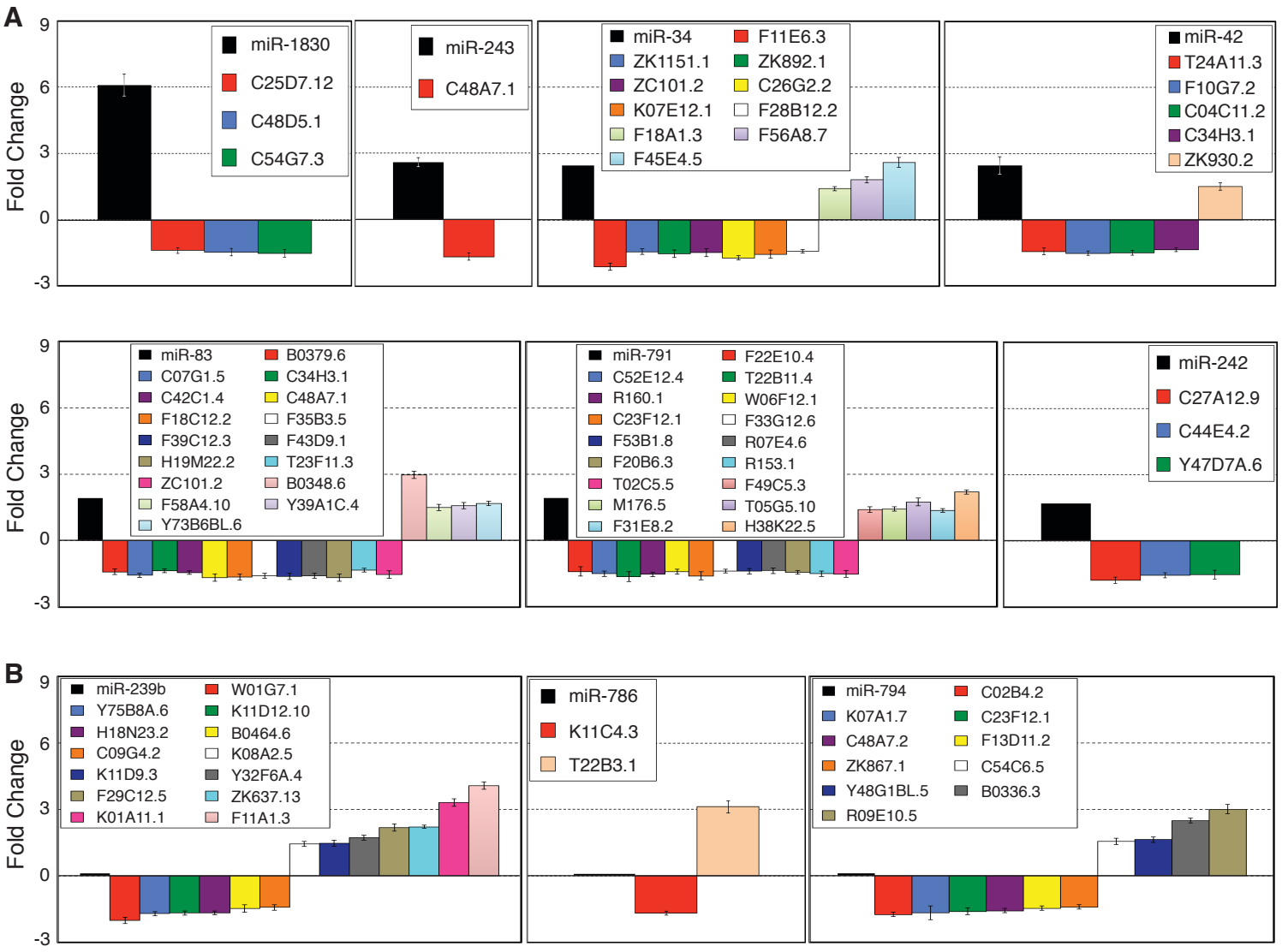
Name	Method	Sequence (5' to 3')
miR-800 probe	northern blot	CGGCAGACAATTTCCGAGTTTG
miR-1830 probe	northern blot	GCCTAGAAAACGTGAAACCTCG
miR-243 probe	northern blot	GATATCCCGCCGCGATCGTACCG
miR-43 probe	northern blot	GCGACAGCAAGTAAACTGTGATA
miR-42 probe	northern blot	TCTGTAGATGTTAACCCGGTGA
lin-4 probe	northern blot	TCCCTGAGACCTCAAGTGTGA
miR-240 probe	northern blot	AGCGAAGATTTGGGGGCCAGTA
U21R-1 probe	northern blot	GCACGGTTAACGTACGTACCA
pri-mir-800 RT primer	qRT-PCR	GATTATAAGAGTGGCCTAACTCGGAAATTGTC
pri-mir-800 Fw primer	qRT-PCR	CATTCATTGCGTCCGGTGTGTAGGATTTTCG
pri-mir-1830 RT primer	qRT-PCR	GAGTTTTCTCATTTCCTAGGCCACAAGCTGGC
pri-mir-1830 Fw primer	qRT-PCR	GAAATGTTCGGTTTTCACTGTCGGTGAGTGGC
pri-mir-253 RT primer	qRT-PCR	GAGAAACGACGTTCACTGTCGCCGCCCTTCCCACAAC
pri-mir-253 Fw primer	qRT-PCR	GAATTGTTCCGATCGCCGCTCTTTTCACAC
gpd-3 Fw primer	qRT-PCR	GGAGGAGCCAAGAAGGTC
gpd-3 RT primer	qRT-PCR	AAGTGGAGCAAGGCAGTT
pri-mir-253 RT primer	RT-PCR/ sequencing	CTGGAACATTGTGAAATTAAAGTATTTTTGATAAATTATACAC
pri-mir-253 Fw primer	RT-PCR/ sequencing	GAATTGTTCCGATCGCCGCTC
gpd-3 Fw primer	qRT-PCR	GGAGGAGCCAAGAAGGTC
gpd-3 Rv primer	qRT-PCR	AAGTGGAGCAAGGCAGTT
C18C4.7 Fw primer	qRT-PCR	GCGACATGATGCCAATTATGATGCACC
C18C4.7 Rv primer	qRT-PCR	CTGAAGCTCGCCTGGAGGAAGTAATACTC
F29F11.6 Fw Primer	qRT-PCR	CGCCGTGTGATGCGACCAACCGATG
F29F11.6 Rv Primer	qRT-PCR	CCTCCGTATTCGAATAAACGGAGCAAG

References

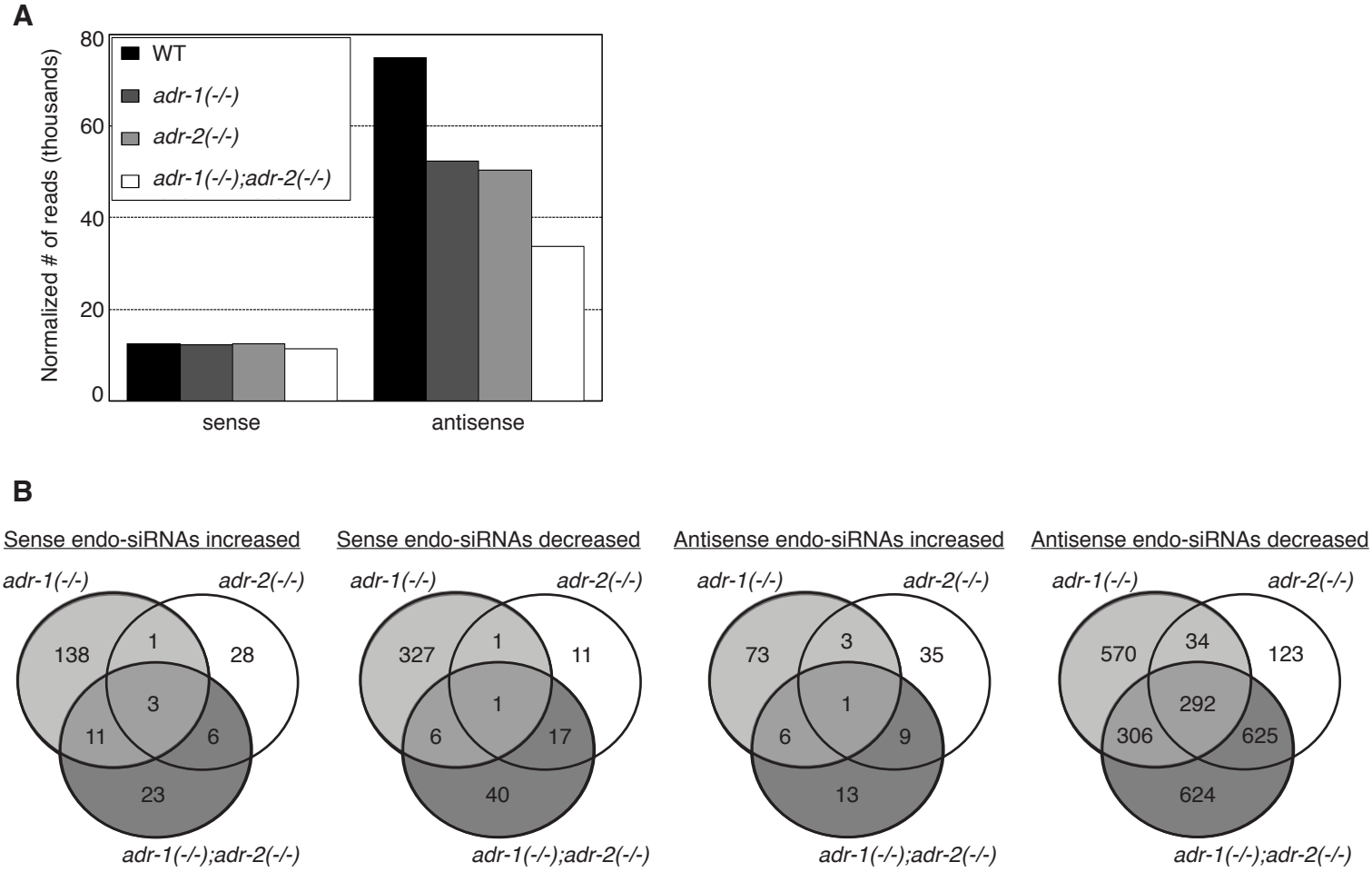
1. Jiang M, Ryu J, Kiraly M, Duke K, Reinke V, et al. (2001) Genome-wide analysis of developmental and sex-regulated gene expression profiles in Caenorhabditis elegans. Proc Natl Acad Sci U S A 98: 218-223.
2. Bolstad BM, Irizarry RA, Astrand M, Speed TP (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19: 185-193.
3. Johnson WE, Li C, Rabinovic A (2007) Adjusting batch effects in microarray expression data using empirical Bayes methods. Biostatistics 8: 118-127.



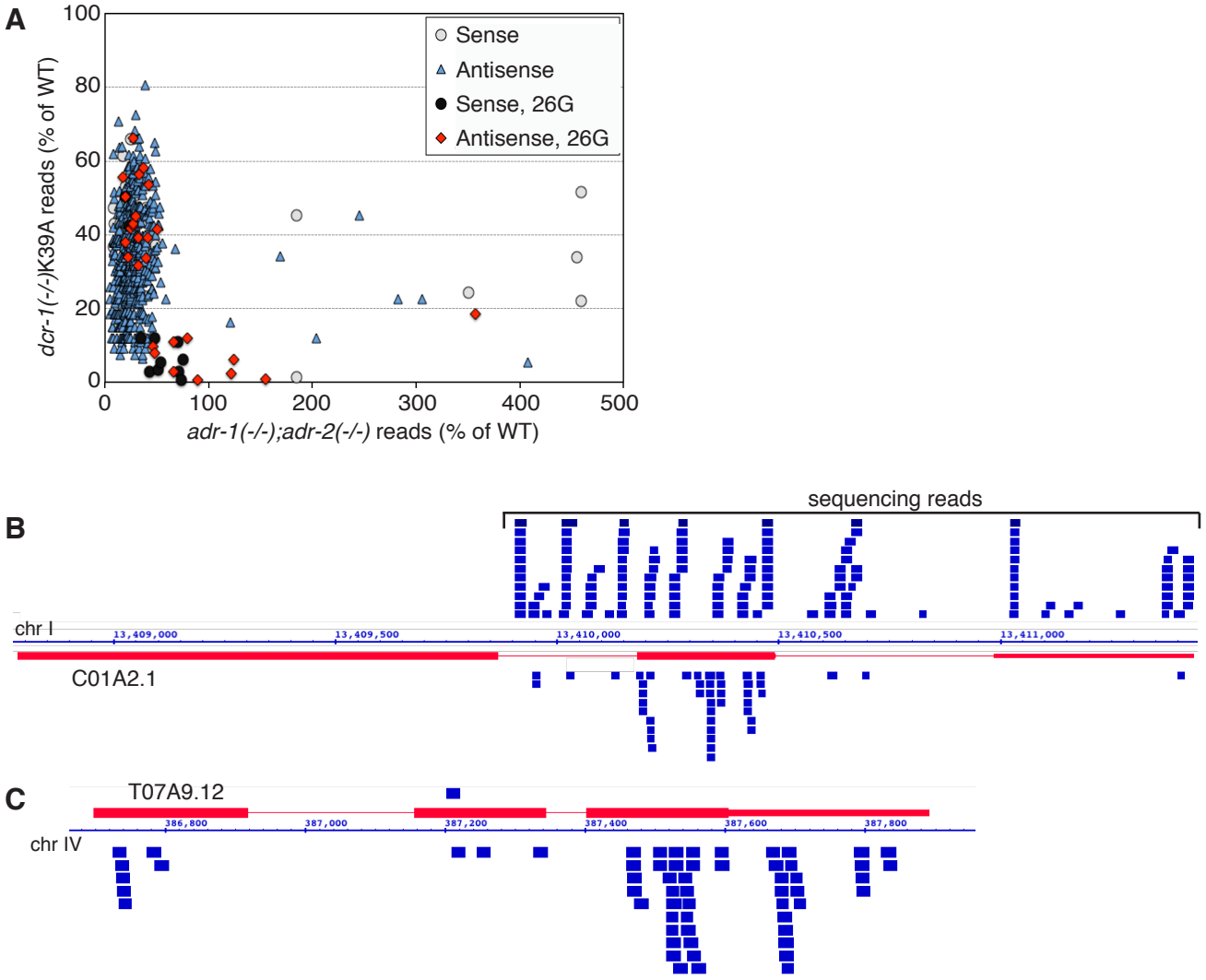
Supplemental Figure 1- ADARs affect the levels of many miRNAs, but few mature miRNAs are edited. *(A-B)* Venn diagrams showing the overlap of miRNA loci with an increased and/or decreased number of sequencing reads in ADAR mutants compared to WT. See also Fig. 1 and Supplemental Table 1. All loci were altered by ≥2-fold in at least a single ADAR mutant compared to WT, or ≥1.2-fold in at least two mutants. *(C)* Structure of pri-mir-1830, with miRNA sequence underlined and bold, and miRNA* sequence italicized. Drosha and Dicer cleavage sites are indicated with single arrowheads. Reads aligned to the genome were re-examined, to determine if mismatched adenosines were edited. Percent editing at indicated adenosines (double arrowhead) is tabulated for each strain. The adenosine with higher editing levels was identified by GNUMAP, but was not statistically significant because its p-value was >0.05. The adenosine with lower editing levels was not identified by GNUMAP.



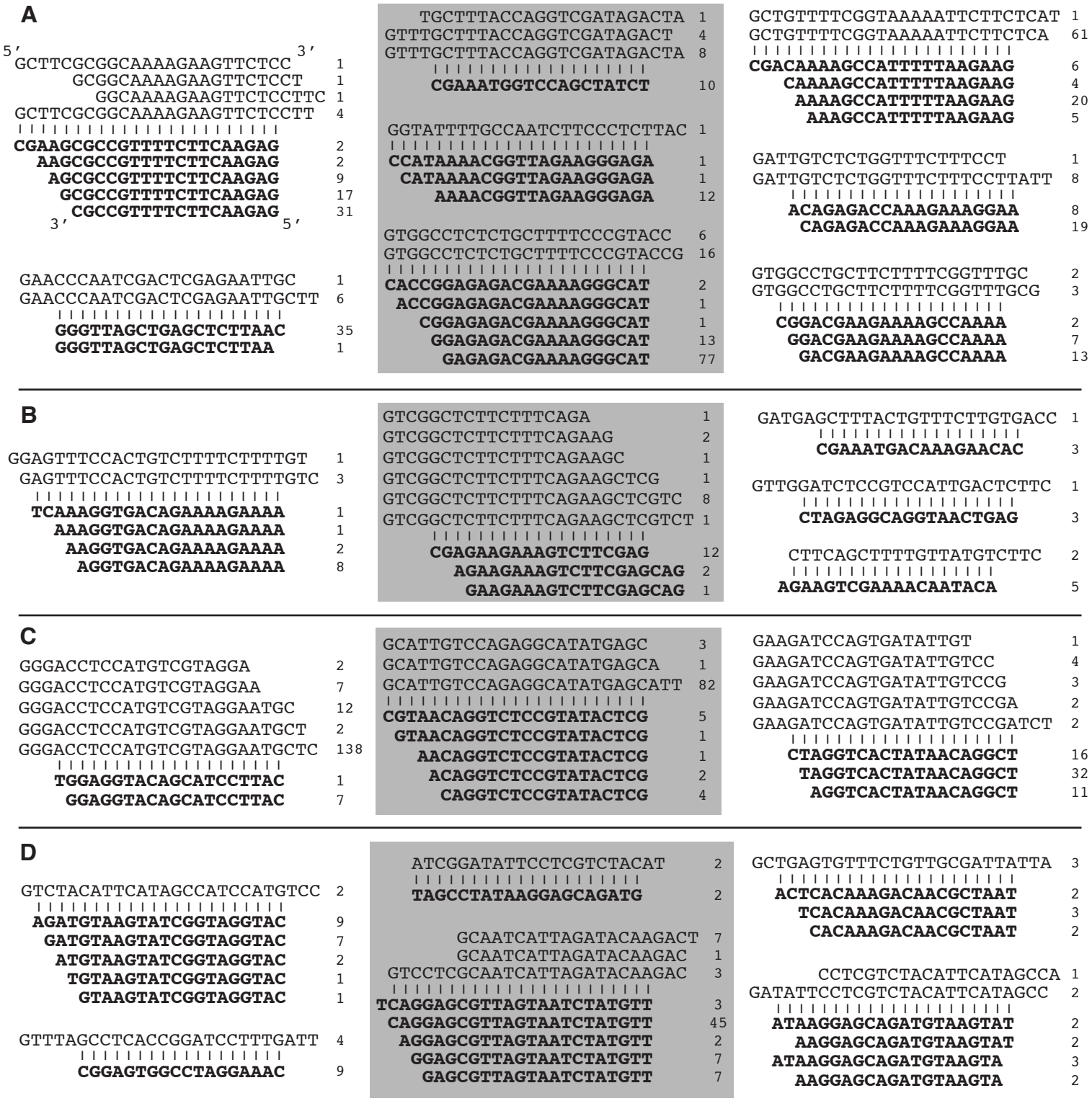
Supplemental Figure 2- miRNAs with altered levels have predicted mRNA targets with correspondingly affected levels. *(A)* Representative data for 7 of the 27 miRNAs whose levels are increased in *adr-1(-/-);adr-2(-/-)* worms compared to WT. For each miRNA, shown are the fold change in the miRNA and predicted mRNA targets. The change in levels shown for miR-1830, miR-243 and miR-42 levels were determined by northern blot, with error bars indicating STD; other changes in miRNAs were determined by sequencing. Error bars of mRNAs indicate SEM. See also Fig. 1E. Only two miRNAs had decreased levels in *adr-1(-/-);adr-2(-/-)* worms compared to WT, of which only miR-253 had predicted mRNA targets on TargetScan (Supplemental Table 2). Five were affected on the microarray, and three showed increased levels, consistent with the decrease in miR-253 levels, although this was not statistically significant at a p-value <0.05. *(B)* Ten miRNAs whose levels were not significantly affected in *adr-1(-/-);adr-2(-/-)* worms were also analyzed, and they were found to have 91 predicted mRNA targets with levels changed on the microarray. These 91 mRNAs had levels similarly affected compared to the overall microarray, where 53% of genes had increased levels and 47% had decreased levels. Shown are three representative miRNAs (miR-239b, miR-786 and miR-794). Also tested, but not shown, were *lys-6*, miR-79, miR-251, miR-254, miR-784, miR-786, miR-792, miR-794 and miR-798.



Supplemental Figure 3- Many endo-siRNA loci had an altered number of sequencing reads in ADAR mutant worms compared to WT. *(A)* The normalized number of total sense and antisense reads in each strain. *(B)* Venn diagrams showing the overlap of endo-siRNA loci with an increased and/or decreased number of sense and/or antisense reads in each ADAR mutant compared to WT. All loci were affected ≥2-fold in at least one ADAR mutant strain compared to WT, or ≥1.2-fold in at least two mutant strains. See also Fig. 2 and Supplemental Table 4.

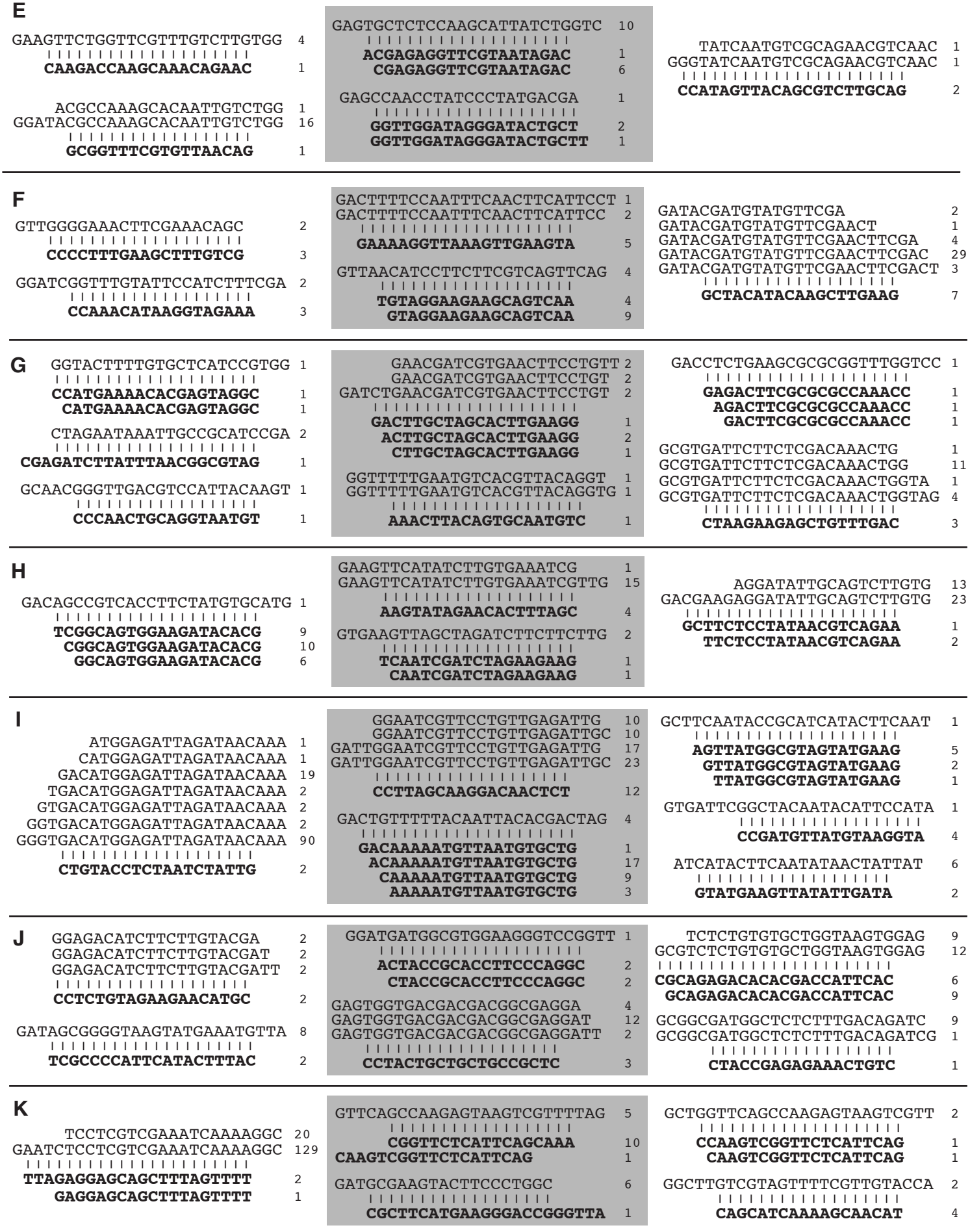


Supplemental Figure 4- Most endo-siRNA loci with an altered number of sequencing reads in a strain with a mutation in Dicer's helicase domain (*dcr-1(-/-)*K39A) also had an altered number of reads in ADAR mutants. *(A)* Endo-siRNA loci with an altered number of reads in both *adr-1(-/-);adr-2(-/-)* and *dcr-1(-/-)*K39A strains. Graphed are the number of reads in each mutant strain, as a percentage of WT reads. See also Fig. 4 and Supplemental Table 4. *(B)* Screen shot of sequence data, as visualized on the Integrated Genome Browser, of WT sequencing reads aligning to the C01A2.1 locus, which is representative of an ADR-2 Inverse 26G locus. The red line indicates the transcribed sequence of the C01A2.1 gene, with a full line representing an exonic sequence, a medium line representing a UTR sequence and a thin line representing an intronic sequence. The chromosomal position is indicated by the thin blue line. Sequencing reads are depicted by a thick blue line. Reads on the same side of the chromosomal line as the transcribed sequence are sense reads, while reads on the opposing side are antisense reads. *(C)* As in B, a screen shot of reads from WT worms aligning to the T07A9.12 locus, which is representative of a Correlated locus.



Supplemental Figure 5- Representative alignments of sense and antisense reads from ADR-2 Inverse 26G endo-siRNA loci. *(A)* Structures of aligned sense and antisense reads from the F39E9.7 locus. Antisense reads are in normal font, on the top of the structure and sense reads are in bold on the bottom of the structure. The 5' and 3' termini of sequences are indicated in the first structure, and are the same in all following structures. The number of reads for each sequence is indicated on the right. All sequences are from WT worms. As with part A, structures of alignments from the *(B)* C01A2.1 locus *(C)* C40A11.10 locus, *(D)* adjacent W04B5.1 and W04B5.2 loci, *(E)* W05H12.2 locus, *(F)* T16A1.8 locus, *(G)* E01G4.5 locus, *(H)* C44B11.6 locus, *(I)* Y37E11B.2 locus, *(J)* Y57G11C.51 locus, and *K)* Y105C5A.14 locus. See also Fig. 5.

Supplemental Figure 5, continued



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atg ggc aag acc aaa gcg gag att caa aaa gag gaa cga gac aac cga tac aac cga aga cac gct gaa aga
 M   G   K   T   K   A   E   I   Q   K   E   E   R   D   N   R   Y   N   R   R   H   A   E   R

gaa agc gct cag gca gag caa gaa aat gta ggc gac gag act gat cgc tcc aat gaa atc gat gag aat tgt
 E   S   A   Q   A   E   Q   E   N   V   G   D   E   T   D   R   S   N   E   I   D   E   N   C

         sense endo-siRNA sequence
gaA gac atc tcA gaA acA tcg gca aat ggc agc aat gat act aac gct atg caa caa aat gtg gct caa gct
 E   D   I   S   E   T   S   A   N   G   S   N   D   T   N   A   M   Q   Q   N   V   A   Q   A

aag act gtt tct gat cac aaa atg gac aac ttc atg ccc atc cca ctt tca aaa cat caa aaa aat tca aag taa
 K   T   V   S   D   H   K   M   D   N   F   M   P   I   P   L   S   K   H   Q   K   I   S   K   *
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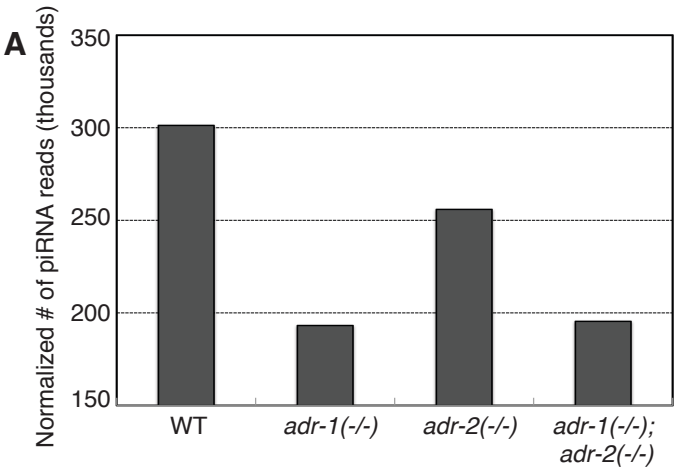
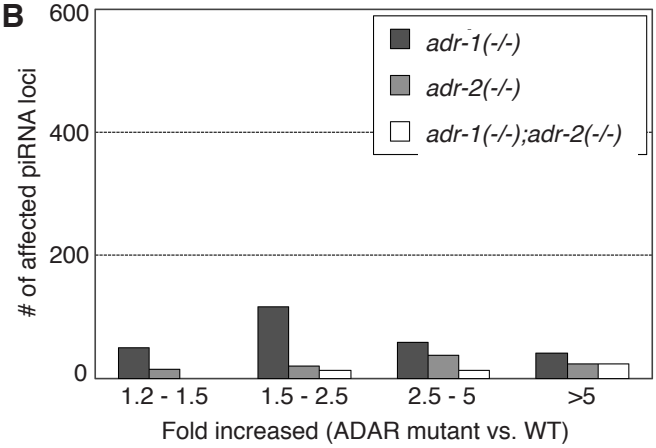
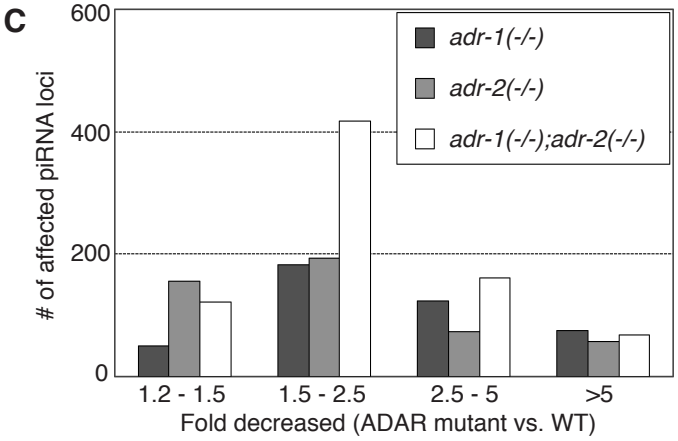
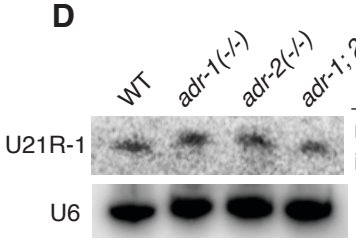
Supplemental Figure 6- Editing in the F43E2.6 endo-siRNA locus. The sequence of the coding region of the F43E2.6 gene, with the in-frame protein translation below the mRNA sequence. The four edited adenosines in sense reads are capitalized and underlined. The sense endo-siRNA sequence is indicated. See also Fig. 6. and Supplemental Table 3.

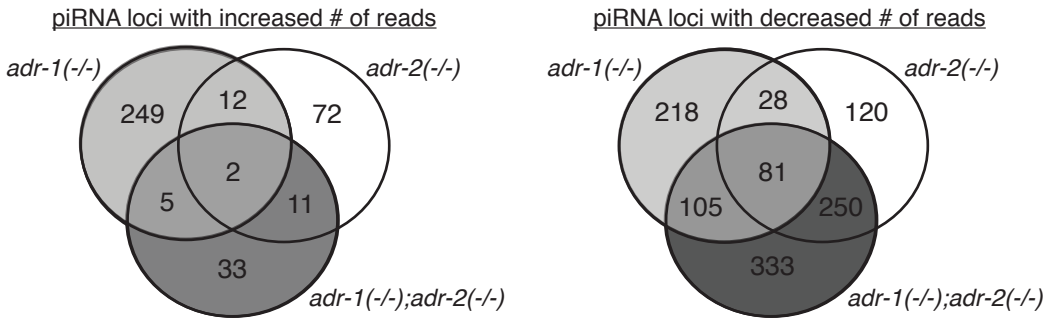


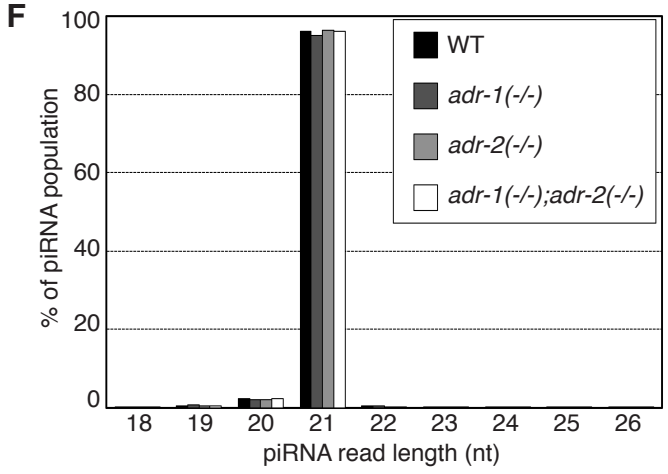


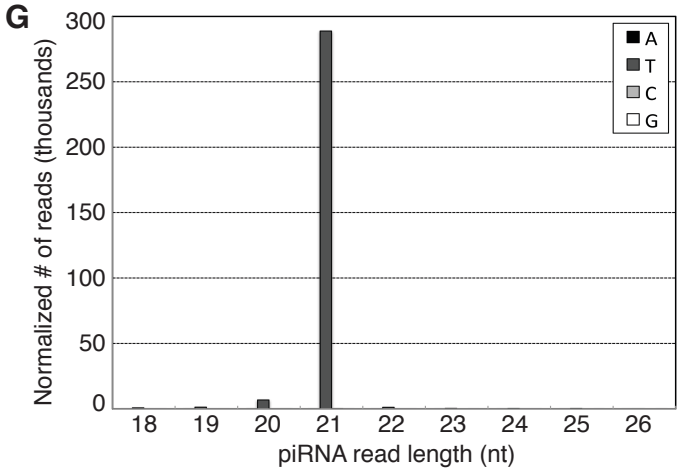




	adr-1(-/-)	adr-2(-/-)	adr-1;2
north.	1.0 ± 0.1	1.3 ± 0.1	0.7 ± 0.1
illum.	1.4		0.8







Supplemental Figure 7, continued next page.

Supplemental Figure 7, continued- Many piRNA loci had a decreased number of sequencing reads in ADAR mutant worms compared to WT. *(A)* The normalized number of total piRNA sequencing reads from each sequenced strain. *(B-C)* piRNA loci with an altered number of reads in ADAR mutants compared to WT, as determined by sequencing. piRNA loci were binned depending on their fold-change. All affected loci were altered ≥2-fold in at least one ADAR mutant strain compared to WT, or ≥1.2-fold in at least two strains. See also Supplemental Table 5. *(D)* Representative northern blot of U21R-1 levels, with U6 as a loading control. The fold change determined by sequencing (illum.) and by northern blot (north.) are tabulated on the right. If the sequencing data did not predict a statistically significant change, it was left blank. *adr-1(-/-);adr-2(-/-)* is abbreviated *adr-1;2*. *(E)* Venn diagrams showing the overlap of piRNA loci with an altered number of reads in each ADAR mutant compared to WT. *(F)* Distribution of piRNA reads, differentiating reads based on length. *(G)* Distribution of piRNA reads from WT worms, differentiating reads by length and 5' nucleotide. All ADAR mutant strains had nearly identical distributions (data not shown).