List of Supplemental Materials

**Figure S1.** Screening for phenotypic miRNA targets in *C. elegans*.

**Figure S2.** Comparison of indel efficiencies across different forms of guide RNAs and Cas9 protein.

**Figure S3.** Direct comparison of labor and readout from one-by-one versus multiplexed screen.

**Figure S4.** Phenotypes of F1 animals isolated from the CRISPR screen across 20 different injection pools.

**Figure S5.** Secondary screen of sites that were not mutated in the primary screen reveals minimal phenotypic impact of these sites.

**Figure S6.** Relationship of seed match-disrupting allele frequency and Cas9 cleavage site position.

**Figure S7.** qRT-PCR of candidate target mRNAs in wild type or seed match mutants.

**Figure S8.** Comparison of gRNA design algorithm scores and observed efficiencies.

**Table S1.** gRNAs and genotyping strategies used in optimizing multiplexing conditions.

**Table S2.** Primary gRNAs and primers used in large-scale screen.

**Table S3.** Alternative gRNAs used in secondary screen of sites with no mutations in primary screen.

**Table S4.** Alleles used in this study.

**Table S5.** Strains used in this study.

**Table S6.** Primers used for qRT-PCR.

**Table S7.** gRNA multiplexing strategy.

**Supplemental Materials and Methods**
Figure S1. Screening for phenotypic miRNA targets in *C. elegans*. (A-D) Schematic of strategy to identify phenotypic binding sites of a microRNA. Orange and green hexagons represent the gene product of the corresponding mRNA. (A) In wild type, both target genes are regulated by the miRNA, resulting in wild type phenotype. (B) In the miRNA mutant, all targets are derepressed, resulting in mutant phenotype. (C) Mutation of the miRNA binding site in the orange gene derepresses only that gene product and recapitulates the miRNA mutant phenotype, defining this as a phenotypic target. (D) Mutation of the miRNA binding site in the green gene derepresses the target but has no phenotypic consequences. (E) Schematic of multiplexed CRISPR screen in *C. elegans*. Injection of multiplexed gRNAs into the germline syncytium allows for the delivery of multiple guide RNAs to different germ cells. Germ cells may receive one or multiple guide RNAs and give rise to mutant progeny carrying indels introduced by CRISPR at different targeted sites.
Figure S2. Comparison of indel efficiencies across different forms of guide RNAs and Cas9 protein. An injection mix of guide RNAs targeting ten different loci (listed in the first column) was injected, and indel efficiency at these sites was assayed by PCR and restriction enzyme digestion (see Table S1 for details). Percent of mutated alleles detected is shown. Second column, gRNAs were injected as plasmids into the transgenic Cas9 strain (EG9615). Third column, Alt-R gRNAs were injected into EG9615. Fourth column, pooled Alt-R guides were mixed with IDT Cas9 to form RNPs prior to injection into N2 hermaphrodites. Fifth and sixth columns, individual Alt-R guides were mixed with IDT Cas9 to form RNPs prior to pooling and injection into N2 hermaphrodites.
**Figure S3. Direct comparison of labor and readout from one-by-one versus multiplexed screen.**

### One-by-one screen

- **89** individual injection mixes
  - Single 30 strains per injection mix
    - **5340** F1 plates
  - Superficially score phenotype of F2/3 population of **5340** plates
  - Harvest and lyse each strain
    - **5340** individual lysates
    - PCR genotyping (poison primer or digest to specifically recognize seed match-disrupting mutations)
      - **5340** PCRs
  - Choose 4 alleles to verify sequence
    - **356** PCR purifications
    - **356** Sanger sequencing runs
  - Identification of 4 alleles with rough phenotypic information
  - Readout of completely lethal effects: mutations not isolated
  - Readout of inefficient gRNAs: mutations not isolated

### Multiplexed screen

- **20** pooled injection mixes
  - Single ~30 strains per injection mix
    - **1362** F1 plates
  - Superficially score phenotype of F2/3 population of **1362** plates
  - Retain 30 wild type plates per pool of **630** plates
  - Harvest and pool lysates into pools of 15 strains
    - **42** pooled lysates
  - 4 lysates per target site
    - **356** PCRs
  - Pool PCRs into 4 samples
    - **6** Illumina library preps
    - **2** paired-end MiSeq runs
  - Identification of all allele sequences yielding wild type strains (on average 12.9 alleles per site in this screen)
  - Readout of sick or lethal effects: low proportion of seed match-disrupting versus seed match-intact alleles
  - Readout of inefficient gRNAs: mutations not isolated

---

**hands-on labor**

**readout**
Figure S4. Phenotypes of F1 animals isolated from the CRISPR screen across 20 different injection pools.
Figure S5. Secondary screen of sites that were not mutated in the primary screen reveals minimal phenotypic impact of these sites. (A) Seed match-disrupting allele frequency was determined by restriction enzyme digestion following CRISPR with alternative guide RNAs to those used in the primary screen (See Table S3 for genotyping details). Number of genotyped alleles is shown in parentheses. Second column: gRNAs were injected in multiplexed pools. Additional gRNAs (not shown) were included to reach a pool size of ten. Each gRNA was assayed in two pools with a partially distinct complement of poolmates. Third and fourth column: Injections were site-specific (non-multiplexed), containing only gRNAs for \textit{dpy-10} and the site of interest. (B) Brood size and embryonic lethality of wild type compared to seed match-disrupting mutations in \textit{Y55F3AM.10} or \textit{dsh-1} (see Table S4 for allele information). Shown are mean and SEM. One-way ANOVA was conducted to determine significance, followed by post hoc pairwise comparisons by Dunnett’s test for comparison of each mutant to wild type with correction for multiple testing. ***p-value < 0.001.
Figure S6. Relationship of seed match-disrupting allele frequency and Cas9 cleavage site position. (A) Legend of Cas9 cleavage positions relative to mir-35 seed binding site CCGGTG. (B) Plot from Figure 3A color-coded by the position of the predicted Cas9 cleavage site relative to the mir-35 seed sequence binding region. (B) Distribution of percent seed match-disrupting alleles for each Cas9 cleavage position relative to the mir-35 seed sequence match as in (A). Squares denote means.
Figure S7. qRT-PCR of candidate target mRNAs in wild type or seed match mutants. Quantification of indicated mRNA in wild type or 3'UTR mutant (scored for phenotype in Figure 3B). Mean and SEM of five or six biological replicates is shown. *p-value < 0.05, unpaired two-tailed Student’s t-test.
Figure S8. Comparison of gRNA design algorithm scores and observed efficiencies.
Correlation of scores predicted by the indicated algorithm for each guide RNA and its observed indel efficiency. Spearman’s correlation test r and p-value are reported.
Supplemental Materials and Methods

CRISPR injections

Alt-R crRNAs and tracrRNAs were resuspended in nuclease-free water. Alt-R gRNA stock solutions were made by annealing Alt-R crRNAs and tracrRNAs in IDT duplex buffer at 10µM (unless otherwise noted). Annealing was done by heating at 95°C for 5 minutes and cooling down to room temperature gradually. IDT Cas9 was diluted to a stock concentration of 20µM in distilled water and stored in aliquots at -80°C. All stock solutions were diluted in distilled water to reach final injection mix concentrations. All injection mixes were centrifuged at 13,000 × g for 15 minutes, and supernatant was used for injections.

For multiplexed gRNA injections, gRNA plasmids were pooled together at a final concentration of 25ng/µl each. Alt-R gRNAs were pooled at a final concentration of 1µM each (Paix et al. 2014). dpy-10 gRNA was included in each injection pool as a co-selection marker. Mixes containing Cas9 protein were injected into N2; mixes containing plasmids or Alt-R gRNAs without Cas9 were injected into EG9615.

For single site CRISPRs, up to three gRNAs were mixed with dpy-10 gRNA at a final concentration of 1µM each and injected into EG9615 or mixed with IDT Cas9 protein (final concentration 2µM) before injection into N2 (Dokshin et al. 2018). In cases where a repair template was provided, a ssDNA oligonucleotide template was included in the injection mix at a final concentration of 100ng/µl.

To pre-load recombinant Cas9 with gRNAs, 20µM annealed gRNAs were incubated with equal volume of either 4µM or 20µM Cas9 protein at room temperature for 20 minutes. Pre-loaded RNPs of each gRNA were pooled together in equal volumes.

Pooling of large-scale screen samples and library preparation

Starved plates were harvested. For each pool of injected gRNAs, two samples were generated from the 30 harvested strains by pooling 15 strains in each sample before genomic DNA extraction. (Sixty strains were harvested for pool 20 since these gRNAs were not included in two pools with distinct poolmates.)

Worm pellets were resuspended in 500µl fresh lysis buffer (100mM Tris pH=8.0, 100mM NaCl, 50mM EDTA, 1% SDS, 1% β-mercaptoethanol, 100µg/ml proteinase K) and placed at -80°C for at least 30 minutes. Buffer was supplemented with 1µl of 10mg/ml RNase A stock solution and incubated at 65°C for 30 minutes with agitation. Samples were spun at 16,000 x g for 10 minutes, and the supernatant was transferred to a new tube. The supernatant was then extracted once with an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol pH=8.0 and once with an equal volume of chloroform. Two volumes of 100% ethanol were added to the final aqueous phase. The solution was mixed and spun at 16,000 x g for ten minutes. The pellet was washed twice with 500µl of 70% ethanol and resuspended in TE buffer.

Primers were designed to generate 300-400bp amplicons with the seed match located in the middle of the amplicon (Table S2). PCR was performed using 50ng of template DNA and Q5 polymerase (NEB), and products were purified using the NEB Monarch PCR purification kit. The eight to ten CRISPR-targeted loci (excluding dpy-10) were PCR-amplified from each sample in
individual PCR reactions. Since each gRNA is present in two pools, this results in four PCR reactions for each targeted site. Amplicons were then pooled into four groups for library preparation, including only one PCR reaction for each site in each group.

The four groups of pooled amplicons were given distinct indexes during library preparation using the NEBNext Ultra II DNA Library Prep Kit for Illumina. A paired end 250bp run was conducted on a MiSeq (generally allowing for overlapping reads in the CRISPR-targeted central region of the amplicon), resulting in two fastq files for each indexed sample.

**Sequencing data analysis**

Raw fastq files were analyzed using the Cas-Analyzer web interface (Park et al. 2017). For each fastq, each amplicon sequence and matching gRNA sequence were entered into the interface, which returned total indel frequency and called all the variant alleles along with the number of reads corresponding to each allele. To rule out background mutations introduced by PCR during amplicon generation and library construction, only mutant alleles that represented at least 1% of all reads for the corresponding amplicon were counted as a true variant allele. This cutoff was established by manual curation which found that variants with mutations distal to the gRNA cut site (likely PCR errors) became prevalent below this cutoff. Because of the pooling of 15 strains into each sample, the allele number may be slightly underestimated. If a particular indel occurs more than once, it will be called as two separate events if it is present in two or more of the pooled samples. Alternatively, multiple occurrences of the same allele will be scored as a single event if they occur in two strains that are pooled into the same sample of 15. All amplicon sequencing data are deposited in the Sequence Read Archive (SRA) under accession number PRJNA644807.

**gRNA editing efficiency calculation**

For calculations in the co-editing experiments with dpy-10, Dpy animals were individually selected and their progeny were genotyped by PCR and Ncol enzyme digestion. The editing efficiency was calculated as alleles with mutations preventing Ncol cutting divided by total number of alleles genotyped. For indel frequency reported in Figures 5 and S8, the indel frequency reported by Cas-Analyzer was averaged across all four samples for each gRNA. (Indel frequency is calculated as reads containing an insertion or deletion divided by total reads.) For Figure 1F, the indel frequency reported by Cas-Analyzer was averaged between the two samples derived from the same gRNA pool (see pool IDs in Table S7), and plotted against the average of the indel frequency in the two samples derived from the other gRNA pool containing that gRNA.

**qRT-PCR**

Animals were grown at 25°C to gravid adult stage, and then treated with alkaline hypochlorite solution to collect embryos. Embryo pellets were suspended in 250ul Trizol solution and frozen at -80°C until use. On the day of RNA extraction, embryo samples were thawed and vortexed for 15 minutes at room temperature. Total RNA was isolated according to Trizol manufacturer’s specifications (Thermo Fisher). qRT-PCR was performed using KAPA SYBR® FAST One-Step qRT-PCR kit (Sigma) according to the manufacturer’s protocol. Primer sequences are available in Table S6. Log2 fold changes were calculated with the -ΔΔC(t) method by using gpd-1 as an internal normalization control across samples.
Construction of \textit{mir-35(cdb2 cdb4 seed reversed)}

The seed mutation of \textit{mir-35} was made by two rounds of CRISPR. First, two gRNAs recognizing the protospacers TTTCCATTAGAACTATCACC and ATTGCTGGTTTCTTCCACAG were used to create a 50bp deletion at the \textit{mir-35} locus. This allele is \textit{mir-35(cdb2)}: GCTGGTTTCTTCCACAGT-50bp_del-CTTTTCCACTTGCTCCAC. The strain carrying \textit{mir-35(cdb2)} was then injected with a homology-directed repair donor generated by PCR, along with a gRNA recognizing the protospacer GGAGCAAGTGGAAAAGACTG (a sequence which is created by the \textit{mir-35(cdb2)} mutation). This resulted in the \textit{mir-35(cdb2 cdb4)} locus of which the sequence is

\begin{verbatim}
ATTCTCGGATCAGATCGAGCCATTGCTGGTTTCTTGTAGACCCGTACTTTTCCATTAGAACTA
TGGGCCACTGGAAAACGTAGCGTGCTCGATCTTTTCCACTTGC
\end{verbatim}

where italic sequences are the 35bp homology arms present in the donor, the bold sequence is the reversed seed of mature \textit{mir-35-3p}, and the underlined sequence is compensatory mutations in \textit{mir-35-5p} to preserve secondary structure of the primary and precursor hairpin.