Nuclear RNAi Contributes to the Silencing of Off-Target Genes and Repetitive Sequences in *Caenorhabditis elegans*

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ABSTRACT Small RNAs recognize, bind, and regulate other complementary cellular RNAs. The introduction of small RNAs to eukaryotic cells frequently results in unintended silencing of related, but not identical, RNAs: a process termed off-target gene silencing. Off-target gene silencing is one of the major concerns during the application of small RNA-based technologies for gene discovery and the treatment of human disease. Off-target gene silencing is commonly thought to be due to inherent biochemical limitations of the RNAi machinery. Here we show that following the introduction of exogenous sources of double-stranded RNA, the nuclear RNAi pathway, but not its cytoplasmic counterparts, is the primary source of off-target silencing in *Caenorhabditis elegans*. In addition, we show that during the normal course of growth and development the nuclear RNAi pathway regulates repetitive gene families. Therefore, we speculate that RNAi off-target effects might not be "mistakes" but rather an intentional and genetically programmed aspect of small RNA-mediated gene silencing, which might allow small RNAs to silence rapidly evolving parasitic nucleic acids. Finally, reducing off-target effects by manipulating the nuclear RNAi pathway *in vivo* might improve the efficacy of small RNA-based technologies.

THE introduction of double-stranded RNA (dsRNA) into organisms elicits a process termed RNA interference (RNAi) that suppresses the expression of genes exhibiting sequence homology (Fire *et al.* 1998). dsRNA molecules are cleaved by the RNase III-like enzyme DICER into ~22-nt small interfering RNAs (siRNAs), which are then loaded onto Argonaute proteins. siRNA guides the Argonaute silencing complex to complementary nucleic acids to silence gene expression via a multitude of mechanisms, including initiating the degradation of the target mRNA, blocking translation, triggering heterochromatin formation, and inhibiting transcription elongation (reviewed by Ketting 2011; Feng and Guang 2013).

The nuclear RNAi defective (Nrde) pathway is necessary for the silencing of nuclear-localized RNAs in *Caenorhabditis elegans*. NRDE-3 is an Argonaute protein that associates with secondary siRNAs in the cytoplasm and escorts them to the nucleus (Guang *et al.* 2008); the siRNA/NRDE-3 complex then binds to complementary nascent transcripts and recruits NRDE-1 and NRDE-2 (Guang *et al.* 2008, 2010; Burkhart *et al.* 2011). With the help of NRDE-4, NRDE-1 associates with chromatin at the targeted genomic locus and elicits histone 3 lysine 9 trimethylation (H3K9me3) at the site (Burkhart *et al.* 2011). The siRNA/NRDE complex, most likely working together with the chromatin modification machinery, pauses RNA polymerase II and inhibits the elongation of transcription (Guang *et al.* 2010).

Although much is known about the molecular mechanism of Nrde-mediated gene silencing, little is known about the biological roles of the nuclear RNAi pathway. *nrde-1/2/4* and *hrde-1* (the germline nuclear Ago) mutants produce fewer progeny and exhibit a mortal germline phenotype, suggesting that nuclear RNAi may be important for fertility and germline immortality (Guang *et al.* 2010; Burkhart *et al.* 2011; Buckley *et al.* 2012). In addition, *hrde-1* and *nrde-2* mutants exhibit high incidence of males (Him) phenotype, suggesting that nuclear RNAi in the germline might promote chromosome segregation during meiosis (Buckley *et al.* 2012). The nuclear Ago NRDE-3 binds endogenous siRNAs

Copyright © 2014 by the Genetics Society of America
doi: 10.1534/genetics.113.159780
Manuscript received November 15, 2013; accepted for publication February 10, 2014; published Early Online February 14, 2014.
Supporting information is available online at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.113.159780/-/DC1.
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termed the 22G siRNAs. Many genes, such as *eri-1*, *eri-6*, *eri-7*, *ergo-1*, *drh-3*, *mut-2*, *mut-7*, and *rrf-1* are required for the production of a small subset, respectively, of endogenous siRNAs that engage NRDE-3 (Guang *et al.* 2008; Fischer *et al.* 2011). RRF-1 is a somatic RNA-dependent RNA polymerase acting upstream but necessary to feed the nuclear RNAi pathway. RRF-1 is guided by the RDE-1/siRNA complex to complementary mRNA targets to generate secondary siRNAs (Pak *et al.* 2012), which bind to NRDE-3 to be escorted to the nucleus (Guang *et al.* 2008).

Although near perfect complementarity between siRNAs and their target RNA sequences is required for efficient gene silencing, the exposure of eukaryotes to siRNA frequently results in the unintended silencing of genes exhibiting <100% sequence identity to the trigger siRNA, a phenomenon termed off-target gene silencing (reviewed by Svoboda 2007). siRNA triggers off-target gene silencing in a variety of ways, including sequence-dependent and sequence-independent mechanisms. For example, siRNA is able to mimic microRNA (miRNA) to inhibit translation or elicit the degradation of mRNAs with partial sequence complementarity (Aleman *et al.* 2007). In addition, high concentrations of siRNA can induce an interferon response or global changes in gene expression by competing with limiting factors involved in the biogenesis and function of the endogenous RNAi pathway (Grimm *et al.* 2006). However, the lack of a genetically tractable system to study RNAi off-target effects has hindered the understanding of the mechanism of off-target gene silencing.

We conducted a genetic screen in *C. elegans* to identify the factors that are specifically required for RNAi off-target gene silencing. This screen identified alleles of *rrf-1*, *nrde-2*, and *nrde-3*. Here we show that following exogenous RNAi, the nuclear Ago NRDE-3 associates with off-target siRNAs and that the nuclear RNAi pathway, but not its cytoplasmic counterparts, is the primary source of off-target silencing in *C. elegans*. In addition, we find that NRDE-3 associates with endo-siRNAs that target repetitive genomic loci and that the nuclear RNAi pathway targets large gene families for silencing. We conclude that nuclear RNAi acts as a major contributor to exogenous and endogenous RNAi off-target effects in *C. elegans*.

Materials and Methods

Strains

Bristol strain N2 was used as the standard wild-type strain. The Hawaiian strain CB4856 was used for snp-SNP mapping. All strains were incubated at 20°: *dpy-13(e458)*, *sqt-3(e2924)*, *eri-1(mg366)*, *ergo-1(gg098)*, *rde-1(ne219)*, *nrde-1(gg088)*, *nrde-2(gg091)*, *nrde-2(ust005)*, *nrde-3(gg066)*, *nrde-3(gg245)*, *rrf-1(pk1417)*, *rrf-1(gg247)*, *ppw-1(pk2505)*, *ppw-2(tm1120)*, *Y49F6A.1(tm1127)*, *sago-1(tm1195)*, *prg-2(tm1094)*, *sur-5::gfp[kuIs54]*, *FLAG::GFP::NRDE-3(ggIs1)*, *MAGO11(−) {wago-1(tm1414); wago-2(tm2686); wago-3(tm1120); wago-4(tm1019); wago-5(tm1113); wago-6(tm894); wago-7(tm914); wago-8(tm1195); wago-9(tm1200); wago-10(tm1186); wago-11(tm1127)}*.

RNAi

RNAi experiments were performed as described previously (Timmons *et al.* 2001). Bacteria expressing dsRNA were mostly obtained from the Ahringer RNAi library and were sequenced to verify their identity (Kamath *et al.* 2003). The *lin-15b* RNAi clone has been described previously (Guang *et al.* 2008). The *dpy-13* RNAi clone was constructed by PCR with the primers 5′-GGGAAGCTTCGTTCGTTACGGACGTGAC-3′ and 5′-GGGAAGCTTTTAGCGGCGAGTTCCG-3′, inserted into the *Hin*dIII site of the L4440 plasmid (a gift from A. Fire), and transformed into the HT115 *Escherichia coli* strain. To generate 76-bp dsRNA, synthetic oligonucleotides were annealed, phosphorylated by T4 polynucleotide kinase, and inserted into the *Hin*dIII site of the L4440 plasmid. The sequences of the primers used to generate each *dpy-13* dsRNA expression clone are available upon request.

To generate GFP dsRNAs with increasing mismatches, the GFP sequence was amplified from pPD95.79 (a gift from A. Fire) by PCR under error-prone conditions with 4 mM $MgCl_2$ and Taq polymerase. The PCR process was repeated 14 times, and the PCR products were cloned into L4440 and sequenced sequentially. The 10% and 20% mismatch levels were achieved at round 8 and round 14, respectively.

RNAi involving a dilution series was performed by mixing overnight cultures of bacteria expressing dsRNA with HT115 control bacteria. HT115 is an RNase III mutant bacterial strain that was modified to express T7 RNA polymerase from an IPTG-inducible promoter, which does not express either empty vector or dsRNA.

Images were collected using Zeiss Imager D1 and Leica DM2500 microscopes.

Genetic screening

To identify the factors specifically required for off-target gene silencing, we screened for cellular factors required for the *dpy-13* dsRNA-induced silencing of off-target RNAs but dispensable for canonical RNAi silencing (see Supporting Information, Figure S2). *dpy-13* RNAi results in a superdumpy phenotype in *eri-1(mg366); dpy-13(e458)* animals. Thus, *eri-1(mg366); dpy-13(e458)* animals were mutagenized by ethyl methanesulfonate (EMS), and the F_2 progeny worms were grown on *E. coli* expressing *dpy-13* dsRNA. Suppressors of the superdumpy phenotype were selected and tested with *pos-1* and *lir-1* RNAi to exclude the Rde and Nrde genes (Guang *et al.* 2008). Animals that suppressed the *dpy-13* RNAi-induced superdumpy phenotype but still responded to *pos-1* and *lir-1* RNAi were selected for further analysis. Nine mutants were isolated from this genetic screening, three of which were identified by snp-SNP mapping and sequencing.

Quantitative RT-PCR

RNAs were isolated from embryos using a dounce homogenizer (pestle B) in TRIzol solution followed by purification with an RNeasy kit including on-column DNase I digestion (Qiagen). cDNAs were generated from RNAs using the iScript cDNA Synthesis kit (Bio-Rad) according to the vendor's

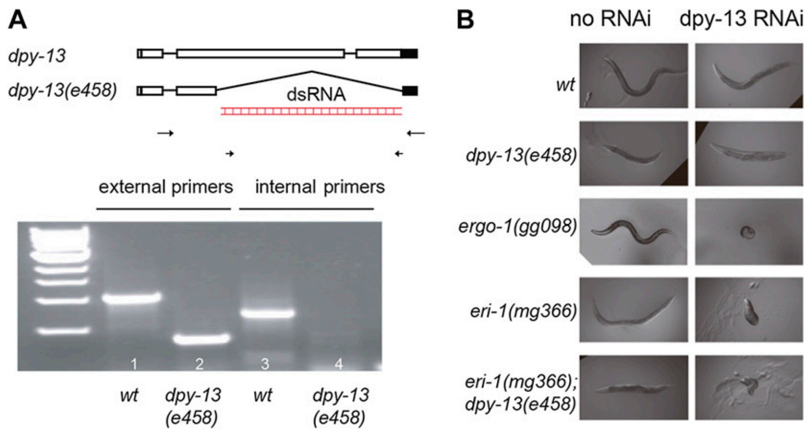


Figure 1 Off-target silencing occurs in enhanced RNAi animals. (A) Gene structure of *dpy-13*. Recessive *dpy-13 (e458)* is a 723-bp deletion, which removed 71% of the protein coding region and the translation stop codon. All of the conserved Gly-X-Y repeats, which are essential for the function of collagens, are deleted. *wt*, wild-type. (B) Feeding RNAi targeting the *dpy-13(e458)*-deleted sequence induced a worm-ball-like superdumpy phenotype in *eri* animals. L1 animals were fed *dpy-13* dsRNA.

protocol. qPCR was performed using an MyIQ2 machine (Bio-Rad) with iQ SYBR Green Supermix (Bio-Rad). The primers for pre-mRNA analysis that were used in qRT-PCR are listed in Table S7. Independent primer pairs were also used in qRT-PCR and gave similar results (data not shown). *eft-3* mRNA was used as an internal control for sample normalization. The data analysis was performed using a $\Delta\Delta$CT approach.

Isolation and deep sequencing of NRDE-3-associated RNAs

NRDE-3-associated siRNAs were isolated from embryo lysates as previously described (Figure S5) (Guang *et al.* 2008). Briefly, the animals were sonicated in lysis buffer [20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 2.5 mM $MgCl_2$, and 0.5% NP-40]; the lysate was precleared with protein G-agarose beads (Roche), and incubated with anti-FLAG M2 agarose beads (Sigma). The beads were washed extensively, and FLAG::GFP::NRDE-3 and associated RNA were eluted with 100 μg/ml 3$\times$ FLAG peptide (Sigma). The eluates were incubated with TRIzol reagent (Invitrogen), followed by isopropanol precipitation. The precipitates were treated with calf intestinal alkaline phosphatase (CIAP, Invitrogen), reextracted with TRIzol, and treated with T4 polynucleotide kinase (T4 PNK, New England Biolabs) in the presence of 1 mM ATP.

The NRDE-3-associated siRNAs were cloned and deep sequenced using an Illumina platform, according to the manufacturer's instructions, by the Beijing Genomics Institute (BGI Shenzhen). sRNAs ranging from 20 to 30 nt were gel purified and ligated to a 3′ adaptor (5′-pUCGUAUGCCGUCUUCUGCUUGidT-3′; p, phosphate; idT, inverted deoxythymidine) and a 5′ adaptor (5′-GUUCAGAGUUCUACAGUCCGACGAUC-3′). The ligation products were gel purified, reverse transcribed, and amplified using Illumina's sRNA primer set (5′-CAAGCAGAAGACGGCATACGA-3′; 5′-AATGATACGGCGACCACCGA-3′). The samples were sequenced using an Illumina Hiseq platform.

The Illumina-generated raw reads were first filtered to remove adaptors, low quality tags, as well as contaminants to get clean reads at BGI Shenzhen. Clean reads ranging from 20 to 30 nt were mapped to the *C. elegans* genome and the transcriptome assembly WS229, respectively, using Bowtie2 with default parameters (Langmead and Salzberg 2012). The number of reads targeting each gene was counted by custom Perl scripts and displayed by IGV (Thorvaldsdottir *et al.* 2013).

AMA-1 (RNA polymerase II) ChIP-seq datasets were retrieved from GSM677643 of the modENCODE project, which identified the binding region of AMA-1 in L4 wild-type animals (Gerstein *et al.* 2010). The targets of WAGO-1, CSR-1, and *eri-6/7* had been published previously (Claycomb *et al.* 2009; Gu *et al.* 2009; Fischer *et al.* 2011).

Results

RNAi triggers off-target gene silencing in C. elegans

dpy-13 is a collagen gene that belongs to a large family of >100 members that share high sequence similarity in *C. elegans*. *dpy-13(e458)* lacks most of the *dpy-13* coding region and is likely to be a null mutant (Figure 1A) (von Mende *et al.* 1988); *dpy-13(e458)* animals exhibit a dumpy phenotype with a length that is approximately half that of wild-type N2 animals (Figure 1B). Exposure of wild-type animals to dsRNA targeting *dpy-13* resulted in the expected dumpy phenotype, consistent with *dpy-13* dsRNA silencing *dpy-13*. Mutant backgrounds have been identified that enhance RNAi silencing. For instance, mutation of the *eri-1*, *rrf-3*, *eri-6*, *eri-7*, and *ergo-1* genes results in an enhanced RNAi (Eri) phenotype (Kennedy *et al.* 2004; Duchaine *et al.* 2006; Fischer *et al.* 2011). Strikingly, RNAi of *dpy-13* in *ergo-1(gg098)* and *eri-1(mg366)* animals elicited a worm-ball-like phenotype that is more severe (superdumpy) than that of the *dpy-13* null phenotype. Furthermore, RNAi of *dpy-13* in *eri-1(mg366)*; *dpy-13(e458)* animals also induced the superdumpy phenotype. Note, the region of *dpy-13* targeted by *dpy-13* dsRNA in this experiment is missing in the *eri-1 (mg366)*; *dpy-13(e458)* strain, indicating that the superdumpy phenotype is not simply the result of highly efficient silencing of *dpy-13* in enhanced RNAi backgrounds.

We asked if the superdumpy phenotype exhibited by Eri animals was caused by off-target gene silencing. We first mapped the minimal sequence in *dpy-13* sufficient to induce a superdumpy phenotype by exposing *eri-1(mg366)* and *eri-1(mg366)*; *dpy-13(e458)* animals to portions of the original *dpy-13* dsRNA. The 76-nt fragment *dpy-13g*, which targets the

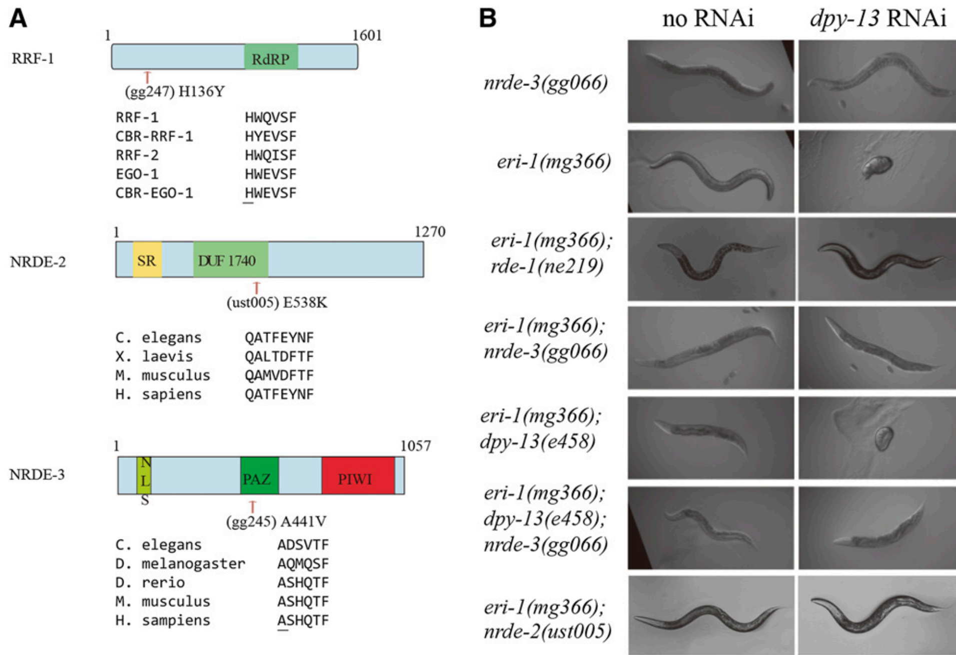


Figure 2 (A) Genetic screening identified the alleles of *rrf-1*, *nrde-2*, and *nrde-3* that are required for off-target gene silencing. *eri-1(mg366)*; *dpy-13 (e458)* animals were chemically mutagenized and screened for mutants that suppress off-target RNAi effect but are still sensitive to *pos-1* and *lir-1* RNAi. The mutants were mapped via the snp-SNP technique and sequenced. Underlining indicates the mutated amino acids. RRF-1, RRF-2, and EGO-1 are RNA-dependent RNA polymerases. CBR-EGO-1 and CBR-RRF-1 are *C. briggsae* genes. (B) Nuclear RNAi is required for *dpy-13* dsRNA-induced off-target silencing. L1 animals of the indicated genotypes were fed *dpy-13* dsRNA, and images were obtained 3 days later. RDE-1 is a primary Argonaute protein that is a prerequisite for feeding RNAi (Steiner *et al.* 2009). *dpy-13* RNAi failed to produce any visible silencing effects in *eri-1(mg366)*; *rde-1 (ne219)* animals.

3′ end of the *dpy-13* mRNA, was sufficient to induce a superdumpy phenotype (Figure S1). The same 76-nt region derived from other collagen genes, including *sqt-3*, *col-43*, *col-93*, and *col-94*, also induced a superdumpy-like phenotype (Table S1). *dpy-13g* does not contain a single 20+ nt sequence that perfectly matches another *C. elegans* gene, suggesting that *dpy-13g*-mediated superdumpy phenotype is due to off-target gene silencing. To test this idea, we introduced mutations into *dpy-13g* and found that these variants of *dpy-13g* were also able to trigger off-target silencing (Figure S1). These data indicate that the superdumpy phenotype elicited by *dpy-13* dsRNAs is caused by RNAi off-target effects. Below, we show that superdumpy is triggered by the off-target silencing of other *C. elegans* collagen genes by *dpy-13* dsRNA (see below).

Nuclear RNAi contributes to off-target silencing

To identify the factors required for off-target gene silencing, we mutagenized *eri-1(mg366)*; *dpy-13(e458)* animals and screened for mutants that failed to exhibit off-target silencing in response to *dpy-13* dsRNA, but retained the ability to initiate RNAi against perfectly complementary cellular RNAs (Figure S2). Nine mutants were isolated that did not exhibit the *dpy-13* RNAi-mediated superdumpy phenotype (off target), but were still fully responsive to *pos-1* and *unc-15* RNAi (on target). We mapped three of our nine mutant alleles by single-nucleotide polymorphism (SNP) mapping and found them to be *rrf-1(H136Y)*, *nrde-2(E538K)*, and *nrde-3 (A441V)* (Figure 2A, Table 1). *rrf-1*, *nrde-2*, and *nrde-3* are components of the *C. elegans* nuclear RNAi pathway, which are required for silencing of nuclear RNAs by dsRNA (Guang *et al.* 2008; Burkhart *et al.* 2011). The identification of *rrf-1*, *nrde-2*, and *nrde-3* in our screen suggests that dsRNAs and siRNAs may guide RRF-1 to off-target mRNAs (partial sequence complementarity to siRNA) to direct unintended secondary siRNA synthesis and, consequently, nuclear RNAi at off-target sites within nuclei. The following results suggest that the alleles of *rrf-1*, *nrde-2*, and *nrde-3* identified in our screen are hypomorphic alleles. Polycistronic RNAs in *C. elegans* are *trans*-spliced to monocistronic messages within nuclei. Therefore, targeting one gene in a polycistron with dsRNA and quantifying silencing of other genes encoded in the polycistron allows for quantification of nuclear RNAi. Two such polycistrons are *lin-15a/lin-15b* and *lir-1/lin-26*. Null alleles of *rrf-1*, *nrde-2*, and *nrde-3* abolish nuclear RNAi at both of these polycistronic RNAs (Guang *et al.* 2008). The alleles of *rrf-1*, *nrde-2*, and *nrde-3* identified in this screen, however, were defective for nuclear RNAi at the *lin-15a/lin-15b* operon, but not at the *lir-1/lin-26* operon, suggesting that these alleles may be hypomorphic alleles of these genes. Consistent with this idea, we tested putative null alleles of *rrf-1*, *nrde-2*, and *nrde-3* and found that these mutations also abolished off-target silencing by *dpy-13* dsRNA (Figure 2B). For the remainder of this manuscript, we use the reference (null) alleles of *rrf-1*, *nrde-2*, and *nrde-3* to explore the role of these nuclear RNAi factors in off-target gene silencing.

Nuclear RNAi is required to couple cosilencing between dpy-13 and sqt-3

siRNA can cause off-target gene silencing through sequence-dependent and sequence-independent mechanisms (Svoboda 2007). To begin to ask if *dpy-13* dsRNA-induced superdumpy is caused by sequence dependent or independent mechanisms, we asked if the nuclear Ago NRDE-3, which is required for off-target silencing, associates with off-target siRNAs after *dpy-13* dsRNA treatment. We fed *eri-1(mg366)*; *dpy-13(e458)*; *FLAG*::*GFP*::*NRDE-3* animals with *dpy-13*

Table 1 Identification of *rrf-1*, *nrde-2*, and *nrde-3* alleles required for RNAi

Genotype	RNAi *pos-1* *no hatch*	*lir-1* *lethal*	*lin-15b* *F1 muv*	*unc-15* *unc*	*dpy-13* *dumpy*	*no RNAi*
N2	++++	−	−	++++	+	−
nrde-3(gg066)	++++	−	−	+++	−	−
nrde-2(E538K)	++++	−	−	++++	−	−
eri-1(mg366)	++++	++++	++++	++++	++++	−
eri-1(mg366); rde-1(ne219)	−	−	−	−	−	−
ergo-1(gg098)	++++	++++	++++	++++	++++	−
*eri-1(mg366); nrde-3(gg066)**	++++	−	−	+++	+	−
eri-1(mg366); rrf-1(H136Y)	++++	++++	−	+++	+	−
eri-1(mg366); nrde-2(E538K)	++++	+++	−	+++	+	−
eri-1(mg366); nrde-3(A441V)	++++	++++	−	+++	+	−
{eri-1(mg366); MAGO12} wago-1(tm1414); wago-2(tm2686); wago-3(tm1120); wago-4(tm1019); wago-5(tm1113); wago-6(tm894); wago-7(tm914); wago-8(tm1195); wago-9(tm1200); wago-10(tm1186); wago-11(tm1127); nrde-3(tm1116); eri-1(mg366)	−	−	−	++	−	−
{eri-1(mg366); MAGO11(−); *nrde-3(+)}*	−	−	−	++	+	−

* *nrde-3(gg066)* is the reference allele.
+ means sensitive to RNAi; − means resistant to RNAi.

dsRNA (dsRNA targets *dpy-13* deleted region). NRDE-3 localizes to the cytoplasm when not bound to siRNA and to nuclei when bound to siRNA (Guang *et al.* 2008). We observed that *dpy-13* dsRNA induced the nuclear accumulation of FLAG::GFP::NRDE-3 in animals lacking the *dpy-13* gene (Figure 3A), suggesting that *dpy-13* dsRNA targeting the deleted region of *dpy-13* induced the generation of off-target siRNAs that associate with NRDE-3. We sequenced small RNAs that associate with NRDE-3 before and after *dpy-13* dsRNA treatment. NRDE-3 associated with few endogenous small RNAs targeting collagens in wild-type animals in the absence of *dpy-13* dsRNA treatment (Figure 3B, Table S2). Following *dpy-13* dsRNA treatment, however, NRDE-3 associated with abundant siRNAs targeting multiple collagen genes. The identification of off-target siRNAs bound to NRDE-3 is consistent with the idea that superdumpy is caused by *dpy-13* RNAi-mediated off-target silencing of other related collagen genes. Interestingly, of all the collagen genes the *sqt-3* gene was targeted by the most off-target siRNAs, suggesting that *sqt-3* may be a major off-target of *dpy-13* RNAi (Figure 3B). *sqt-3* is a cuticle collagen essential for viability and body morphology, and the molecule is likely to form higher order structures with other cuticle collagens (Novelli *et al.* 2006). Two lines of evidence support the idea that *sqt-3* is a major off-target of *dpy-13* RNAi. First, we found that *dpy-13(e458)*; *sqt-3(e2924)* double mutant animals exhibited a superdumpy-like phenotype (Figure 3C, Figure S3). Second, exposure of *eri-1(mg366)* and *eri-1(mg366)*; *dpy-13(e458)* animals to dsRNA, targeting either *dpy-13* or *sqt-3*, elicited superdumpy phenotypes (Figure 3D). *eri-1(mg366)*; *nrde-3(gg066)* animals were only mildly sensitive to dsRNA, targeting either *dpy-13* or *sqt-3*. However, *eri-1(mg366)*; *nrde-3(gg066)*; *dpy-13(e458)* animal displayed the superdumpy-like phenotype after *sqt-3* RNAi, but not *dpy-13* RNAi, suggesting that NRDE-3 likely couples the cosilencing of these two genes (Figure 3D). We conclude that the off-target effects of *dpy-13* RNAi are the result of sequence-dependent off-target silencing of related collagen genes.

Nuclear RNAi is required for off-target silencing induced by gfp dsRNA

Two lines of evidence argue that the role of nuclear RNAi in off-target silencing is not limited to the collagen genes. First, we subjected animals to RNAi targeting 196 genes (168 operon genes and 28 multigene families), respectively, and asked if the phenotypes induced by these various RNAi treatments depended on the nuclear RNAi pathway. Most RNAi treatments had similar effects in both nuclear RNAi competent and nuclear RNAi defective animals (Table S3). Interestingly, many RNAi treatments that targeted multigene families such as the histone genes, homeobox genes, and G-protein-coupled receptor (GPCR) genes were strongly dependent on a functional nuclear RNAi pathway; animals lacking a functional nuclear RNAi pathway exhibited dramatically reduced phenotypes than animals with a functional RNAi pathway (Table S3, Figure S4). We speculate that the reason that nuclear RNAi is preferentially required for generating phenotypes following RNAi targeting multigene families is because these phenotypes are actually due to off-target silencing of other members of these large gene families. Second, we generated plasmids that express *gfp* dsRNA harboring randomly generated mutations introduced using error-prone PCR (Figure 4A). Animals expressing the *sur-5*::*gfp* transgene were fed wild-type, 10%, or 20% mismatched *gfp* dsRNA (Figure 4B). Wild-type *gfp* dsRNA silenced GFP expression in wild-type animals and *nrde* mutants efficiently, whereas the silencing effect of dsRNA with 10% mismatch was weakened in the *nrde* animals. *gfp* dsRNA with 20% mismatch efficiently silenced GFP expression in wild-type animals but showed diminished

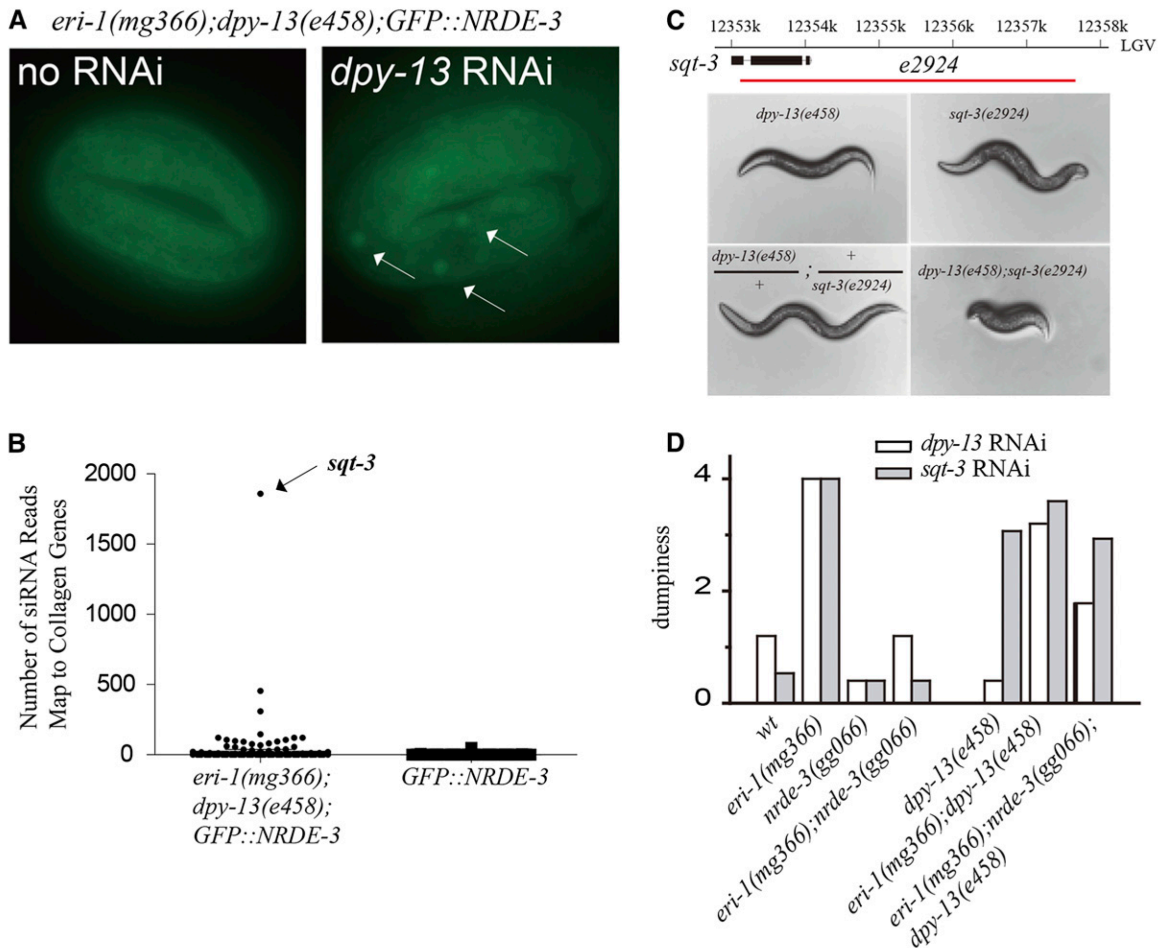


Figure 3 Nuclear RNAi couples cosilencing between *sqt-3* and *dpy-13*. (A) Off-target RNAi elicits the nuclear accumulation of NRDE-3. L4 animals were fed bacteria expressing *dpy-13* dsRNA, and images of embryos were obtained 2 days later. Arrows indicate nuclei. (B) Feeding *dpy-13* dsRNA leads to small RNAi targeting of other collagens. NRDE-3-associated small RNAs were immunoprecipitated from embryo lysates with an anti-FLAG antibody and subjected to deep sequencing. Reads of the small RNAs targeting collagens were compared. (C) A hypostasis interaction between *dpy-13* and *sqt-3*. *dpy-13(e458)*; *sqt-3(e2924)* double mutant leads to a superdumpy-like phenotype. (D) NRDE-3 is required to couple cosilencing between *sqt-3* and *dpy-13*. L1 animals were fed dsRNAs targeting *dpy-13* or *sqt*-3. The phenotypes were scored 3 days later. 0, no dumpy; 4, superdumpy. The experiments were performed in triplicate (*N* = 3).

silencing in *nrde* mutants, suggesting that the nuclear RNAi machinery may be important for off-target silencing of the *gfp* gene. We note, however, that imperfectly complementary *gfp* dsRNA did not work as well as perfectly complementary dsRNA in either wild-type or *nrde* mutant animals, suggesting that the nuclear RNAi machinery is required for both on- and off-target gene silencing. To exclude the possibility of dose-response effects of the mismatched *gfp* in *nrde* mutants, we measured the silencing efficacy of a *gfp* dsRNA dilution series. We found that wild-type *gfp* dsRNA diluted 10-fold induced silencing in *sur-5*::*gfp* and *nrde-2 (gg091)*; *sur-5*::*gfp* animals in a similar fashion as the undiluted dsRNA (Figure 4C). Similar responses were also observed when wild-type and *nrde-3*(*gg066)* animals were fed serially diluted *unc-15* dsRNA (Figure 4D). Therefore, the off-target silencing deficiency of *nrde* mutants is unlikely due to a dose-response effect of mismatched dsRNA, but, rather, may be an inherent characteristic of the nuclear RNAi pathway. Together, these data suggest that the nuclear RNAi machinery is required for off-target silencing following RNAi targeting many, if not all, genes in *C. elegans*.

Nuclear RNAi is a primary source of off-target silencing in C. elegans

We identified components of the *C. elegans* nuclear RNAi pathway in our genetic screen for RNAi off-target factors. One of these nuclear RNAi factors was the Ago NRDE-3. *C. elegans* expresses 12 secondary Argonaute proteins, including NRDE-3 (Yigit *et al.* 2006). We tested whether off-target gene silencing specifically requires the nuclear RNAi pathway by examining the role of other secondary Argonaute mutants, including *ppw-1*, *ppw-2*, *Y49F6A.1*, *sago-1*, and *prg-2*, in off-target gene silencing. We found that, unlike NRDE-3, these four secondary Argonautes were not required for off-target silencing (Table 2). In addition, the duodecuple mutant strain MAGO12 harbors deletion alleles in all 12

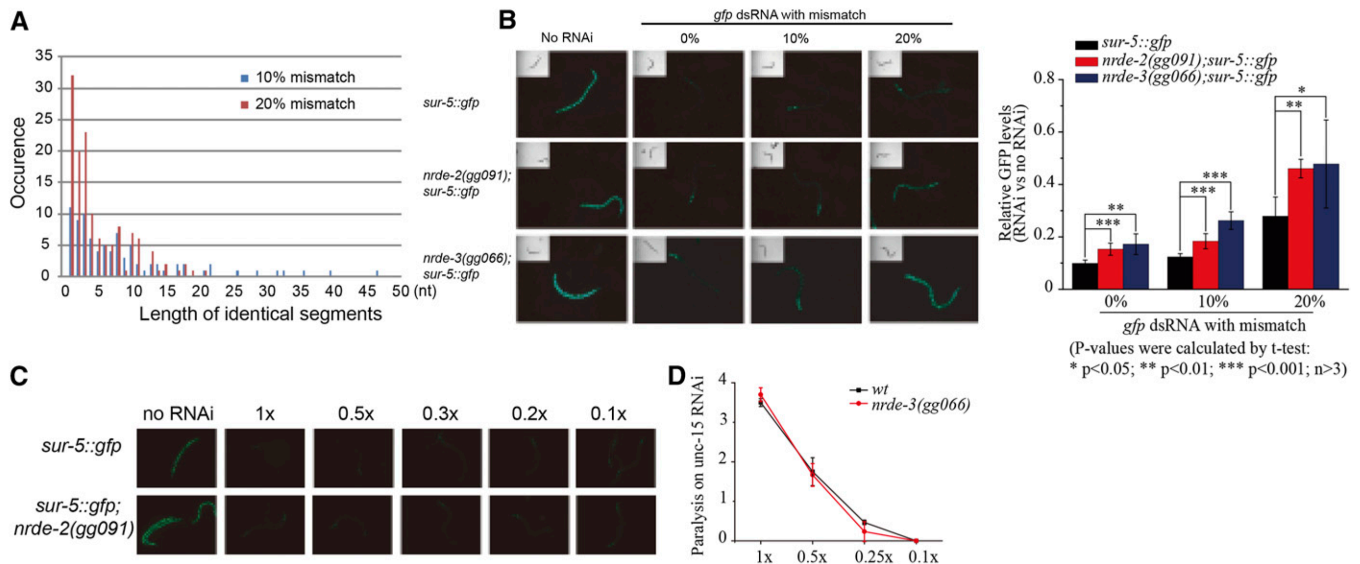


Figure 4 Nuclear RNAi is required for *gfp* dsRNA-induced off-target silencing. (A) Sequence comparison of mutagenized *gfp* dsRNAs that were generated by error-prone PCR. At the 20% mismatch level, one 21-nt segment is identical in sequence to wild-type GFP. (B) Left: L1 animals expressing *sur-5*::*gfp* were fed *gfp* dsRNAs with 0, 10, or 20% sequence mismatch. Right: GFP levels were quantified by ImageJ and normalized to control animals. (C) L1 animals expressing *sur-5*::*gfp* were fed a diluted series of *gfp* dsRNA. The dilution series was performed by mixing overnight cultures of bacteria expressing dsRNA with HT115 control bacteria. (D) L1 animals were fed a diluted series of *unc-15* dsRNA, and their phenotypes were scored 3 days later.

secondary Argonaute genes and is resistant to feeding RNAi (Table 1) (Gu *et al.* 2009). We crossed *eri-1(mg366)*; *MAGO12(−)* with *eri-1(mg366)* and isolated eight independent F_2 animals that were *eri-1(mg366)* but suppressed *dpy-13* RNAi-induced off-target silencing. We genotyped the 12 Argonaute genes in these eight lines (Table S4). *nrde-3 (tm1116)*, but not the other 11 *wago* deletion alleles, was consistently linked to superdumpy suppression. These data suggest that NRDE-3 is the major secondary Argonaute that is responsible for the off-target silencing effects of *dpy-13* dsRNA. We introduced a wild-type copy of *nrde-3* into the MAGO12 strain to generate *eri-1(mg366)*; *MAGO11(−)*; *nrde-3(+)*. We found that this strain was moderately defective for *dpy-13*-induced off-target silencing (Table 1). Thus, unknown combinations of other secondary Argonaute proteins may also contribute redundantly to RNAi off-target effects. We conclude that the nuclear Ago NRDE-3 is the primary, but not the sole, Ago-mediating off-target silencing. These results are consistent with the idea that nuclear RNAi is the major contributor of off-target silencing in *C. elegans*.

NRDE-3 interacts with 22G-RNAs

Previously, we sequenced endogenous siRNAs that bind to NRDE-3 on a small scale. To better understand the endogenous roles of the nuclear RNAi pathway, and to ask if nuclear RNAi drives off-target silencing during the normal

Table 2 Off-target gene silencing specifically requires Nrde pathway

Genotype	*dpy-13 RNAi*
	dumpy
eri-1(mg366)	++++
eri-1(mg366); *rde-1(ne219)*	−
eri-1(mg366); *nrde-1(gg088)*	+
eri-1(mg366); *nrde-2(gg091)*	+
eri-1(mg366); *nrde-3(gg066)*	+
eri-1(mg366); *ppw-1(pk2505)*	++++
eri-1(mg366); *ppw-2(tm1120)*	++++
eri-1(mg366); *Y49F6A.1(tm1127)*	++++
eri-1(mg366); *sago-1(tm1195)*	++++
eri-1(mg366); *prg-2(tm1094)*	++++
eri-1(mg366); MAGO11(−); *nrde-3(+) {wago-1(tm1414)*; *wago-2(tm2686)*; *wago-3(tm1120)*; *wago-4(tm1019)*; *wago-5(tm1113)*; *wago-6(tm894)*; *wago-7(tm914)*; *wago-8(tm1195)*; *wago-9(tm1200)*; *wago-10(tm1186)*; *wago-11(tm1127)*; *eri-1(mg366)}*	+

+ means sensitive to RNAi; − means resistant to RNAi.

course of growth and development, we immunoprecipitated NRDE-3 from the FLAG::GFP::NRDE-3 strain and deep sequenced the associated small RNAs from embryo lysates (Figures 5, Figure S5). *eri-1(mg366)*; *dpy-13(e458)*; *FLAG*::*GFP*::*NRDE-3* fed *dpy-13* RNAi was included as a control. We used a small RNA-cloning method compatible with cloning monophosphorylated and triphosphorylated small RNAs (*i.e.*, 22G-RNAs). Small RNAs were isolated, pretreated with calf intestinal alkaline phosphatase to remove phosphate groups, treated with polynucleotide kinase to add a monophosphate group to the 5′ ends, and subjected to deep sequencing using the Illumina platform. Small RNAs were aligned to the *C. elegans* transcriptome (WS229 assembly) using Bowtie2 software with the default parameters, and the number of small RNAs targeting each gene was counted.

We first focused on the small RNA reads that were complementary to annotated genes to avoid sense mRNA or sense pre-mRNA sequences that were likely to have coimmunoprecipitated with NRDE-3 (Guang *et al.* 2008, 2010). An analysis of both the length and first nucleotide distribution of the reads revealed that NRDE-3-associated siRNAs belong to the 22G-RNA class, which is consistent with previous results (Figure 5B; Guang *et al.* 2008; Zhang *et al.* 2011). NRDE-3-associated 22G-RNAs were dependent upon *eri-1* (Figure 5C). We searched for NRDE-3 target genes by selecting genes that had >10 raw read counts (not normalized) and twofold enrichment (normalized) between *FLAG*::*GFP*::*NRDE-3* and *eri-1(mg366)*; *dpy-13(e458)*; *FLAG*::*GFP*::*NRDE-3*; *(dpy-13 RNAi)* animals. Among the set of 178 genes identified as NRDE-3 targets (Table S5), E01G4.5 showed the most siRNA reads, in accordance with our previous result (Guang *et al.* 2008). Consistently, the expression of NRDE-3 targets was increased in the *nrde-2(gg091)* mutant, as determined by real-time PCR (Figure 5D).

Nuclear RNAi silences repetitive genomic loci

Interestingly, many of the NRDE-3 reads did not map to annotated genes (Figure 6A). We aligned all NRDE-3 associated small RNAs to the *C. elegans* genome and, surprisingly, found that many small RNAs are derived from intergenic genomic loci (Figure 6, A and B). Despite the lack of annotation, these sequences exist in clusters and are oriented on one strand as if they target expressed transcripts. These loci bind RNA polymerase II to produce nascent transcripts, suggesting that active transcription occurs at these sites (Figure 6B). We speculate that nuclear RNAi may target incompletely annotated transcripts at these genomic loci (Gu *et al.* 2009).

To analyze the extent of homology between nuclear RNAi target loci, we identified 63 genomic loci that were complementary to small RNA reads (Figure 6A, Table S6). The sequences of these loci were retrieved and aligned to the genome using BLASTn with a cutoff of >90% nt identity over a stretch of >200 nt of sequence. Most of the 63 loci are homologous to another region in the genome (Table S6). We also examined the NRDE-3-targeted genes and found that they are broadly repetitive with each other (Figure 6, C and D). Consistent with this idea, it was recently reported that *eri-6/7*-targeted genes are repetitive in sequence and that NRDE-3-bound siRNAs depend on *eri-6/7* (Fischer *et al.* 2011). To ask if the repetitiveness of NRDE-3 target loci was unique to NRDE-3 or a general characteristic of small RNA-targeted genes in *C. elegans*, we compared the repetitiveness of the targets of three secondary Argonaute proteins, including WAGO-1, CSR-1, and NRDE-3 (Figure 6E). As described above, more than 60% of the targets of NRDE-3 were repetitive, yet only 10% of WAGO-1 and CSR-1 targets were repetitive. Within a set of randomly selected genes, ~7.5% (3 of 40) share this degree of sequence similarity. Thus, NRDE-3-associated 22G siRNAs preferentially target repetitive genomic loci and the repetitiveness of these target loci is particular to NRDE-3-associated siRNAs. We note, however, that this correlation is not perfect: there are very few NRDE-3-associated endogenous siRNAs targeting multigene families such as the collagen genes or other protein coding genes from multigene families and most CSR-1-associated endo-siRNAs target genes are not repetitive (Table S2, Table S5) (Claycomb *et al.* 2009).

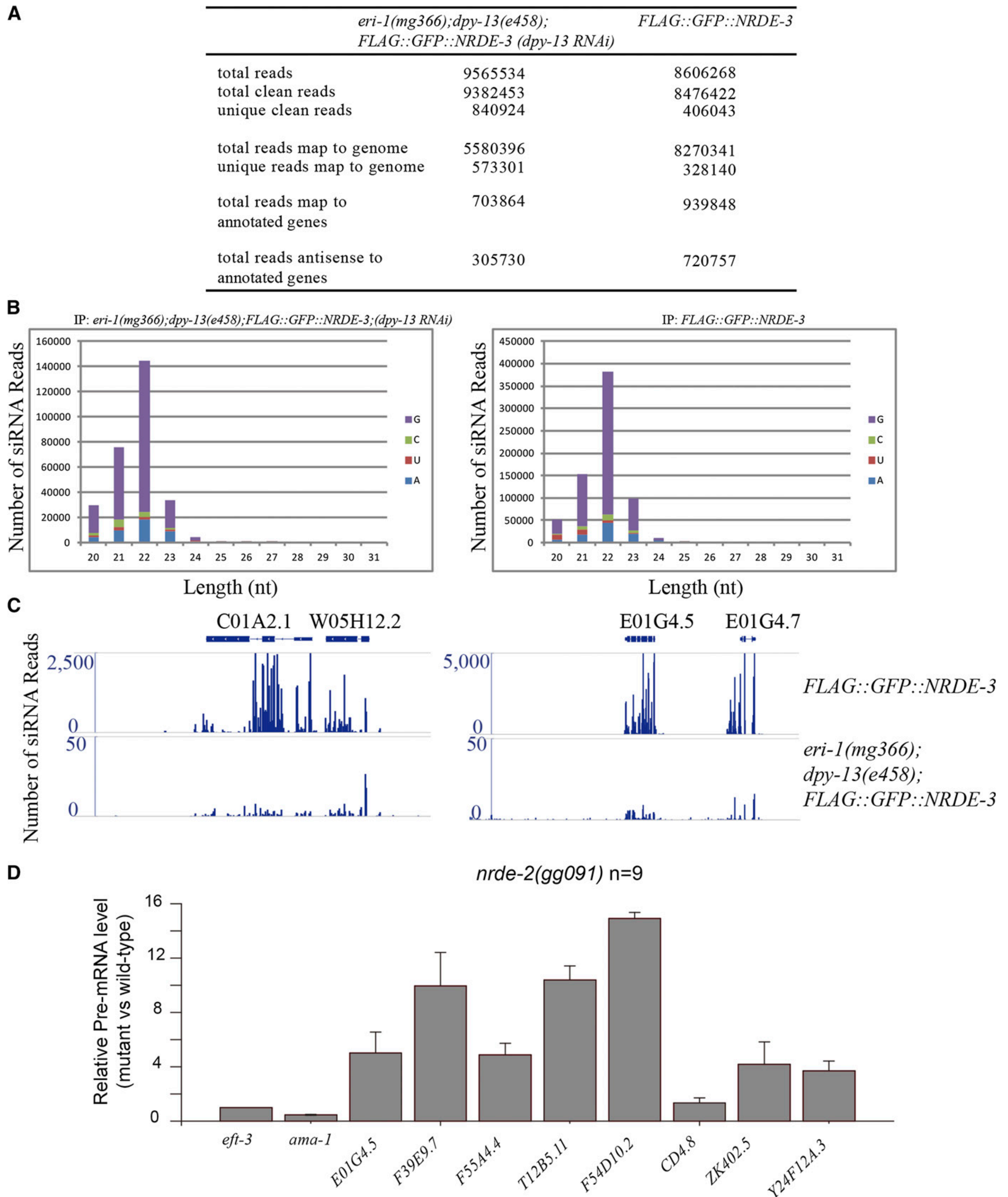
Discussion

Here, we report that components of the *C. elegans* nuclear RNAi pathway are required for off-target silencing. We also show that members of large gene families are prone to silencing by the nuclear RNAi pathway. Our data suggest that nuclear RNAi, but not its cytoplasmic counterparts, is the primary source of off-target silencing. We also identified endogenous nuclear RNAi targets by deep sequencing and found that NRDE-3 binds to 22G-RNAs and targets distinct repetitive genomic loci. These data revealed unexpected roles for nuclear RNAi in gene silencing.

Why off-target silencing?

Invasive parasitic nucleic acids can evolve rapidly, which results in highly similar and heterogeneous sequences. To protect the host genome, allowing RNAi off-target silencing can enable the host to efficiently recognize, memorize, and defend against these sequences. Consistent with this idea, nuclear RNAi is involved in the inheritance of RNAi silencing (Buckley *et al.* 2012; Feng and Guang 2013). In addition, many of the NRDE-3 targets are pseudogenes and unlikely express functional proteins (Fischer *et al.* 2011). *C. elegans* expresses many pseudogenes that exhibit sequence similarity to other protein coding genes. Pseudogenes can also evolve rapidly and interfere with the normal function of the homologous protein coding genes, therefore requiring an efficient silencing by nuclear RNAi.

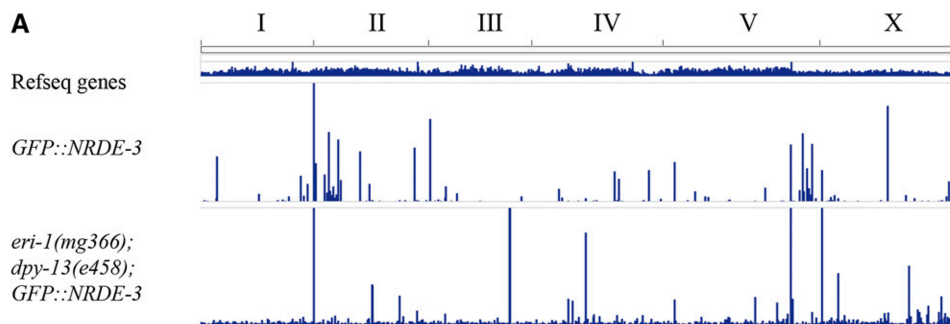
Off-target gene silencing is one of the major concerns for the application of RNAi-based technology. Although most people assume that the off-target effects of RNAi are simply due to inherent and unavoidable biochemical limitations of the RNAi machinery, our data suggest that off-target silencing is a genetically programmed aspect of RNAi.

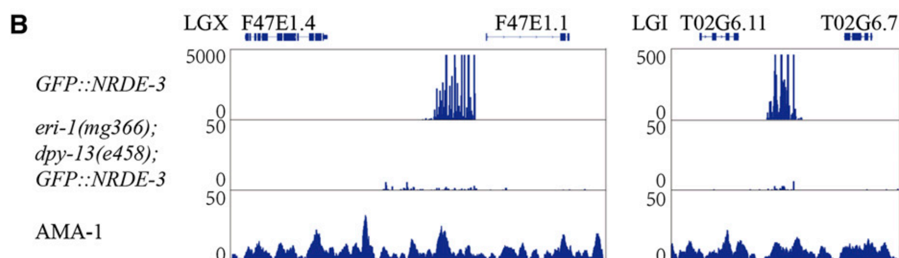


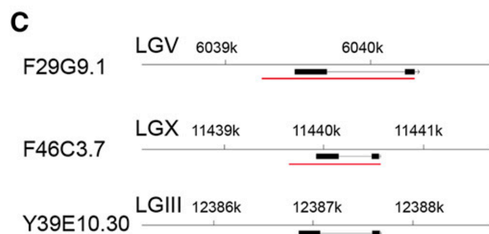
A

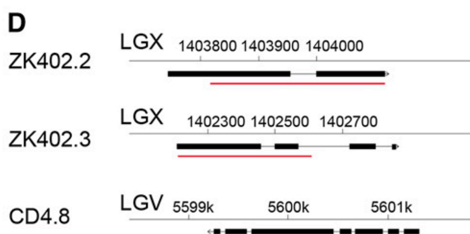
	eri-1(mg366);dpy-13(e458); FLAG::GFP::NRDE-3 (dpy-13 RNAi)	*FLAG::GFP::NRDE-3*
total reads	9565534	8606268
total clean reads	9382453	8476422
unique clean reads	840924	406043
total reads map to genome	5580396	8270341
unique reads map to genome	573301	328140
total reads map to annotated genes	703864	939848
total reads antisense to annotated genes	305730	720757

Figure 5 Deep sequencing indicates that NRDE-3 associates with 22G-RNAs. (A) Summary of the deep-sequenced NRDE-3-associated small RNAs from *eri-1(mg366)*; *dpy-13(e458)*; *FLAG*::*GFP*::*NRDE*-3; *dpy-13(RNAi)* and *FLAG*::*GFP*::*NRDE-3* animals. (B) Length and first letter distribution of NRDE-3-associated siRNAs. (C) *eri-1* is required for the production of NRDE-3-associated siRNAs. (D) The expression of NRDE-3 targets is misregulated in *nrde-2* mutants. Total RNAs were isolated from embryos and subjected to qRT-PCR to detect the pre-mRNA level of the indicated genes. Independent primer sets were applied, and similar results were obtained (data not shown). *eft-3* mRNA was used as an internal control for normalization. $N = 9$.









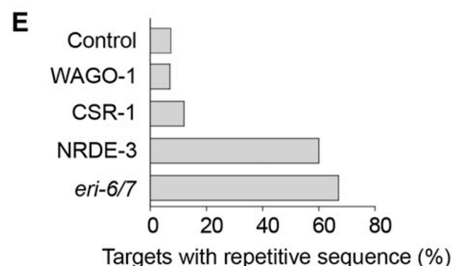


Figure 6 Nuclear RNAi targets distinct genomic loci. (A) NRDE-3-associated small RNAs were mapped to the *C. elegans* genome and displayed using the integrative genomics viewer (IGV). (B) NRDE-3-associated small RNAs target intergenic regions. The left panel indicates X-cluster siRNAs on chromosome X, which has the most NRDE-3-bound small RNA reads (Guang *et al.* 2008). AMA-1 (RNA polymerase II) ChIP-seq datasets were retrieved from GSM677643 of the modENCODE project to identify the binding region of AMA-1 in L4 wild-type animals (Gerstein *et al.* 2010). (C and D) The genes targeted by NRDE-3-associated siRNAs were blasted against the genome. Shown are examples of the gene groups that share high sequence similarity with a cutoff of >90% nt identity over a stretch of >200 nt of sequence. The red bars indicate the regions with sequence homology. (E) The repetitiveness of the targets of three secondary Argonaute proteins, including WAGO-1, CSR-1, and NRDE-3, and the targets of *eri-6/7* were compared by using a criteria of >90% nt identity in a stretch of >200 nt sequence to one or more of the other targeted genes or genomic loci. A set of randomly selected genes (n = 40) was used as a control.

Nuclear RNAi machinery is the major contributor to RNAi off-target effects in *C. elegans*, implying its potential application in clinics. Off-target silencing can be alleviated via the use of low concentrations of siRNA, adding methyl groups, and/or modifying siRNA with locked nucleic acids (LNAs) (Svoboda 2007). Our data suggest that off-target gene silencing could also be reduced by abrogating the nuclear RNAi pathway. As NRDE-2 is a conserved protein that has only one homolog in mammals (Guang *et al.* 2010), it will be interesting to explore how NRDE-2 is involved in nuclear RNAi and off-target silencing in higher eukaryotes.

RNAi in eri animals induces unintended silencing effects

eri mutant animals respond more robustly than wild-type animals to exogenously provided dsRNA (Kennedy *et al.* 2004; Duchaine *et al.* 2006; Fischer *et al.* 2011). This effect could be partially explained by a competition model in which the exogenous RNAi pathway competes with the endogenous RNAi pathway for limiting factors (Lee *et al.* 2006; Yigit *et al.* 2006). Indeed, the overexpression of SAGO-1, SAGO-2, or NRDE-3 enhances feeding RNAi, indicating that some secondary Argonaute proteins could be the limiting factors (Yigit *et al.* 2006; Zhuang *et al.* 2013). On the other hand, our data suggest that at least some of the enhanced RNAi effect could arise from off-target silencing, particularly for dsRNAs targeting large gene families.

RRF-1 is required for off-target silencing

Off-target silencing can happen either by imperfect complementation between siRNA with its targets or by synthesizing off-target secondary siRNA in *C. elegans*. In the latter case, Argonaute-mediated silencing may be a targeted process *per se*, directed by RdRP-generated off-target secondary siRNAs. The identification of siRNAs that target collagens other than *dpy-13* in *eri-1(mg366)*; *dpy-13(e458)*; *FLAG*::*GFP*::*NRDE-3* after *dpy-13* RNAi supports the second model.

It is unknown why nuclear RNAi is special in contributing to off-target silencing. Previous genetic screening to search for nuclear RNAi defective (Nrde) mutants had isolated many *rrf-1* alleles, suggesting an inherent connection between *rrf-1* and nuclear RNAi (Guang *et al.* 2008; Burkhart *et al.* 2011). RRF-1 is required for NRDE-3 to bind both endogenous siRNA and siRNA elicited by feeding RNAi (Guang *et al.* 2008). However, we have conducted proteomic analysis of NRDE-2 and NRDE-3 but failed to identify RRF-1 interaction with either of them (data not shown). It is possible that RRF-1-synthesized off-target secondary siRNAs can preferentially bind to NRDE-3 and trigger silencing in the nucleus. Alternatively, off-target siRNAs can bind either NRDE-3 or other cytoplasmic Argonautes; yet, nuclear RNAi can be more permissive than cytoplasmic silencing systems. It will be very interesting to sequence and compare siRNAs from all secondary Argonaute proteins after *dpy-13* RNAi to answer this question.

Nuclear RNAi mediates off-target silencing

We observed that *nrde-3*, but not *ppw-1*, *ppw-2*, *Y49F6A.1*, *sago-1*, or *prg-2*, is phenotypically critical for off-target silencing, suggesting that some of the effects of off-target silencing strongly depend on nuclear RNAi machinery. Alternatively, cytoplasmic Argonaute proteins might be more redundant in *C. elegans*; therefore, knocking out one cytoplasmic Ago is not sufficient to elicit visible RNAi effects. The *eri-1(mg366)*; *MAGO11(−)*; *nrde-3(+)* strain is nearly completely RNAi defective in the germline but partially RNAi defective in the soma, and NRDE-3 is only expressed in soma (Guang *et al.* 2008), suggesting that NRDE-3 likely functions in parallel with cytoplasmic Argonaute proteins to perform silencing. However, the observation that *dpy-13* RNAi of *eri-1(mg366)*; *MAGO11(−)*; *nrde-3(+)* animals does not induce superdumpy and the *MAGO12(−)* strain is defective in small RNA accumulation (Vasale *et al.* 2010), suggest that cytoplasmic Argonaute proteins may also act as a whole to stabilize siRNAs and indirectly modulate nuclear RNAi.

NRDE-3 binds to 22G-RNAs

In *C. elegans*, endogenous siRNAs are mostly categorized according to their length and 5′ nucleotide: 22G-RNAs are predominantly 22 nt in length and contain a triphosphorylated 5′-G. These siRNAs bind to particular subtypes of the 27 *C. elegans* Argonaute proteins to confer their unique functionality. The majority of endogenous siRNAs are derived from RdRP transcripts and likely undergo additional nuclease-mediated processing. 22G-RNAs are antisense to >50% of annotated genes (Gu *et al.* 2009). Two major 22G-RNAs exist in *C. elegans*: those that interact with CSR-1 and those that interact with worm-specific AGO (WAGO) proteins. It has been suggested that the WAGO/22G-RNA system provides surveillance against transposable elements and aberrant transcripts. ERGO-1-dependent 26G-siRNAs were derived from unannotated intergenic regions (Vasale *et al.* 2010), and NRDE-3 targets repetitive intergenic loci, suggesting that nuclear RNAi suppresses aberrant transcripts at these sites. Further deep sequencing and annotation to identify long intervening noncoding RNAs (lincRNAs) will facilitate an understanding of these intergenic loci in *C. elegans*.

Acknowledgments

We are grateful to Scott Kennedy, Guang Yao, Yupeng Yang, Ge Shan, Qingfa Wu, and Ying Liu and members of the Kennedy and Guang labs for comments. We are grateful to the *Caenorhabditis* Genetics Center for providing the strains. This work was supported by grants from the Chinese 973 Program (no. 2011CBA01100), the National Natural Science Foundation of China (nos. 31171254 and 31371323), the Fundamental Research Funds for Central Universities (nos. WK2060190018 and WK2070000034), and the research foundation of Chinese Academy of Science, KJZD-EW-L01-2.

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Communicating editor: B. Meyer

GENETICS

Supporting Information
http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.113.159780/-/DC1

Nuclear RNAi Contributes to the Silencing of Off-Target Genes and Repetitive Sequences in *Caenorhabditis elegans*

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DOI: 10.1534/genetics.113.159780

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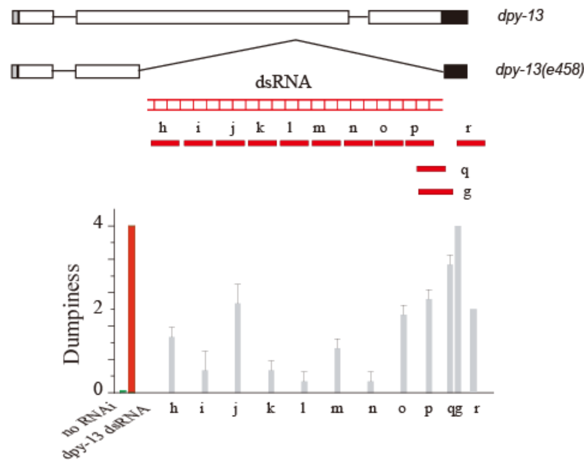


B

```
                      ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....
                              10         20         30         40         50         60         70
dpy-13                AGGAGAGCGT GGAATCTGTC CAAAGTACTG CGCCATCGAT GGAGGAATCT TCTTCGAGGA CGGAACTCGC CGC---TAA
sqt-3                 AGGAGAGAAG GGAATCTGTC CAAAGTATTG CGCTCTCGAT GGAGGAGTCT TCTTCGAGGA CGGAACCAGA CGA---TAA
col-34                AGGAGAGCGT GGAATCTGTC CAAAATACTG TGCTATCGAC GGAGGAGTCT TCTTCGAAGA CGGAACTCGT CGT---TAA
col-43                AGGAGAGAAG GGAATCTGTC CAAAGTACTG CGCTCTCGAT GGAGGAGTCT TCTTCGAGGA CGGAACTCGC CGC---TAA
col-93                CGGAGAGAAG GGAATCTGTC CAAAGTACTG CGCCATCGAC GGAGGAGTAT TCTTCGAGGA CGGAACCCGC AGA----AA
col-94                CGGAGAGAAG GGAATCTGTC CAAAGTACTG CGCCATCGAC GGAGGAGTCT TCTTCGAGGA CGGAACCCGC AGAAAGTAA
col-122               AGGAGAGAAG GGAATCTGTC CAAAATACTG TGCTATCGAT GGAGGAGTCT TCTTCGAGGA CGGATCCAGA CGC---TAG
                      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
                      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
                      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
dpy-13g (RNAi)        AGGAGAGCGT GGAATCTGTC CAAAGTACTG CGCCATCGAT GGAGGAATCT TCTTCGAGGA CGGAACTCGC CGC---TAA
dpy-13g(m1) (RNAi)    ---------- ---------- --G------- ---------- ---------- ---------- ---------- ---------
dpy-13g(m2) (RNAi)    ---------- ---------- ---------- ---------- ---------- ---------G ---------- ---------
dpy-13g(m1m2) (RNAi)  ---------- ---------- --G------- ---------- ---------- ---------G ---------- ---------
```

C

RNAi	*eri-1(-);dpy-13(-)*	*eri-1(-);dpy-13(-);nrde-3(-)*
dpy-13 (723mer)	++++	++
g(76mer)	++++	++
g(m1)	++++	++
g(m2)	++++	++
g(m1m2)	++++	++

Fig. s1

Figure S1 (A) Identification of a 76-nt sequence, *dpy-13g*, which targets the 3' end of the *dpy-13* mRNA, induced the superdumpy-like phenotype after RNAi. A series of *dpy-13* sequences were cloned in the L4440 dsRNA expression plasmid and fed L1 animals of *eri-1(mg366)*. Dumpiness was scored based on their relative length to that of N2 animal. Without RNAi, N2 and *eri-1(mg366)* were scored as 0 at the scale of 1 to 4 or – at the scale of – to ++++. After *dpy-13* RNAi, *eri-1(mg366)* exhibited a worm-ball-like phenotype, which was scored as 4 or ++++. The length of *dpy-13(e458)* animal is approximately half of that of N2, which is denoted as "dumpy" and scored as 2 or ++. Animals that exhibited a length less than 25% of that of N2 were usually classified as superdumpy or superdumpy-like and scored as 3 / 4 or +++ / ++++. (B) The 76-nt sequence in *dpy-13* is highly similar between collagen genes. Shown below are mutations introduced into the *dpy-13g*. (C) Nuclear RNAi was required for *dpy-13g*-mediated off-target silencing.

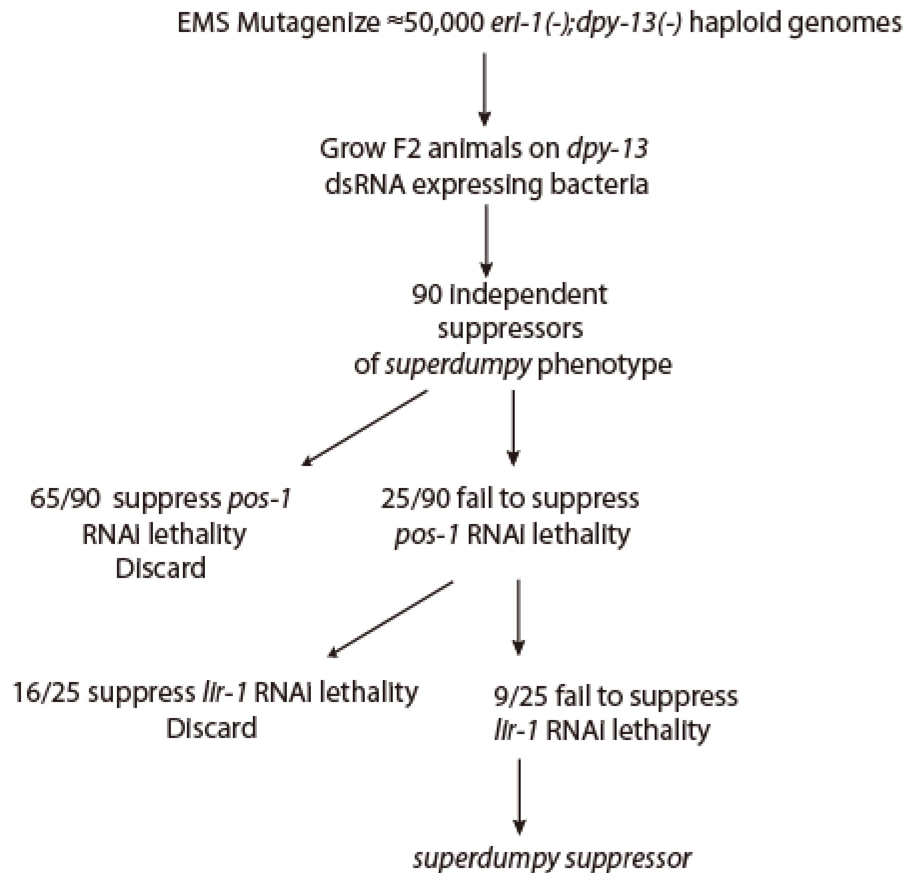
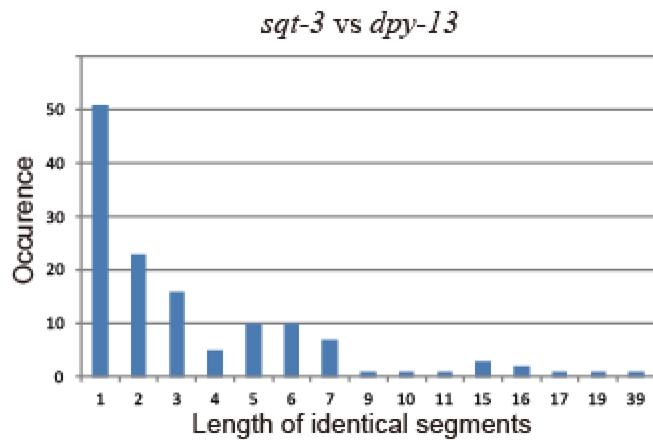


Figure S2 Schematic diagram of genetic screening to search for factors required for off-target silencing. *eri-1(mg366);dpy-13(e458)* animals were chemically mutagenized and searched for mutants that suppressed RNAi off-target effect but were dispensable for *pos-1* and *lir-1* RNAi. *pos-1* RNAi leads to unhatched F1 embryos; animals that are resistant to *pos-1* RNAi likely carry mutations in the core components of RNAi machinery. *lir-1/lin-26* genes are expressed in an operon; they are co-transcribed as a polycistronic pre-mRNA, which is spliced into distinct mRNAs in the nucleus before export to the cytoplasm. *lir-1(−)* mutant animals are viable. Loss of function alleles of *lin-26* are inviable. RNAi targeting *lir-1* induces a lethal phenotype by silencing the nuclear-localized *lir-1/lin-26* RNA. *nrde* mutant animals are viable following *lir-1* RNAi, because *nrde* genes are required to silence the nuclear-localized *lir-1/lin-26* RNA (Guang, 2008). Similarly, *nrde* genes are required to silence the nuclear-localized *lin-15b/lin-15a* polycistronic RNA. Nine mutants that were specifically defective in off-target silencing were isolated. Mutants were mapped by snp-SNP mapping and candidate genes are sequenced.

A



B

strain	phenotype
dpy-13(e458);col-34(RNAi)	dumpy
dpy-13(e458);col-43(RNAi)	dumpy
dpy-13(e458);sqt-3(RNAi)	superdumpy-like
sqt-3(e2924);dpy-13(RNAi)	superdumpy-like

Figure S3 *sqt-3* is a major off-target of *dpy-13* RNAi. (A) Sequence comparison between *sqt-3* and *dpy-13*. There is one identical sequence fragment longer than 21 nt. (B) dsRNA targeting *sqt-3,* but not *col-34* or *col-43,* in the *dpy-13(e458)* animal elicits the superdumpy-like phenotype.

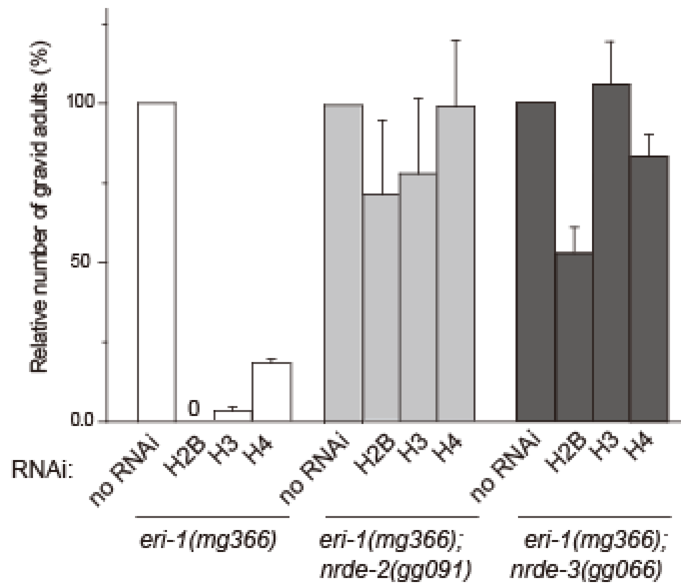


Figure S4 The silencing of histone genes requires nuclear RNAi. Synchronized L1 animals (>100) were fed with indicated dsRNAs and the numbers of gravid adults were scored 3 days later. dsRNAs from the Ahringer RNAi library were applied in this experiment. The experiments were performed in triplicate (N=3).

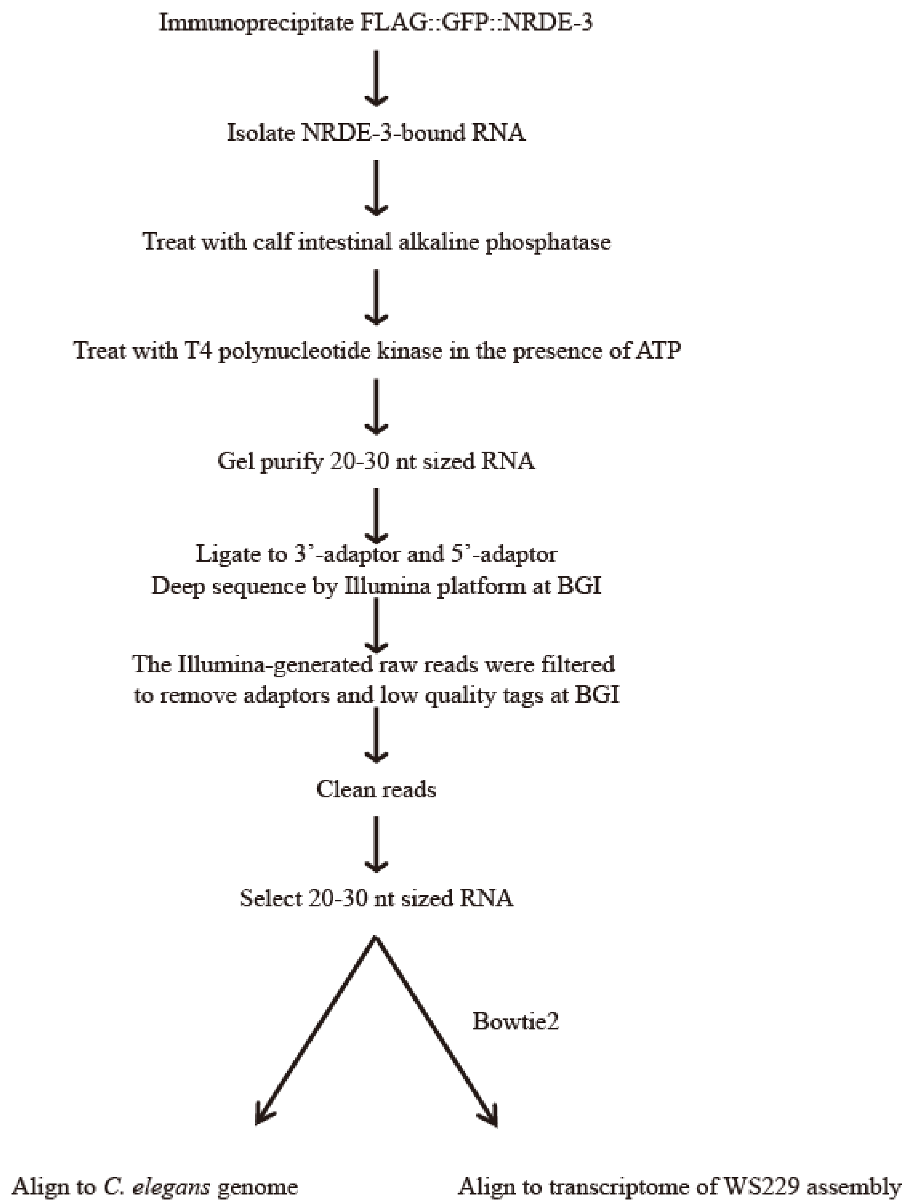


Figure S5 A flowchart of the procedure to isolate, sequence, and analyze NRDE-3-associated small RNAs.

Table S1 dsRNAs targeting the 76 nt sequence of collagen genes elicit the superdumpy phenotype in *eri-1(mg366)* animals. L1 animals of *eri-1(mg366)* mutants were fed with dsRNAs and the phenotypes were scored 3 days later. ++++, superdumpy; -, no dumpy.

RNAi (76mer)	*superdumpy*
no RNAi	-
dpy-13	++++
sqt-3	++++
col-93a	++++
col-93c	++++
col-43	++++
col-94	++++
col-122	++
col-179	-

Tables S2-S7

Available for download as Excel files at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.113.159780/-/DC1

Table S2 The number of reads of NRDE-3-associated siRNAs targeting collagen genes.

Table S3 Candidate-based RNAi screening to search for genes by which the silencing were strongly depended on *nrde* pathway. 168 genes present in operons were selected from the Ahringer RNAi library with reported phenotype in either N2 or *rrf-3* strain. The 168 operon genes are unlikely to be repetitive based on the criteria of >90% nt identity in a stretch of >200 nt sequence to one or more of the other genes or genomic loci. 28 RNAi clones targeting multi-gene families were selected from the Ahringer RNAi library. NRDE-dependent silencing was observed when feeding animals with dsRNA targeting of histones, homeobox genes, and GPCR.

Table S4 Off-target gene silencing preferentially requires the Nrde pathway. *eri-1(mg366);MAGO12* was crossed with *eri-1(mg366)*. Eight independent F2 animals were selected that suppressed the *dpy-13* RNAi-induced superdumpy. The F2 suppressors were then genotyped for the twelve Argonaute genes. *nrde-3(tm1116)*, but not other eleven *wago* alleles, is linked to superdumpy suppression.

Table S5 Deep sequencing identified 178 NRDE-3 targets that exhibit at least ten raw reads and two fold enrichment between *FLAG::GFP::NRDE-3* and *eri-1(mg366);dpy-13(e458);FLAG::GFP::NRDE-3;(dpy-13 RNAi)* strains.

Table S6 Sixty-three genomic loci were identified to display most NRDE-associated siRNA reads. Most of these loci are homologous in sequence to another region in the genome.

Table S7 The primers used for quantitative real time PCR analysis.