Supporting Online Material:

Materials and Methods:

RNAi. RNAi experiments were conducted as described previously (*1*). dsRNA expressing bacteria, including *lir-1, pos-1, unc-40, wrt-2, dpy-7, unc-3, unc-14, unc-22, unc-15,* and *unc-73,* were obtained from the Ahringer RNAi library and were sequenced to verify their identities. *lin-15b* RNAi clone was constructed by PCR amplification of *lin-15b* exon 5 from genomic DNA with primers (5'-tttaagctt AGCGCCTTCTTCAACAATGC-3' and 5'-tttaaaggg GCGGATGATTGTTGGAGAG-3'), inserted into HindIII site of L4440 plasmid (a gift from A. Fire), and transformed into HT115 *E. coli* strain. This *lin-15b* dsRNA resulted in ≈95% multi-vulva progenies when fed to *eri-1(-)* animals (Table 1). For unknown reasons, dsRNA targeting several other regions of *lin-15b* only induced ~5% of animals to exhibit a multi-vulva phenotype, presumably due to differential RNAi efficiencies (data not shown) (*2*).

Phenotypes elicited by each RNAi clone were scored on a scale of zero to four, where phenotypes of *eri-1(mg366)* animals exposed to dsRNA were defined as four and phenotypes of *eri-1(mg366)* animals fed control bacteria were defined as zero (Table 1 and Supplemental Fig. S5C). An *eri-1(-)* genetic background was utilized in these experiments to maximize our ability to score RNAi induced phenotypes.

Images were collected on a Zeiss Imager D1 microscope. Seam cell pictures were taken from L3 stage animals.

Construction of plasmids and transgenic strains. For FLAG::GFP::NRDE-3 (referred to as GFP::NRDE-3 when assaying NRDE-3 localization or FLAG::NRDE-3 when referring to NRDE-3 immunoprecipitation or western blotting) and its mutant variants, a 3xFLAG::GFP coding region was PCR amplified from pPD95.79 (gift from A. Fire) with primers (5'- tttgtcgac-ATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGATATCGATTACAAGGATGACGATGACAAG-atgagtaaaggagaagaacttttc-3' and 5'-aaagtcgacTTTGTATAGTTCATCC-3'), digested with SalI, and inserted into XhoI site of the pPD##MM051 plasmid. Then, a 2.2 kb Nrde-3 promoter region was PCR amplified from genomic N2 DNA with primers (5'- tttAAGCTTTTTTCCATACATTGCTCTTCG-3' and 5'- aaaaagcttGAGATCCATGATTAGTTGTGC-3'), and inserted into the HindIII site. Nrde-3 coding region (including the predicted *nrde-3* 3'-UTR) was PCR amplified from genomic N2 DNA with primers (5'-tttggtaccATGGATCTCCTAGACAAAGTAATG-3' and 5'-aaaggtaccTTACATAGGATGTACTCTGATGC-3'), and inserted into the KpnI site of the plasmid to make a Nrde-3p::3xFLAG::GFP::NRDE-3 fusion gene. Mutant variants of NRDE-3 were constructed by standard PCR-based mutagenesis methods. SV40 NLS sequence was constructed from synthesized oligonucleotides (5'-cCCAAAGAAGAAGCGTAAGGTAgggcc-3' and 5'-cTACCTTACGCTTCTTCTTTGGgggcc-3'), annealed, and inserted in-frame into ApaI

site between Nrde-3 promoter and 3xFLAG::GFP::NRDE-3(*NLS) protein coding sequences, to make a N-terminal SV40 NLS::3xFLAG::GFP::NRDE-3(*NLS) fusion. Low-copy integrated lines of these constructs were obtained by biolistic transformation. 7/7 independent lines expressing wild type (WT) NRDE-3, 5/5 lines expressing NRDE-3(*NLS), and 2/3 lines expressing SV40NLS::NRDE-3(*NLS) behaved similarly for *nrde-3(-)* rescue. One SV40NLS::NRDE-3(*NLS) expressing line failed to rescue *nrde-3(gg066)* animals for *lir-1* RNAi mediated lethality (not shown). For unknown reasons, 0/7 wild type NRDE-3 constructs rescued *nrde-3(gg066)* animals for *lin-15b* RNAi driven multi-vulva formation (not shown).

Isolation of NRDE-3 associated RNAs. To isolate NRDE-3 associated siRNAs, animals (or hypochlorite isolated embryos, as indicated) 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). Lysates were clarified by centrifuging at 14k rpm for 15 min. Supernatants were pre-cleared with Protein G agarose beads (Roche) and incubated with anti-FLAG M2 agarose (Sigma) beads for 1 hour at 4ºC. Beads were washed extensively and FLAG::GFP::NRDE-3 was eluted with 100 ug/ml 3xFLAG peptide (Sigma). Eluates were incubated with 5 volumes of TRIzol reagent (Invitrogen), followed by isoproponal precipitation. Precipitates were treated with calf intestinal alkaline phosphatase (CIAP, Invitrogen) at 37^{o}C for 30 min, re-extracted with TRIzol, and treated with T4 polynucleotide kinase (T4 PNK, New England Biolabs) together with γ-[32]p-ATP at 37ºC for 30 min. FLAG::NRDE-3 western blot was performed with anti-FLAG M2 antibody (Sigma), and detected by western blotting detection system (Amersham, GE).

To isolate NRDE-3 associated total RNAs after RNAi, hypochlorite isolated embryos were sonicated in lysis buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 2.5 mM $MgCl_2$, 0.5% NP-40, and 10% glycerol, with proteinase inhibitor tablet (Roche) and RNaseOUT (Invitrogen)). FLAG::GFP::NRDE-3 was immunoprecipitated and eluted as described above. Eluates were first incubated with Turbo DNase I (Ambion) at 37^{o}C for 20 minutes. Then RNAs were purified by TRIzol reagent (Invitrogen), and followed by isoproponal precipitation. NRDE-3 associated RNAs were quantified by qRT-PCR (see below).

NRDE-3 localization in *mut-7(pk204)* animals. *mut-7(pk204)* animals exhibit partial defects in NRDE-3 endo-siRNAs binding and partial defects in NRDE-3 nuclear localization (Fig. 1B). Thus, siRNA-mediated redistribution of NRDE-3 to the nucleus appears to be, at least somewhat, quantitative.

siRNA-mediated NRDE-3 nuclear localization. siRNA-mediated redistribution of NRDE-3 to the nucleus may result from retention of NRDE-3/siRNA complexes in the nucleus, and/or siRNA binding by NRDE-3 may trigger a conformational shift in NRDE-3 (or NRDE-3 associated factor), resulting in NRDE-3 NLS exposure and nuclear localization of NRDE-3.

Tissue-specific redistribution of NRDE-3. Prolonged exposure (> 3 days) to all four dsRNAs described in Fig. 4C resulted in nuclear localization of NRDE-3 in most, if not all, cells (not shown). The delayed, organism-wide, redistribution of NRDE-3 may reflect systemic spreading of an RNAi silencing signal.

***lir-1* RNAi**. *lir-1* lies in an operon with *lin-26*. Animals harboring loss of function mutations in *lir-1* are viable. Loss of function alleles of *lin-26* are inviable (*3*). L1 *eri-1(mg366)* animals exposed to *lir-1* dsRNA arrest at ≈L3 stage of larval development, consistent with somatic silencing of the nuclear-localized *lir-1/lin-26* polycistronic RNA. Wild-type L1 animals exposed to bacteria expressing *lir-1* dsRNA grow to adulthood, but fail to produce viable progeny. *nrde-3(gg066),* (eri-1+), P0 and F1 animals appear wild-type following exposure to *lir-1* RNAi (not shown).

Small RNA cloning. NRDE-3 associated endo and exo siRNAs were cloned following the protocol designed by Lau et al (*4*) with minor modifications. NRDE-3 co-precipitating small RNAs were isolated by TRIzol extraction, CIAP treated, and T4 PNK kinased with γ-[32]p-ATP. Labeled small RNAs were then cloned as described previously (*4*).

Quantitative RT-PCR. RNAs were isolated from embryos by first douncing with pestle B in TRIzol solution followed by purifying with RNeasy kit (Qiagen). cDNAs were generated from RNAs with iScript cDNA synthesize kit (Bio-Rad) according to vendor's protocol. qPCR was performed on an iCycler machine (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). The primers used for qRT-PCR are as follows:
eft-3-mRNA-(acttgatctacaagtgcggagga) and (aaagatcccttacccatctcctg).
eft-3-pre-mRNA-(acttgatctacaagtgcggagga) and (cgggtgagaaaatctttcaaacta).
lin-15b-mRNA-(gccctccgtccacacattattgc) and (ttcttcaattccgctggcgtttc).
lin-15b-pre-mRNA-(gccctccgtccacacattattgc) and (caaaatgtgcaattttaaaaacg).
lin-15a-mRNA-(gcattggaacggatgctctgcag) and (tggagtttggctctcttttgaca).
lin-15a-pre-mRNA-(gcattggaacggatgctctgcag) and (gggagaaaatccttaaaaagcca).
E01G4.5-mRNA-(ctcaagaaagtttcacagcaggcc) and (cacttacacaaaacatttctc).
E01G4.5-pre-mRNA-(ctcaagaaagtttcacagcaggcc) and (gattgcaatccgaaaaaatttacac).
unc-40-mRNA-(actggaaggcatcagccgattc) and (gaggcggatccaattgcagatg).
unc-40-pre-mRNA-(ctggaaggcatcaggttggagtc) and (gaggcggatccaattgcagatg).

For E01G4.5, *lin-15b, lin-15a,* and *eft-3* genes, qRT-PCR experiments were performed with independent primers pairs that gave similar results (not shown). No independent primer set was attempted for *unc-40* mRNA and pre-mRNA quantification.

Northern Blot. Total RNA samples were prepared from purified embryos by TRIzol extraction. Small RNAs were enriched utilizing *mir*Vana miRNA Isolation Kit according to vendor's protocol (Ambion). 10 ug of RNAs was separated on a 15% denaturing PAGE gel and transferred to hybond-N^+ membrane (GE-Amersham) with a semi-dry apparatus (Hoefer SemiPhor TE 70, Amersham Pharmacia), and blotted in Ultrahyb-Oligo hybridization buffer (Ambion). The membranes were washed in 2xSSC + 0.5%

SDS, and exposed on a PhosphorImager screen (Molecular Dynamics). E01G4.5 probe of the following sequence (5'-GACCAAACCGCGCGCTTCAGAGGTCATTGGCTTCATACACTCAAAAGC/3StarFire/-3') was synthesized by IDT and labeled with α-[32]p-dATP utilizing StarFire Kit (IDT). The non-modified, 5'-end phosphor-modified, and 3'-end 2'-O-methyl-modified E01G4.5 siRNAs were synthesized by IDT.

In situ hybridization. The pes-10::GFP transgenic array: axIs36[pJH1.16(*pes-10*::*gfp*);pMH86(*dpy-20*)]X was provided by M. Wallenfang and G. Seydoux. To generate probes for *in situ* hybridization, a 3' portion of *gfp* was PCR amplified with primers (5'-tttaagcttGTGCTGAAGTCAAGTTTGAAGG-3' and 5'-aaaaagcttGCTATTTGTATAGTTCATCCATGCC-3'). Digoxigenin (DIG)-labeled single-stranded DNA probes were synthesized by multiple cycles of primer extension in the presence of DIG-dUTP. GFP dsRNA expression plasmid was generated by inserting 5' portion of *gfp* ((generated with primers (5'-gggaagcttATGAGTAAAGGAGAAGAACTTTTC-3' and 5'-gctaaagcttTGTCTTGTAGTTCCCG-3')) into L4440 plasmid. Whole-mount *in situ* hybridizations were performed essentially as previously described (*5*). Bleached embryos were freeze-cracked and fixed in 1% formaldehyde for 20 min and incubated with an α-digoxigenin antibody conjugated to alkaline phosphatase (Roche) to visualize the probe.

Author Contributions. S.G. performed genetic screening, mapped *nrde-3*, generated constructs, and contributed Figs. 1c, 1d, 2a, 3c, 3d, 4a, 4b and Supplemental Figs. S2-S4, and S5b. A.B. generated all stable transgenic lines and contributed Figs. 1b, 3b, and S5a. D.P contributed Fig. 2c. K.B. and J.L contributed Table 1 and Supplemental Fig. S5c. S.H. contributed Fig. 2b. S.K. contributed Figs. 1a, 1d, 2d, 4c, 4d and Supplemental Table S1.

Supporting Materials Bibliography:

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Table S1. FLAG::NRDE-3 co-precipitating small RNAs were cloned and sequenced as described previously (*4*). 65 reads were matched to ribosomal genes (F31C3) with sense orientation. These siRNAs likely represent degradation products of rRNA and thus were not included for further analysis (*7*). 465 siRNAs that matched the *C. elegans* genome were retained for further characterization. These small RNAs represent endogenous (endo) siRNAs; they map to similar chromosomal loci as previously identified endo siRNAs (*7, 8*). The siRNAs cloned in this experiment mapped predominantly (>3 reads) to forty genomic loci. The chromosome, cosmid name, cosmid coordinates, and number of NRDE-3 associated siRNA within each loci, are shown.

Fig. S1. Genetic screen for factors specifically required for nuclear RNAi. **(A)** Hypothetical bifurcation of RNAi pathway. dsRNA is converted to siRNAs by the core RNAi machinery including factors such as Dicer and Rde-4. The canonical RNAi pathway silences cytoplasmic RNAs exhibiting sequence homology to the trigger dsRNA. A subset of siRNAs are directed to the nucleus and silence nuclear localized RNAs. **(B)** To identify nuclear RNAi specific factors, we screened for cellular factors required for silencing of nuclear localized RNAs but dispensable for silencing of cytoplasmically localized RNAs. *lir-1* RNAi results in a lethal phenotype due to silencing of the nuclear-localized *lir-1/lin-26* polycistronic RNA (see online Materials and Methods and Fig. 3B). In order to identify factors required for nuclear RNAi, *eri-*

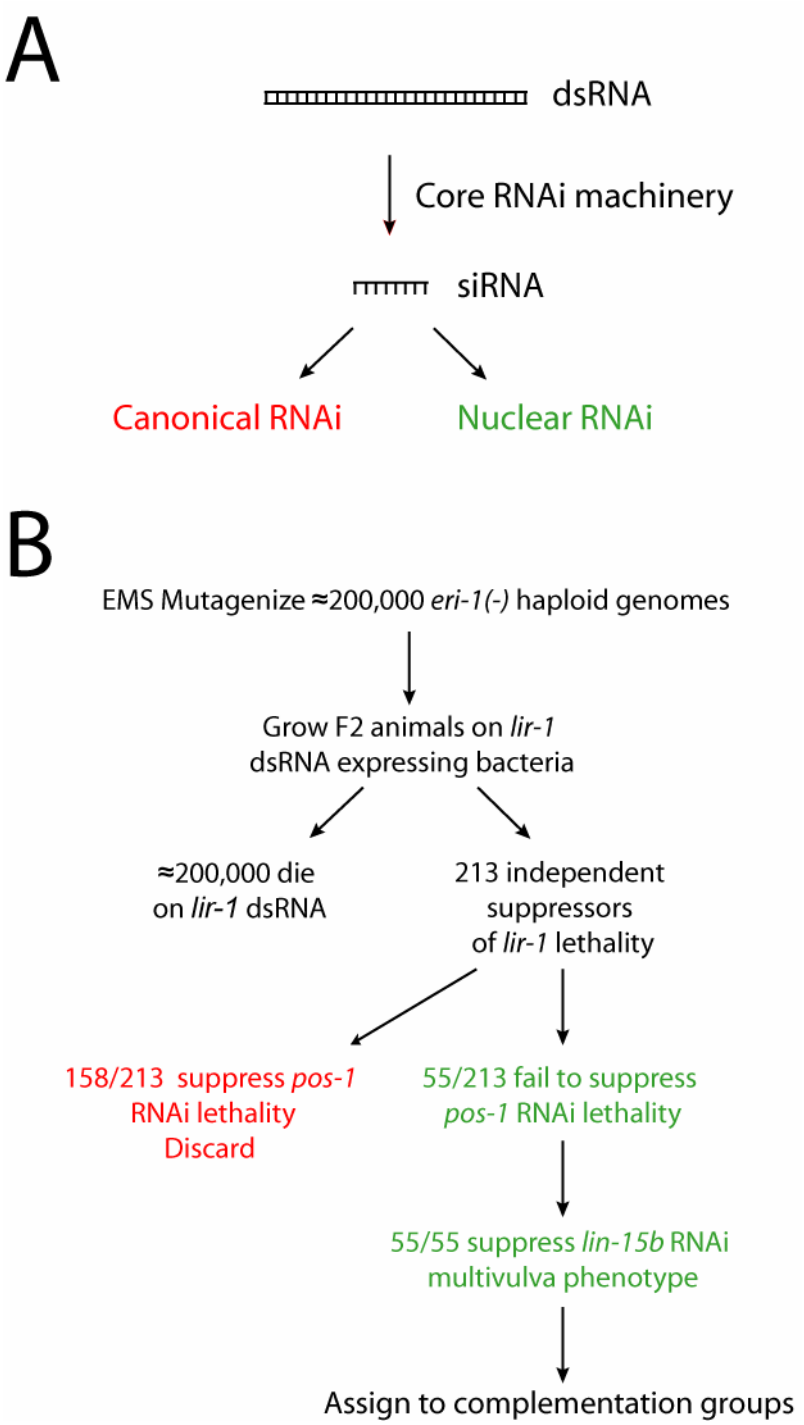
1(mg366) animals (utilized in this screen due to their enhanced sensitivity to dsRNAs) were mutagenized with ethyl methanesulphonate and F2 progenies were grown on *E. coli* expressing *lir-1* dsRNA. 213 suppressors of *lir-1* dsRNA mediated lethality were identified. In order to distinguish between animals harboring mutations in nuclear RNAi specific factors and animals carrying mutations in the core RNAi machinery we subjected the 213 mutants animals to *pos-1* RNAi. Unlike the *lir-1/lin-26* polycistron, the *pos-1* RNA does not localize exclusively to the nucleus (*6*). 158/213 mutant animals failed to respond to *pos-1* RNAi. These animals likely carry mutations in components of the core RNAi machinery and consequently were not analyzed further. The remaining mutant animals were scored for an independent measure of nuclear RNAi: *lin-15b* mediated multi-vulva formation (see main text and Table 1). 55 of these 55 mutant animals failed to exhibit a multi-vulva phenotype in response to *lin-15b* RNAi supporting the notion that they represent components of a dedicated nuclear RNAi pathway. 46 of these 55 mutant alleles failed to complement for *lir-1* RNAi mediated lethality and were assigned to a single complementation group termed *nrde-3*.

Fig. S2. NRDE-3 localizes to the nucleus. Embryos expressing GFP::NRDE-3 were isolated by hypochlorite treatment, freeze-cracked, fixed with methanol and formaldehyde, and incubated with α-FLAG M2 antibody (Sigma), followed by fluorescein-conjugated goat anti-mouse secondary antibody (Molecular Probes). Embryos were co-stained with DAPI and analyzed on a spinning disk confocal microscope. A portion of a representative 300-cell embryo is shown.

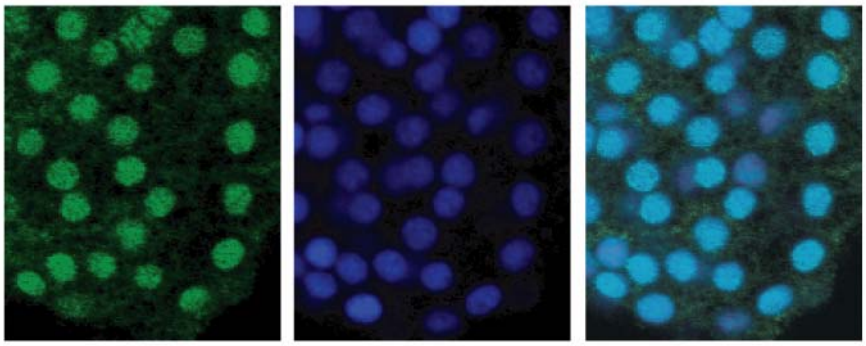
Fig. S3. *pes-10::gfp* RNA accumulates in nuclei during early embryogenesis. *gfp* RNA was detected in 6 cell embryo by whole-mount *in situ* hybridization (left panel) and DAPI (right panel). For unknown reasons, nuclei stained weakly by alkaline phosphatase exhibit strong DAPI staining (long arrows), while cells exhibiting strong alkaline phosphatase staining fail to stain strongly with DAPI (short arrows) (*2*).

Fig. S4. NRDE-3 associates with a subset of E01G4.5 endo siRNAs. Northern blot hybridization analysis of total RNA obtained from (1) wild type animals, (2) *eri-1(mg366)* animals, (3) FLAG::NRDE-3 co-precipitating siRNAs (4) and a synthetic 22 nt E01G4.5 siRNA (IDT). 5S RNA levels are shown as loading controls. Northern blot hybridization was conducted essentially as described in Fig. 2C. Interestingly, *C. elegans* express two size classes of E01G4.5 endo siRNAs of distinct lengths (lane 1) of which NRDE-3 interacts with only the shorter class (lane 3). **(B)** NRDE-3 associating E01G4.5 endo siRNAs carry 3' *cis-diols*. FLAG::NRDE-3 associated siRNAs were mock treated or subjected to periodate oxidation and Beta-elimination was performed as described (*9*). Synthetic small RNA carrying a 3' *cis-diol*, or a 2'-CH3 served as positive and negative controls, respectively. The longer class of E01G4.5 endo siRNAs, that fail to associate with NRDE-3, exhibit distinct 3' modifications; they are resistant to Beta-elimination (not shown).

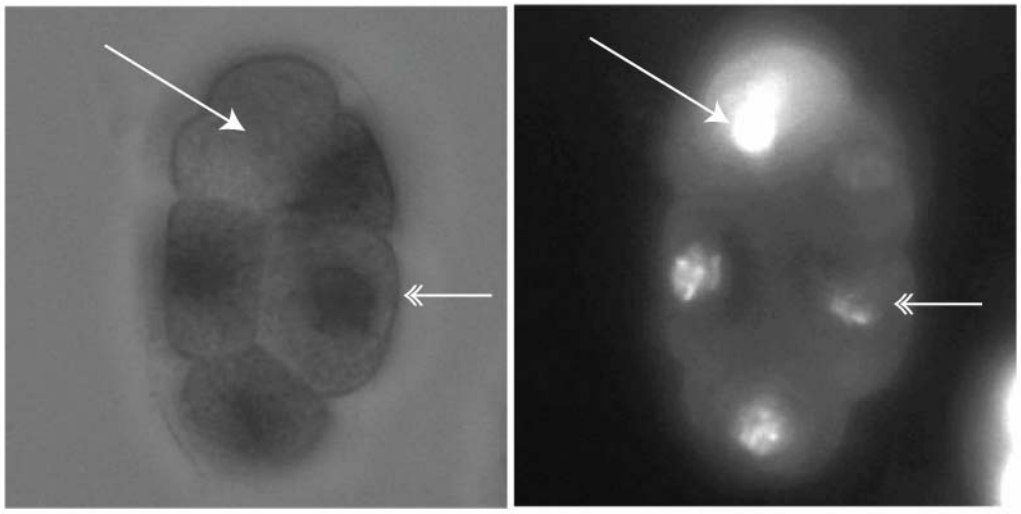
Fig. S5. Forced expression of NRDE-3 in the cytoplasm does not significantly alter organismal responses to RNAi. **(A)** RNAi triggers an association of NRDE-3 with spliced and unspliced RNAs. FLAG::NRDE-3 variants were immunoprecipitated from animals exposed to (+/-) *unc-40* dsRNA. qRT-PCR analysis of cDNA generated from associating RNAs (n=4, +/- s.d.). It is possible that the RNAi-driven NRDE-3 association with cytoplasmic mRNAs facilitates loading of NRDE-3 with RNA-dependent RNA Polymerase products. **(B)** Forced localization of NRDE-3 to the cytoplasm does not affect organismal responses to RNAi. *nrde-3(-)* and *nrde-3(-)*;FLAG::NRDE-3(*NLS) animals were fed bacteria expressing *unc-40* dsRNAs. Total RNA was isolated via TRIzol extraction and cDNA was generated as described in Materials and Methods. *unc-40* mRNA levels were analyzed by qRT-PCR (n=4, +/- s.d.). **(C)** *nrde-3(-)* and *nrde-3(-)*;GFP::NRDE-3(*NLS) animals were fed bacteria expressing dsRNAs derived from the indicated genes (dsRNA). Phenotypes were scored on a scale of zero to four as described in Table 1 (n=3, +/- s.d.).



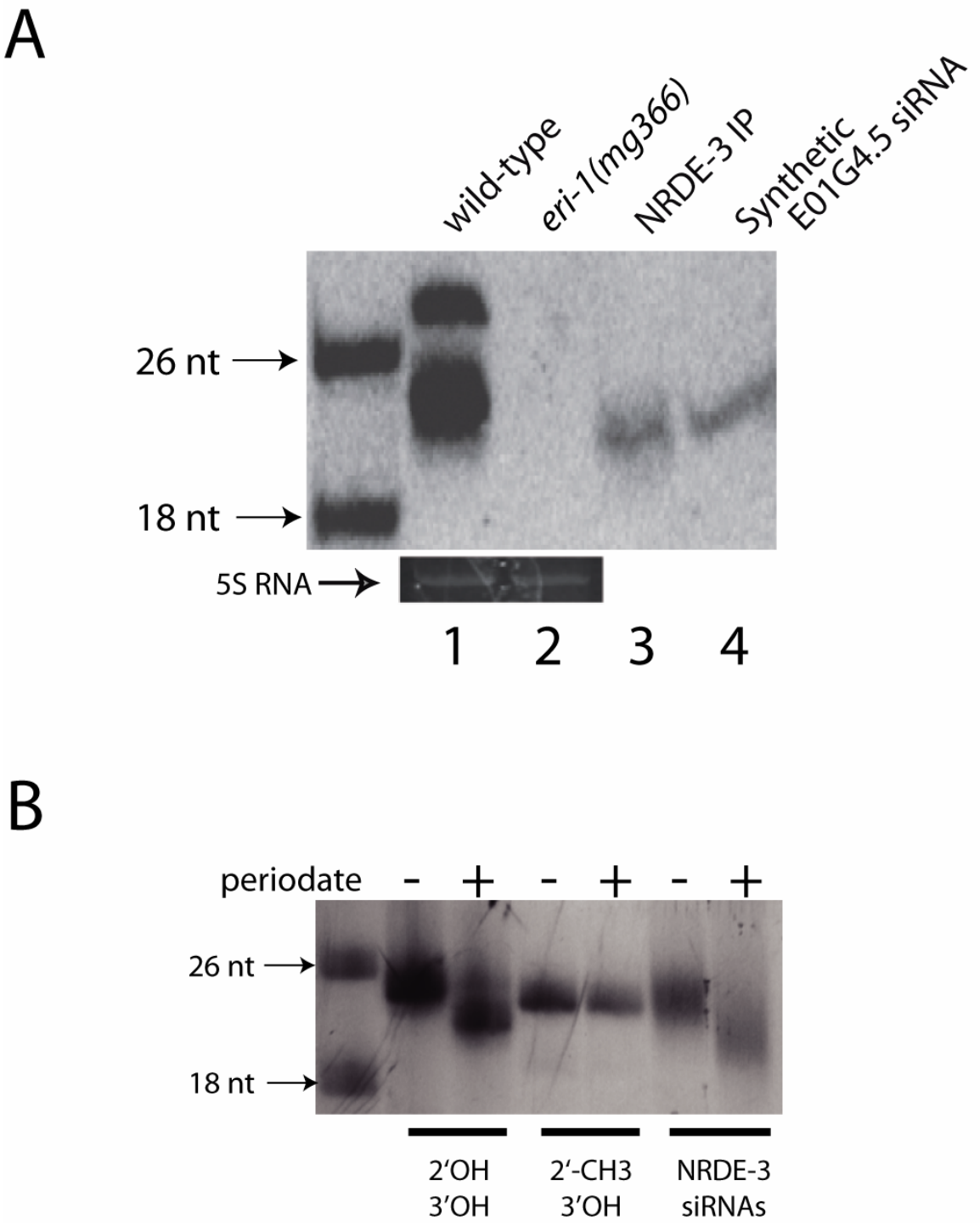
Supplemental Fig. S1



Supplemental Fig. S2

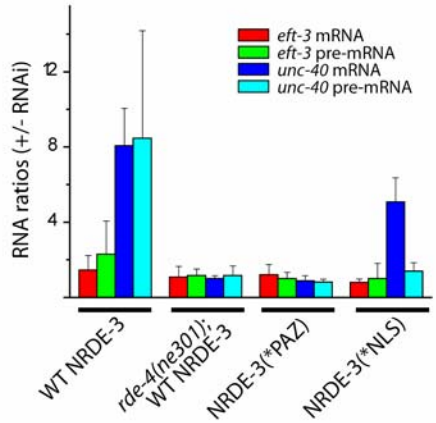


Supplemental Fig. S3



Supplemental Fig. S4

A



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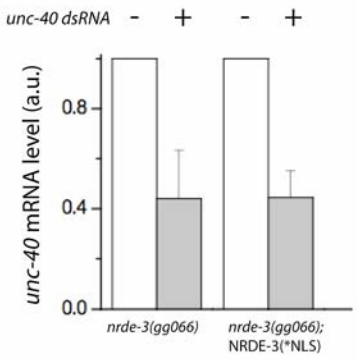

C

Genotype	dsRNA						
	unc-22 (twitcher)	*unc-15* (paralysis)	*pos-1* (lethality)	*unc-73* (paralysis)	*dpy-13* (dumpy)	*lir-1* (lethality$^{\alpha}$)	*lin-15b* (multi-vulva$^{\alpha}$)
nrde-3(gg066)	2.1 +/- 0.2	0.6 +/- 0.2	3.9 +/- 0.2	0	0.9 +/- 0.2	0	0
nrde-3(gg066); NRDE-3(*NLS)	2 +/- 0.3	1.1 +/- 0.5	4	0	1.1 +/- 0.2	0	0

$^{\alpha}$ indicates phenotype predicted to be elicited by silencing of nuclear-localized RNA

Supplemental Fig. S5