The Nuclear Argonaute NRDE-3 Contributes to Transitive RNAi in *Caenorhabditis elegans*

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ABSTRACT The *Caenorhabditis elegans* nuclear RNA interference defective (*Nrde*) mutants were identified by their inability to silence polycistronic transcripts in enhanced RNAi (*Eri*) mutant backgrounds. Here, we report additional *nrde-3*-dependent RNAi phenomena that extend the mechanisms, roles, and functions of nuclear RNAi. We show that *nrde-3* mutants are broadly RNAi deficient and that overexpressing *NRDE-3* enhances RNAi. Consistent with *NRDE-3* being a dose-dependent limiting resource for effective RNAi, we find that *NRDE-3* is required for *eri*-dependent enhanced RNAi phenotypes, although only for a subset of target genes. We then identify *pgl-1* as an additional limiting RNAi resource important for *eri*-dependent silencing of a nonoverlapping subset of target genes, so that an *nrde-3*; *pgl-1*; *eri-1* triple mutant fails to show enhanced RNAi for any tested gene. These results suggest that *nrde-3* and *pgl-1* define separate and independent limiting RNAi resource pathways. Limiting RNAi resources are proposed to primarily act via endogenous RNA silencing pathways. Consistent with this, we find that *nrde-3* mutants misexpress genes regulated by endogenous siRNAs and incompletely silence repetitive transgene arrays. Finally, we find that *nrde-3* contributes to transitive RNAi, whereby amplified silencing triggers act in *trans* to silence sequence-similar genes. Because *nrde*-dependent silencing is thought to act in *cis* to limit the production of primary transcripts, this result reveals an unexpected role for nuclear processes in RNAi silencing.

GENETIC analysis of RNA interference (RNAi) in *Caenorhabditis elegans* initially identified and characterized genes and activities important for post-transcriptional gene silencing (PTGS) in response to exogenous double-stranded RNA (dsRNA) (Tabara *et al.* 1999, 2002). Because these gene products target mature RNAs (mRNAs), they were presumed to act in the cytoplasm (Fire *et al.* 1998). However, a genetic screen for mutants specifically defective for pan-operon RNAi silencing (Bosher *et al.* 1999) identified genes important for transcriptional gene silencing (TGS) processes that operate in the nucleus (Guang *et al.* 2008, 2010; Burkhart *et al.* 2011; Burton *et al.* 2011). These genes were termed nuclear RNAi defective (*nrde*).

The best-characterized *nrde* is *NRDE-3*, an Argonaute that shuttles secondary short-interfering RNAs (siRNAs) from the cytoplasm to the nucleus (Guang *et al.* 2008). When

siRNA are absent or reduced, *NRDE-3* is primarily detected in the cytoplasm, and when siRNAs are abundant, *NRDE-3* is readily detected in the nucleus. In the nucleus, the siRNA-bound *NRDE-3* forms a complex with the nuclear restricted *NRDE-1*, *-2*, and *-4* (Burton *et al.* 2011). The associated siRNA guides the NRDE complex to cognate nascent mRNA transcripts, which impedes RNA polymerase II transcription elongation and further initiates histone methylation-dependent TGS (Guang *et al.* 2010; Burton *et al.* 2011). This RNA-directed epigenetic mechanism also enables multigenerational silencing (Gu *et al.* 2012); the recently identified germline nuclear Argonaute *HRDE-1* functions like the soma-restricted *NRDE-3* to enable this multigenerational silencing (Buckley *et al.* 2012). *hrde-1* is also important for germline immortality. As these enlightening studies on mechanisms and downstream endogenous roles of nuclear RNAi have progressed, broader identification of other nuclear RNAi-dependent upstream processes and their interactions with cytoplasmic RNAi therefore becomes ever more important.

Enhanced RNAi (*Eri*) mutant backgrounds have been important for the discovery and characterization of *nrde* functions. *Eri* mutants identify genes important for the production or stability of endogenous short-interfering RNAs (endo-siRNAs) (Simmer *et al.* 2002; Kennedy *et al.* 2004; Duchaine *et al.* 2006; Fischer

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et al. 2008; Pavelec *et al.* 2009). Genes complementary to endo-siRNAs are misregulated in *Eri* mutants (Gent *et al.* 2010). Interestingly, these endo-siRNAs also make up the bulk of siRNAs associated with *NRDE-3* *in vivo* (Guang *et al.* 2008). Thus, a role for *nrde-3* in the enhanced RNAi silencing associated with the *eri* mutants is expected, although the scope and significance of this role has not been investigated.

Here, we report our identification of additional *nrde-3*-dependent processes. We define a significant and broad contribution of *nrde-3* to exogenous RNAi silencing and determine that *NRDE-3* is a distinct limiting RNAi resource. As expected, *nrde-3* is important for enhanced RNAi phenotypes, but unexpectedly only for a subset of targeted genes. We then identify the perinuclear protein *PGL-1* as important for the enhanced RNAi phenotypes of a nonoverlapping subset of target genes. These results thus define separate and independent limiting RNAi resource pathways. We also provide evidence that *nrde-3* is important for endo-siRNA regulated gene expression and multicopy transgene silencing. Finally, we show that *nrde-3* contributes to transitive RNAi, revealing an unexpected role for nuclear processes in exogenous RNAi.

Materials and Methods

Strains

The following strains were used: Bristol N2 wild type, (FX1917) *eri-6/7(tm1917)*, (GR1373) *eri-1(mg366)*, (HC195) *nrIs20* (*sur-5::NLS-GFP*), (HC745) *eri-1(mg366); nrIs20*, (HC758) *eri-1(mg366); nrde-3(gg66)*, (HC759) *eri-1(mg366); nrde-3(gg64)*, (HC760) *rrf-3(pk1426); nrde-3(gg66)*, (HC761) *eri-6/7(tm1917); nrde-3(gg66)*, (HC762) *eri-8(gg100); nrde-3(gg66)*, (HC763) *eri-1(mg366); vpls9*, (HC764) *eri-1(mg366); nrde-3(gg66); vpls9*, (HC765) *nrde-3(gg66); nrIs20*, (HC766) *eri-1(mg366); nrde-3(gg66); nrIs20*, (HC767) *rrf-3(pk1426); nrde-3(gg66); nrIs20*, (HC774) *lin-15b(n765); nrde-3(gg66)*, (HC778) *lin-35(n745); nrde-3(gg66)*, (HC792) *eri-1(mg366); nrde-2(gg91)*, (HC794) *nrde-3(gg66); ggIs01(nrde-3p::FLAG3x-gfp-nrde-3)*, (HC824) *nrde-2(gg91) nrIs20*, (HC825) *eri-1(mg366); pgl-1(bn101)*, (HC826) *eri-6/7(tm1917); pgl-1(bn101)*, (HC831) *nrde-3(gg66); vpls9*, (HC832) *lin-15ab(n765); pgl-1(bn101)*, (HC833) *eri-1(mg366) nrde-2(gg91) nrIs20*, (HC838) *eri-1(mg366); nrde-3(gg66); (nrde-3p::FLAG3x-gfp-nrde-3; *NLS)*, (HC839) *nrde-3(gg66); (nrde-3p::FLAG3x-gfp-nrde-3; *NLS); vpls9*, (HC840) *rrf-3(mg373); nrde-3(gg66)*, (HC841) *eri-1(mg366); pgl-1(bn101); nrde-3(gg66)*, (HC842) *rrf-3(pk1426); pgl-1(bn101)*, (HC843) *eri-8(tm1860); pgl-1(bn101)*, (HC844) *lin-35(n745); pgl-1(bn101)*, (HC848) *nrde-3(gg66); (nrde-3p::FLAG3x-gfp-nrde-3; *NLS, SV40NLS); vpls9*, (HC860) *nrde-3(gg66); nels11*, (HC861) *eri-1(mg366); nrde-3(gg66); [nrde-3p::FLAG3x-gfp-nrde-3; *NLS, SV40NLS]*, (HC862) *eri-1(mg366); pgl-1(bn102)*, (HC888) *rrf-3(pk2042)*, (HC889) *rrf-3(pk2042); nrde-3(gg66)*, (HC891) *(nrde-3p::FLAG3x-gfp-nrde-3; *NLS)*, (HC895) *rrf-3(mg373); nrIs20; nrde-3(gg66)*, (HC896) *rrf-3(pk1426); (rrf-*

3p::rrf-3; myo-3p::dsRed), (JG33) *vpls9 (unc-119(+)) + elt-1::GFP*, (MT8189) *lin-15b(n765)*, (MT10430) *lin-35(n745)*, (NL2099) *rrf-3(pk1426)*, (SS2) *pgl-1(ct131) him-3(e1147)*, (SS579) *pgl-1(bn101)*, (SS580) *pgl-1(bn102)*, (WM107) *pgl-1(bn101); nels5[Y43B11::pgl-1::gfp + pRF4(rol-6(su1006))]*, (WM120) *Mago; nels11(myo-3p::GFP::sago-1 + pRF4(rol-6(su1006)))*, (WM158) *eri-8/ergo-1(tm1860)*, (YY13) *rrf-3(mg373)*, (YY158) *nrde-3(gg66)*, (YY168) *eri-8(gg100)*, (YY174) *ggIs01(nrde-3p::FLAG3x-gfp-nrde-3)*, (YY186) *nrde-2(gg91)*, (YY238) *nrde-3(gg64)*, (YY298) *nrde-3(gg66); (nrde-3p::FLAG3x-gfp-nrde-3; *NLS)*, and (YY362) *nrde-3(gg66); (nrde-3p::FLAG3x-gfp-nrde-3; *NLS,SV40NLS)*.

All strains tested wild type for the *mut-16(mg461)* background *Rde* allele. All strains and assays were maintained and performed at 20° as previously described (Brenner 1974), except where indicated. The YY298 and YY362 strains that affect the nuclear localization signal of *nrde-3* were previously characterized (Guang *et al.* 2008) and used in the same context as our experiments. The YY174 strain that rescues *nrde-3(gg66)* was previously characterized (Guang *et al.* 2008) and used in the same context as our experiments. All three transgenes were documented to be expressed at comparable levels as wild-type *nrde-3*. The WM120 strain that causes an *Eri* phenotype by *sago-1* overexpression was also previously characterized (Yigit *et al.* 2006) and used in the same context as our experiments.

RNAi

RNAi assays were performed as previously described (Timmons and Fire 1998). Bacteria engineered to express dsRNA against genes listed in Supporting Information, Table S1 were obtained from the Ahringer library (Kamath and Ahringer 2003) and verified by sequencing. Bacteria engineered to express dsRNA against *gfp* were prepared as previously described (Winston *et al.* 2002). All RNAi assays involving the dilution series were performed as previously described (Zhuang and Hunter 2011), unless explicitly stated otherwise. Briefly, individual L3 animals were placed on RNAi food at the indicated concentrations and their progeny were scored for previously published knockdown phenotypes. Feeding RNAi assays from the L1 were performed by hypochlorite treating gravid adults on RNAi plates and scoring the surviving embryos in the same generation. All feeding RNAi assays were performed in triplicate and repeated three times. The penetrance shown is representative of all the assays performed.

The (JG33) *elt-1-gfp* strain was previously described (Smith *et al.* 2005). In performing the transitive RNAi assay (see Figure 3), knockdown of *gfp* levels was consistent across all strains tested, ensuring that the intake of dsRNA trigger was unaffected. In previously published transitive RNAi assays, the *elt-1-gfp* transgene was deemed the most efficacious in causing transitive RNAi (Alder *et al.* 2003). The *vpls9 (unc-119(+)) + elt-1::GFP* insertion is <2 cM from *nrde-2*, and we were thus not able to make the *nrde-2(gg91); elt-1-gfp* double mutant.

Reverse transcription quantitative PCR

Hatch-synchronized (within 1 hr) mid-L4 worms from five normal growth (NG) large plates grown at 15° for 91 hr were pooled, washed extensively (M9), and allowed to swim for 20 min to clear gut content. RNA was isolated with Trizol (Invitrogen) followed by phenol:chloroform extraction (Amresco). The RNA pellets were subjected to DNase I (Roche) treatment, removed by RNeasy (Qiagen) per manufacturer's instruction. All RNA stock concentrations were adjusted to 150 ng/ μ l.

Reverse transcription was performed using 300 ng of input RNA by Thermoscript RT (Invitrogen), using gene-specific RT primers (available upon request). cDNA quantification was performed using 2 μ l of the 20 μ l RT reaction in a 50 μ l QuantiTect SYBR Green (Qiagen) reaction with nested PCR primers. The PCR reaction cycles were: 15 min 95°, 15 sec 94°, 30 sec 52°, 1 min 72°, read, cycle to step 2 for 45 cycles, all performed using an Eppendorf Mastercycler Realplex4 with Noiseband quantification. Subsequent analysis was performed using a $\Delta\Delta$ CT approach (Livak and Schmittgen 2001), using *gpd-3* RNA levels for normalization (Welker *et al.* 2010), as previously described (Zhuang and Hunter 2012).

Cross-progeny assays

Males were marked by the *sur-5::gfp* transgene while hermaphrodites were unmarked. Only green cross-progeny were scored in all assays.

Transgene silencing assay

Hatch-synchronized (within 1 hr) young adult worms grown at 20° for 53 hr were imaged, at the same magnification and exposure for all strains indicated in Figure 4. Outlined individual worms were analyzed using ImageJ (National Institutes of Health) by first “subtract background” and then “measure” functions for determining *gfp*-intensity units.

Penetrance calculations and statistical notes

All penetrance results were representative of at least three independent trials performed in triplicate. All assays with binary phenotypic penetrances (*i.e.*, *Dpy* or WT) are represented by individual trials and their standard deviations or a representative trial set. All assays with trinomial phenotypic penetrances (*i.e.*, embryonic lethal, developmental arrest, or WT) are represented by sums of all trials; breakdown of individual trials are available upon request. All error bars indicate standard deviation. *P*-values calculated by *t*-test; **P* < 0.05 and ***P* < 0.01.

Results

Nuclear RNAi mutants are broadly deficient for dsRNA-induced silencing

RNAi effectiveness in wild-type animals is dependent on dsRNA dose. Consequently, high concentrations of dsRNA can compensate for, or mask, weak RNAi-defective (*Rde*) phenotypes (Zhuang and Hunter 2011). To determine whether *nrde-3*

mutants have a weak *Rde* phenotype when dsRNA is limiting, we measured RNAi efficacy in *nrde-3* mutants in response to a dsRNA dilution series. We found that the silencing responses to *dpy-11*(RNAi), *par-1*(RNAi), *qua-1*(RNAi), and *unc-22*(RNAi) were significantly reduced in *nrde-3*(*gg66*) mutants (Figure 1A; Table S1, Table S2, and Table S3). Because reduced RNAi efficacy could be caused by background mutations (Zhang *et al.* 2011), we confirmed the above results with a second *nrde-3* allele, *gg64* (Figure S1; Table S3) and showed that an *nrde-3-gfp* transgene construct completely rescued the RNAi defects (Figure S1). Therefore, in addition to RNAi targeting operons, *nrde-3* is also required for efficient RNAi targeting conventionally transcribed and processed genes. However, RNAi remains effective in *nrde-3* mutant backgrounds when the trigger dsRNA is not limiting (Figure 1A), indicating that nuclear-dependent RNAi silencing is particularly important when dsRNA is limited.

NRDE-3 is an Argonaute that has been proposed to shuttle siRNA from the cytoplasm to the nucleus where they associate with nuclear-restricted factors, including NRDE-2, to effect transcriptional gene silencing (Guang *et al.* 2010; Burton *et al.* 2011). To determine whether these broad RNAi deficiencies reveal an undetected role for NRDE-3 in the cytoplasm, we similarly tested *nrde-2*(*gg91*) mutants. The results show that NRDE-2 is similarly required for an efficient response to exogenous RNAi (Figure 1A; Figure S2; Table S2). Because NRDE-2 is thought to be completely nuclear localized (Burton *et al.* 2011), these results support a nuclear-acting step for efficient exogenous RNAi. To provide additional support for this conclusion, we compared the rescue of *nrde-3*(*gg66*) mutant animals by *nrde-3-gfp* transgene constructs that either lack a nuclear localization signal (“*NLS-*nrde-3*”) or in which the NRDE-3 NLS has been replaced with a Simian virus 40 nuclear localization signal (“SV40NLS-*nrde-3*”) (Guang *et al.* 2008). We found that the *NLS-*nrde-3* construct failed to rescue the *nrde-3*(*gg66*) RNAi defect, while the SV40NLS-*nrde-3* construct provided at least a wild-type response to *dpy-11*(RNAi) (Figure 1A). These results show that nuclear localized activities broadly contribute to RNAi triggered by exogenous dsRNA.

nrde-3 contributes to inter- and intragenerational responses to exogenous RNAi

The *nrde* genes have recently been implicated in the transmission of silencing to the progeny (Burton *et al.* 2011; Gu *et al.* 2012). A remarkable feature of RNAi in *C. elegans* is that silencing initiated in the mother can be efficiently transmitted via the germline to the progeny, and once established, can persist for at least 80 generations (Vastenhouw *et al.* 2006). Analysis of this multigenerational silencing demonstrated that PTGS mechanisms are required to initiate silencing and implicated TGS mechanisms in its maintenance (Grishok *et al.* 2000). The most convenient methods for performing exogenous RNAi in *C. elegans* involve exposing both the mother and her progeny to the silencing trigger, *i.e.*, feeding dsRNA-expressing *E. coli* to a mother and scoring her progeny brood

Figure 1 *nrde-3* is required for robust exogenous RNAi. (A) The penetrance of *dpy-11(RNAi)* relative to dose of feeding RNAi bacteria for the indicated strains is shown. The progeny of L3 larvae placed on the RNAi food were scored as L4 larvae. (B) Schematic of *trans*-generational RNAi assay. Dpy or Unc L4 larvae grown on either *dpy-11* or *unc-22* dsRNA expressing bacteria were transferred to non-RNAi bacteria. The nonexposed progeny were scored for Dpy-11 or Unc-22 phenotypes as L4 larvae. (C–F) The fraction of N2 and *nrde-3(gg66)* broods that transmitted *dpy-11(RNAi)* (C) or *unc-22(RNAi)* (E) silencing to nonexposed progeny and the penetrance of the Dpy (D) and Unc (F) phenotypes within individual N2 and *nrde-3(gg66)* broods. (G–I) The penetrance of Dpy phenotypes within individual broods from the indicated crosses. (J) The penetrance of *dpy-11(RNAi)* relative to dose of feeding RNAi bacteria for the indicated strains is shown. Embryos from unexposed mothers hatched in the presence of RNAi food and were scored as L4 larvae. The phenotypes scored for all assays are listed in Table S1. The *dpy-11(RNAi)* bacteria OD_{600nm} = 2.0 and the *unc-22(RNAi)* bacteria OD_{600nm} = 1.0. The numbers of animals scored for A and J are listed in Table S2. *P*-values are calculated by *t*-test; **P* < 0.05 and ***P* < 0.01.

for phenotypes on that same plate. Therefore, the requirement for *nrde* function for efficient silencing (Figure 1A) may reflect a requirement for TGS-dependent maintenance mechanisms in the progeny rather than PTGS mechanisms in the mother. To investigate whether *nrde-3* function is required in the mother or the progeny for silencing, we exposed either mothers or their progeny to the dsRNA trigger and compared in both cases RNAi silencing efficiency in the progeny.

First, we asked whether *nrde-3* is important for transmission of silencing from dsRNA-exposed mothers to nonexposed progeny. Specifically, we grew wild-type and *nrde-3(gg66)* worms from hatching to the fourth larval stage (L4) on either *dpy-11* or *unc-22* dsRNA expressing bacteria. We then transferred the RNAi-affected *Dpy* or *Unc* L4 worms to control bacteria and scored their nonexposed progeny for *Dpy-11* or *Unc-22* phenotypes (Figure 1B). We found that the fraction of broods inheriting RNAi silencing was two- to fourfold higher in the wild-type background (Figure 1, C and E; Table S4). Furthermore, the penetrance of *Dpy* or *Unc* within each RNAi-inheriting brood was also higher in the wild-type background (Figure 1, D and F; Table S4). Thus, in the absence of continued exposure to the dsRNA trigger, NRDE-3 is important for the transmission of RNAi silencing.

We next asked whether introducing wild-type NRDE-3 to the progeny of *nrde-3(gg66)* mothers could restore the penetrance of the inherited silencing. Specifically, we crossed dsRNA-exposed *nrde-3(gg66)* hermaphrodites to wild-type males and scored the penetrance of inherited silencing in the heterozygous wild-type progeny. In wild-type by wild-type positive control crosses, almost all mothers transmitted the *Dpy-11* RNAi phenotype to their progeny (Figure 1G), while in *nrde-3(gg66)* by *nrde-3(gg66)* negative control crosses, almost none of the mothers transmitted the phenotype (Figure 1I). However, in only 1 of 10 crosses did *nrde-3(gg66)* mothers robustly (>80% penetrant) transmit the *Dpy-11* RNAi phenotype to their wild-type cross progeny, while most *nrde-3(gg66)* mothers only partially transmitted the *Dpy-11* RNAi phenotype (Figure 1H; Table S5). These results indicate that, although NRDE-3 activity in the mother is important for the production of a transmissible silencing signal or state, NRDE-3 function in the progeny is also important.

To directly determine whether nuclear processes, in the absence of maternal contributions, are important for silencing, we hatched L1 larvae in the presence of *dpy-11* or *qua-1* dsRNA expressing bacteria and scored the resulting adults for *Dpy-11* or *Qua-1* phenotypes. We found that *nrde-2(gg91)*, *nrde-3(gg66)*, and **NLS-nrde-3-rescued nrde-3(gg66)* mutant strains all showed reduced silencing compared to wild type and *SV40NLS-nrde-3-rescued nrde-3(gg66)* mutant strains (Figure 1J; Table S2 and Table S3). Together, these results suggest that the weak Rde effect observed in *nrde-3* mutants is due to both reduced transmission of a trans-generational silencing signal or state, likely silenced chromatin, and decreased *de novo* silencing within dsRNA-exposed animals.

nrde-3* overexpression causes enhanced RNAi independent of *sago-1

While analyzing the weak Rde phenotype of *nrde-3* mutants, we noticed that the transgenic rescue strains often exhibited a stronger RNAi response than the wild-type animals (Figure S1, E and G). Because *C. elegans* multicopy transgenes are often overexpressed (Praitis *et al.* 2001), we hypothesized that NRDE-3 overexpression may enhance RNAi. We therefore crossed the transgene array into a wild-type background (so that there are at least two wild-type copies of *nrde-3*) and compared the RNAi effectiveness of this strain to wild type. We found that this strain exhibited enhanced RNAi to *dpy-13* (RNAi) (Figure 2, A–C; Table S6), as well as to *unc-15* (RNAi) and *unc-73* (RNAi) (Figure 2C; Table S6). As a control, we then crossed the **NLS-nrde-3-gfp* transgene array into a wild-type background and found that this array did not robustly enhance RNAi compared to wild type (Figure 2C; Table S6), suggesting again that it is predominantly the nuclear functions of *nrde-3* that are contributing to exogenous RNAi.

A previous report had analogously overexpressed the cytoplasmic secondary Argonautes SAGO-1 and SAGO-2, necessary for endogenous secondary RNAi, and found that those particular strains enhanced RNAi (Yigit *et al.* 2006). The SAGOs and NRDEs function in distinct cellular compartments and thus likely function in independent pathways. However, it is possible that *nrde-3* activity is required for the SAGO overexpression-associated Eri phenotype. Therefore, we crossed *nrde-3(gg66)* into a strain containing the *myo-3p::gfp-sago-1* construct used previously to demonstrate overinduced enhanced RNAi in muscle cells (Figure 2, D–G). We observed that the *nrde-3(gg66); myo-3p::gfp-sago-1* strain remained Eri (Figure 2H; Table S7). Thus, SAGO-1-enhanced RNAi is independent of *nrde-3*. Furthermore, these results suggest that *sago-1* and *nrde-3* may define complementary silencing pathways.

***nrde-3* is partially required for Eri mutants' enhanced RNAi**

The availability of enzymes utilized by both endogenous and exogenous RNAi pathways, like SAGO-1 and SAGO-2 (Yigit *et al.* 2006), plays a large role in determining the efficacy of exogenous RNAi. For example, mutations that reduce the abundance of endogenous siRNAs competing for SAGOs are thought to enhance exogenous RNAi silencing (Figure S3) (Duchaine *et al.* 2006). If this model is true, then mutations to these limiting RNAi resources should attenuate the Eri phenotype. Several observations indicate that *nrde-3* is a candidate for such a limiting RNAi resource. First, NRDE-3 nuclear localization is sensitive to endo- and exo-siRNA levels (Guang *et al.* 2008). Second, like the SAGOs, *nrde-3* overexpression enhances RNAi (Figure 2C). Third, *nrde-3* was identified as a mutant that abolished the *eri-1*-dependent phenotype of panoperon silencing (Guang *et al.* 2008). Fourth, *nrde-3* is auxiliary to RNAi silencing: the Rde phenotype is only apparent when dsRNA trigger is limiting (Figure 1A).

Figure 2 *nrde-3* overexpression causes enhanced RNAi independent of *sago-1*. (A and B) Representative images of progeny from L3 hermaphrodites of the indicated strains grown on *dpy-13(RNAi)* bacteria. (C) Penetrance of *dpy-13(RNAi)*, *unc-15(RNAi)*, and *unc-73(RNAi)* phenotypes for the indicated strains. (D–G) Representative images of progeny from L3 hermaphrodites of the indicated strains grown on *myo-3(RNAi)* bacteria. (H) Penetrance of *myo-3(RNAi)*-induced paralysis for the indicated strains. The *dpy-13(RNAi)* bacteria OD_{600nm} = 1.5, *unc-15(RNAi)* bacteria OD_{600nm} = 1.0, *unc-73(RNAi)* bacteria OD_{600nm} = 4.0, and *myo-3(RNAi)* bacteria OD_{600nm} = 3.0. The penetrances scored for C are listed in Table S6 and for H in Table S7. *P*-values are calculated by *t*-test; **P* < 0.05 and ***P* < 0.01.

To determine whether *nrde-3* is important for enhanced RNAi, we compared the penetrance and expressivity of RNAi silencing between *eri* single mutants and *eri; nrde-3(gg66)* double mutants. For these tests, we used each of the four widely conserved *C. elegans eri* genes (*eri-1*, *rif-3*, *eri-6/7*, and *ergo-1/eri-8*). *nrde-3* is known to be important for RNAi targeting the *lir-1-lin-26* operon via *lir-1(RNAi)* (Guang *et al.* 2008), and indeed in all four tested *eri; nrde-3* double mutants, silencing of *lin-26* by *lir-1(RNAi)* was eliminated (Table 1; Table S8 and Table S9). However, the *nrde-3* dependence for enhanced RNAi is not limited to operons; all four *eri; nrde-3* double mutants also showed reduced RNAi efficacy targeting a variety of genes (Table 1; Table S1, Table S8, Table S9; Figure S4). A second allele of *nrde-3*, *gg64*, in an *eri-1(mg366)* double mutant behaved similarly (Table S10). Furthermore, in an *eri-1(mg366); nrde-3(gg66)* background, the cytoplasmic-only *NLS-*nrde-3* construct failed to rescue the enhanced RNAi, while the nuclear localized SV40NLS-*nrde-3* construct rescued the enhanced RNAi

(Table 1; Table S9). Consistent with these results, *nrde-2(gg91)* mutants also disrupted enhanced silencing of non-operon genes (Table S10). These results suggest that *nrde-3* is a key factor whose accessibility is a rate-limiting factor for exogenous RNAi and that nuclear RNAi plays an important role in enhanced RNAi silencing phenomena.

Interestingly, the enhanced RNAi phenotype of some genes was completely dependent on *nrde-3*, while that of others was almost completely independent of *nrde-3* (Table 1). This suggests that expression of some genes may be more sensitive than others to nuclear RNAi silencing. In our limited analysis, *dpy-13* and *ifc-2* showed the most pronounced *nrde-3*-dependent enhanced RNAi phenotype (Table 1; Figure S4). These two genes are members of conserved and expansive gene families that share significant nucleotide identity (Cox 1985; Woo *et al.* 2004). One possibility is that *nrde-3*-dependent nuclear RNAi processes may preferentially silence multiple related genes.

Table 1 *eri-1* mutant partially requires *nrde-3* for enhanced RNAi

RNAi target	Operon or homologous sequences	N2	<i>nrde-3</i> (gg66)	<i>eri-1</i> (mg366)	<i>eri-1</i> (mg366); <i>nrde-3</i> (gg66)	<i>eri-1</i> (mg366); <i>nrde-3</i> (gg66); [*NLS- <i>nrde-3</i>]	<i>eri-1</i> (mg366); <i>nrde-3</i> (gg66); [SV40-NLS- <i>nrde-3</i>]
<i>lir-1</i>	Operon	+++	—	+++++	—	—	+++++
<i>dpy-13</i>	91 homologs	+	—	+++++	+	+	+++++
<i>ifc-2</i>	3 homologs	+	—	+++++	+	++	+++++
<i>unc-15</i>	0 homologs	—	—	+++++	+++	+++	+++++
<i>hmr-1</i>	1 homolog	+	—	+++++	+++	+++	+++++
<i>dpy-28</i>	0 homologs	—	—	+++++	+++++	+++++	+++++
<i>unc-73</i>	0 homologs	—	—	+++++	+++++	+++++	+++++

The severity of RNAi phenotypes, from “—” to “+++++,” based on expressivity described in Table S1 (illustrated for select mutants in Figure S4) and penetrances tabulated in Table S9 of indicated strains for indicated RNAi gene targets are shown. All scores are normalized to the strongest Eri response for a particular RNAi assay as “+++++” and no response as “—.” The enhanced RNAi response against genes in operons or with many homologs (*lir-1*, *dpy-13*, or *ifc-2*) is most dependent on *nrde-3*. The number of homologous sequences was determined by the number of unique genes with at least a 26mer match of perfect identity in mRNA. The bacteria OD_{600nm} concentrations used were *lir-1*(RNAi) = 3.0, *dpy-13*(RNAi) = 1.5, *ifc-2*(RNAi) = 3.5, *unc-15*(RNAi) = 1.0, *hmr-1*(RNAi) = 1.0, *dpy-28*(RNAi) = 4.0, and *unc-73*(RNAi) = 4.0.

nrde-3 contributes to transitive RNAi

Intriguingly, a mechanism that could explain the *nrde-3*-dependent enhanced RNAi targeting sequence-related multigene families is transitive RNAi. Transitive RNAi is the observation that RNAi silencing specificity can spread in *cis* along a target mRNA to target sequences not in the original trigger dsRNA. Mechanistically, this occurs during RNA-directed RNA polymerase (RdRP) production of secondary siRNAs. Subsequently, and most importantly, these secondary siRNAs can then target other mRNA transcripts in *trans* (such as homologous transcripts), which is essential for the potency of RNAi in *C. elegans* (Pak *et al.* 2012). Transitive RNAi can be detected by sequencing small RNAs in response to exogenous RNAi (Pak and Fire 2007), but it is more readily detected in *C. elegans* using transgenes that artificially fuse separate genes, resulting in secondary siRNAs that independently target both sequences (Hannon 2002; Alder *et al.* 2003).

To determine whether *nrde-3* can contribute to transitive RNAi, we used a strain with an *elt-1-gfp* fusion transgene that when exposed to *gfp* dsRNA causes *elt-1*-related developmental defects (Figure 3A) (Alder *et al.* 2003; Smith *et al.* 2005). Specifically, we exposed wild-type, *nrde-3*(gg66), and *eri-1*(mg366) strains that express the *elt-1-gfp* transgene to *gfp* dsRNA and scored the progeny for *elt-1*-related developmental defects. In a wild-type background, feeding *gfp* dsRNA to worms expressing the *elt-1-gfp* fusion causes mild levels of developmental deformities and embryonic lethality (Figure 3, B and E), while in an *eri-1*(mg366) background, *gfp*(RNAi) causes near complete *elt-1*-related embryonic lethality (Figure 3, C and E). In contrast, *gfp*(RNAi) in the *nrde-3*(gg66) single mutant and the *eri-1*(mg366); *nrde-3*(gg66) double mutant caused almost no *elt-1*-related phenotypes (Figure 3, D and E). Furthermore, the *NLS-*nrde-3* transgene failed to rescue transitive RNAi in an *nrde-3*(gg66) mutant, while the SV40NLS-*nrde-3* transgene-rescued *nrde-3*(gg66) strain behaved like wild type (Figure 3E), indicating that only nuclear localized NRDE-3 can contribute to transitive RNAi. Importantly, we also observed that *elt-1*(RNAi) was fully penetrant in all strains,

thus *nrde-3* is not simply required for efficient silencing of the *elt-1* locus. Therefore, these results show that nuclear NRDE-3 contributes to transitive RNAi, which may in turn be the mechanism behind *nrde-3*'s contributions to exogenous RNAi silencing.

nrde-3 is required for endogenous gene regulation

Previous reports indicate that NRDE-3 nuclear localization is dependent on *eri* endo-siRNA levels and it co-immunoprecipitates with *eri* endo-siRNAs (Guang *et al.* 2008). Furthermore, we observed that it is necessary and sufficient for enhanced RNAi (Figure 2C, Table 1). These observations suggest that *nrde-3* may function endogenously to regulate endo-siRNA targeted genes. To test this, we used qPCR to measure the expression level of five target genes of endo-siRNAs that are up-regulated in *rrf-3*(pk1426) mutants (Gent *et al.* 2010). If NRDE-3 uses *rrf-3*-dependent endo-siRNAs to repress gene expression, then the expression levels of these five genes should increase in *nrde-3* mutants. Specifically, we compared transcript abundances in wild type, *eri-1*(mg366), *rrf-3*(mg373), *rrf-3*(pk1426), and *nrde-3*(gg66) L4 larvae. We found that expressions of all five *rrf-3* siRNA target genes were increased in both *rrf-3* mutant alleles and the *nrde-3*(gg66) mutant (Figure 4A). To our knowledge, this is the first report of an endogenous function for *nrde-3*, as previous reports on *nrde* roles in germline maintenance implicated only *nrde-1*, *nrde-2*, and *nrde-4*. Consistent with previous reports, we observed that *eri-1* and *rrf-3* mutants had overlapping but incompletely shared effects on endogenous gene expression (Figure 4A) (Lee *et al.* 2006; Asikainen *et al.* 2007).

nrde-3 is required for transgene silencing

Enhanced RNAi mutant backgrounds often enhance the frequency and penetrance of spontaneous silencing of multi-copy repetitive transgenes (Kim *et al.* 2005). To determine whether *nrde-2* and *nrde-3* are required for *eri*-enhanced silencing of repetitive transgene arrays, we compared the extent of silencing of the ubiquitously expressed *sur-5p::gfp*

Figure 3 *nrde-3* contributes to transitive RNAi. (A) Schematic for transitive RNAi showing the 5' spread of secondary siRNAs triggered by the introduced *gfp* dsRNA. The secondary siRNAs, which now include siRNAs complementary to *elt-1*, can act in *trans* to target the endogenous *elt-1* locus. (B–D) Representative phenotypes of (B) wild type (N2), (C) *eri-1(mg366)*, and (D) *nrde-3(gg66)* and *eri-1(mg366); nrde-3(gg66)* strains expressing an *elt-1-gfp* fusion transgene grown on *gfp* RNAi bacteria. Wild-type animals had modest amounts of *elt-1*-related developmental defects while *eri-1(mg366)* animals exhibited *elt-1*-related embryonic lethality. In contrast, *nrde-3(gg66)* and *eri-1(mg366); nrde-3(gg66)* animals had very few, if any, *elt-1*-related developmental defects. (E) The penetrance of *elt-1*-like phenotypes for the indicated strains expressing an *elt-1-gfp* fusion transgene grown on *gfp*(RNAi) bacteria.

transgene in *eri-1(mg366)* single mutant to the silencing in the *eri-1(mg366); nrde-2(gg91)* and *eri-1(mg366); nrde-3(gg66)* double mutants. We found that both *nrde* genes are required for *eri-1*-induced silencing (Figure 4, E–H). To determine whether this *nrde*-dependent transgene silencing activity represents an endogenous *nrde* function, or reflects only the unusual circumstances present in *eri*-mutant backgrounds, we examined *nrde*-dependent transgene expression in non-*Eri* backgrounds. Specifically, we compared the extent of silencing of *sur-5p::gfp* in wild-type vs. *nrde-2(gg91)* and *nrde-3(gg66)* backgrounds. We found that the loss of *nrde-2* and *nrde-3* also increased transgene expression in non-*Eri* backgrounds (Figure 4, B–D and H), indicating that silencing repetitive transgenes is likely another endogenous function of *nrde-3*.

pgl-1 acts in parallel to *nrde-3*

Mutants that disrupt core RNAi functions, such as *rde-1* and *dcr-1*, have near 100% *Rde* penetrance (Tabara *et al.* 1999; Tabara *et al.* 2002), while the *nrde-3* *Rde* phenotype is only apparent at limiting dsRNA concentrations (Figure 1A). Furthermore, *nrde-3* is only essential for the enhanced RNAi phenotypes of a subset of target genes (Table 1). These observations indicate that *nrde-3* likely acts after the core RNAi components and that one or more additional postcore activities may act in parallel with *nrde-3* to mediate the full spectrum of enhanced RNAi phenotypes. The SAGOs are

candidates for this parallel activity, as their overexpression enhances exo-RNAi independent of *nrde-3*. However, their functional redundancy (Yigit *et al.* 2006) challenges analysis of *eri(-); sago(-)* double mutants. Therefore, to discover what other secondary RNAi pathways may act in parallel to *nrde-3*, we screened available mutants with functions associated with the nucleus for RNAi phenotypes analogous to *nrde-3(-)* mutants: weak *Rde* to exogenous RNAi and enhanced RNAi when overexpressed as a transgene (Table S11).

Among these mutants, the perinuclear P-granule component *pgl-1* emerged as a promising candidate. Like *nrde-3*, three different *pgl-1* mutant alleles were weakly *Rde*, while worms carrying a *pgl-1-gfp* transgene showed enhanced RNAi (Figure 5, A–C; Table S12). To analyze the effects of *pgl-1* loss of function on the *eri-1* phenotype, we constructed and tested an *eri-1(mg366); pgl-1(bn101)* double mutant and an *eri-1(mg366); pgl-1(bn101); nrde-3(gg66)* triple mutant. Surprisingly, the *eri-1; pgl-1* double mutants maintained enhanced RNAi phenotypes for the target genes that require *nrde-3*, but failed to show enhanced RNAi phenotypes for the *nrde-3*-independent target genes (Figure 5, D and E). The complementary nature of this pattern was confirmed by analyzing the *eri-1; pgl-1; nrde-3* triple mutant, which was non-*Eri* for all tested target genes (Figure 5, D and E). A second allele, *pgl-1(bn102)*, behaved the same way (Table S13). The effect *pgl-1* loss had on enhanced RNAi was also observed for the other conserved *Eri* mutants: *rff-3*, *eri-6/7*, and *ergo-1/eri-8*

Figure 4 *nrde-3*-dependent silencing of endogenous RNAi targets and transgenes. (A) *eri*- and *nrde-3*-dependent changes in relative mRNA levels for select endo-siRNA target genes. Log2 ratio (relative to N2 = 0) of qPCR determined mRNA levels for the indicated genes from L4 larvae of the indicated genotypes. Expression of these five genes, which are targets of *rrf-3*-dependent endo-siRNAs, was previously reported to be up-regulated in *rrf-3* mutants (Gent et al. 2010). Transcript abundances are normalized to *gpd-3*. *P*-values are calculated by *t*-test; **P* < 0.05; ***P* < 0.01. (B–G) Representative photomicrographs of *sur-5p::gfp* expression in (B) wild type, (C) *nrde-2(gg91)*, (D) *nrde-3(gg66)*, (E) *eri-1(mg366)*, (F) *eri-1(mg366); nrde-2(gg91)*, and (G) *eri-1(mg366); nrde-3(gg66)* strains. The extensive transgene silencing that is readily apparent in intestinal cells (yellow arrows) in the *eri-1(mg366)* background is dependent on *nrde-2* and *nrde-3*. Compared to *nrde-2(gg91)* and *nrde-3(gg66)*, wild-type animals show reduced *gfp* levels. (H) Average *gfp* fluorescence of hatch-synchronized 24-hr adult (20 °C) worms. For each genotype the average whole body fluorescence (0.1-sec exposure) of 10 worms is shown. Error bars represent standard deviation. *P*-values are calculated by *t*-test; red asterisks are *P* < 0.01 compared to wild type (N2) and blue asterisks are *P* < 0.01 compared to *eri-1(mg366)*.

(Table S13). Finally, because *PGL-1* is overexpressed in the *rb* mutants that enhance RNAi (Wang et al. 2005), we tested both *lin-15ab* and *lin-35* for *pgl-1*-dependent *Eri* phenotypes. We found that *pgl-1(bn101); lin-15ab(n765)* and *pgl-1(bn101); lin-35(n745)* double mutants were non-*Eri* for the same subset of target genes as the *pgl-1(bn101); eri-1(mg366)* double mutants (Table S13). These results thus suggest *pgl-1* and *nrde-3* act as parallel activities that seemingly account for all *eri* mutant enhanced RNAi effects.

pgl-1 acts to regulate endo-RNAi target gene expression

NRDE-3 interacts with endo-siRNAs and is required to regulate *rrf-3* endo-siRNA target genes (Figure 4A) (Guang et al.

2008). To determine whether *pgl-1* is also required for endo-siRNA target gene regulation, we used qPCR to measure the RNA levels for seven endo-siRNA regulated genes in embryos, L4 larvae, and mixed stage worms, comparing wild type, *rrf-3*, and *pgl-1* mutants. In all three conditions, a subset of these genes showed significant changes in the *pgl-1* mutant compared to wild type (Figure 6, A–C), although we failed to discern a definitive pattern among these changes. Furthermore, *pgl-1* was not required for a variety of RNAi-dependent phenomena that could provide insight into how *PGL-1* contributes to enhanced RNAi (File S1; Figure S5). However, despite our lack of mechanistic insight, our results suggest that *pgl-1* is important for endo-siRNA regulated

Figure 5 Complementary contributions of *pgl-1* and *nrde-3* to *eri*-dependent enhanced RNAi phenotypes. (A) RNAi phenotypic penetrance for the indicated genes in N2 and three *pgl-1* alleles. The progeny of L3 larvae placed on the RNAi food were scored as L4 larvae. The RNAi bacteria concentrations, phenotypes, and penetrances are listed in Table S12. *P*-values are calculated by *t*-test; ***P* < 0.01. (B and C) Representative images of progeny from L3 hermaphrodites of the indicated strains grown on *dpy-13(RNAi)* bacteria. (D) The severity of RNAi phenotypes, as described in Table 1, is based on expressivity measurements presented in Table S1. Representative examples are presented in E. Complete penetrances are tabulated in Table S13. The enhanced RNAi response targeting genes in operons or with many homologs (highlighted in yellow) is most dependent on *nrde-3*. The enhanced RNAi response targeting genes with few or no homologs (highlighted in green) is most dependent on *pgl-1*. The *eri-1; nrde-3; pgl-1* triple mutant (highlighted in salmon) is non-Eri. (E) Representative images of the expressivity of *dpy-13(RNAi)*, *unc-15(RNAi)*, and *unc-73(RNAi)* for the indicated strains. The progeny of L3 larvae placed on the RNAi food were scored as L4 larvae. The stars correspond to RNAi responses as scored in D. The *eri-1(mg366); nrde-3(gg66)* double mutant is most like wild type in RNAi targeting genes with many homologs, like *dpy-13(RNAi)* (bracketed in yellow). The *eri-1(mg366); pgl-1(bn101)* double mutant is most like wild type in RNAi targeting unique genes, like *unc-73(RNAi)* (bracketed in green). The RNAi bacteria concentrations used in D and E were the same as Table 1.

Figure 6 *pgl-1* mutant misexpression of *rrf-3* siRNA targets. *rrf-3(pk1426)* and *pgl-1(bn101)* dependent changes in relative mRNA levels for select endo-siRNA target genes. Log2 ratio (relative to N2 = 0) of qPCR determined mRNA levels for the indicated genes from (A) mixed-stage worms, (B) bleached embryos, and (C) L4 worms of the indicated genotypes. These seven genes were previously reported to be targets of *rrf-3*-dependent endo-siRNAs (Gent et al. 2010). Transcript abundances are normalized to *gpd-3*. P-values are calculated by t-test; **P* < 0.05 and ***P* < 0.01.

gene expression via a process that may compete with exogenous RNAi.

***rrf-3(pk1426)* is likely associated with a nonallelic background mutation**

During this analysis, we noticed that the well-characterized deletion allele of *rrf-3*, *pk1426*, behaved differently in several assays from the missense *rrf-3(mg373)* allele, which is roughly indistinguishable from *eri-1(mg366)*. First, the *rrf-3(pk1426); nrde-3(gg66)* double mutant was, like the *eri-1; nrde-3; pgl-1* triple mutant, non-Eri for all tested genes. This is at odds with all the other *eri; nrde-3(gg66)* double mutants, including the *rrf-3(mg373); nrde-3(gg66)* and *rrf-3(pk2042); nrde-3(gg66)* strains (Table 2; Table S14). Second, genes that were previously shown to be down-regulated in an *rrf-3(pk1426)* background (Lee et al. 2006; Asikainen et al. 2007) showed a significantly different expression profile in an *rrf-3(mg373)* background (Figure 7A). Previous gene expression profile analyses also show little overlap between *rrf-3(pk1426)* and *eri-1(mg366)* (Lee et al. 2006; Asikainen et al. 2007). This indicates either a different mechanism or set of targets for endogenous gene regulation. Third, qPCR designed to measure *dpy-13* mature and pre-mRNA transcript levels showed that *dpy-13(RNAi)* in *rrf-3*

(*pk1426*) mutants more effectively reduced both mature and pre-mRNA levels than RNAi in *eri-1(mg366)* and *rrf-3(mg373)* mutants (Figure 7B). Given that two of the three *rrf-3* alleles are phenotypically similar to the other *eri* mutants, we hypothesize that these apparently allele-specific phenotypes are due to a *pk1426* allele-associated background effect (File S1).

To test this hypothesis, we determined whether a wild-type *rrf-3* genomic fragment could rescue each distinct *rrf-3(pk1426)* phenotype. We found that *rrf-3(pk1426); [rrf-3(wild type)]* transgenic animals were rescued for the temperature-sensitive sterility associated with all *rrf-3* alleles (Table S14) (Gent et al. 2009; Pavelec et al. 2009). In addition, these animals were rescued for the Eri phenotypes against the *nrde-3*-dependent target genes (e.g., *lir-1(RNAi)*, *dpy-13(RNAi)*, *ifc-2(RNAi)*), but not against the *pgl-1*-dependent target genes (e.g., *dpy-28(RNAi)*, *unc-73(RNAi)*) (Table 2). Thus, the wild-type *rrf-3* transgene specifically failed to rescue a phenotype that is unique to the *pk1426* allele, implicating a non-*rrf-3* lesion in the background of the *pk1426* strain. We also constructed and scored an *rrf-3(mg373/pk1426); nrde-3(gg66)* strain (Table 2). Consistent with a semidominant effect, the *rrf-3* trans-heterozygote strain was, at a very reduced level, Eri for some *pgl-1*-dependent targets. In

Table 2 *rnf-3(pk1426)* has unique dependence on *nrde-3* for enhanced RNAi

RNAi target	N2	<i>nrde-3</i> (gg66)	<i>rnf-3</i> (mg373)	<i>rnf-3</i> (mg373); <i>nrde-3</i> (gg66)	<i>rnf-3</i> (pk2042)	<i>rnf-3</i> (pk2042); <i>nrde-3</i> (gg66)	<i>rnf-3</i> (pk1426)	<i>rnf-3</i> (pk1426); <i>nrde-3</i> (gg66)	<i>rnf-3</i> (pk1426) + [WT <i>rnf-3</i>]	<i>rnf-3</i> (mg373/pk1426) het	<i>rnf-3</i> (mg373/pk1426); <i>nrde-3</i> (gg66)het
<i>lir-1</i>	+	+	+	+	+	+	+	+	+	+	+
<i>dpy-13</i>	+	+	+	+	+	+	+	+	+	+	+
<i>ifc-2</i>	+	+	+	+	+	+	+	+	+	+	+
<i>hmr-1</i>	+	+	+	+	+	+	+	+	+	+	+
<i>unc-15</i>	+	+	+	+	+	+	+	+	+	+	+
<i>dpy-28</i>	+	+	+	+	+	+	+	+	+	+	+
<i>unc-73</i>	+	+	+	+	+	+	+	+	+	+	+

The severity of RNAi phenotypes as described in Table 1 is based on penetrance measurements presented in Table S14. The RNAi bacteria concentrations used were the same as in Table 1. The enhanced RNAi response targeting genes most dependent on *nrde-3* (*lir-1*, *dpy-13*, or *ifc-2*) holds the same pattern of RNAi responses as the other *Eri* mutants, while the enhanced RNAi response targeting genes most dependent on *pgl-1* (*dpy-28* or *unc-73*) holds the same pattern of RNAi responses as the other *Eri* mutants for the *mg373* and *pk2042* alleles, but not the *pk1426* allele.

summary, our results indicate that an *rnf-3(pk1426)*-associated background effect, which may act via a *pgl-1*-dependent pathway, accounts for the unusual properties associated with the *rnf-3(pk1426)* strain.

Discussion

We show that the nuclear RNAi Argonaute *nrde-3* contributes broadly and potently to RNAi triggered by exogenous dsRNA. Specifically, we found that nuclear RNAi contributes to silencing of genes that are members of multigene families and multicopy transgenes, as well as the phenomena of transitive RNAi. Particularly intriguing was the finding that overexpression of *NRDE-3* enhances RNAi, suggesting that, like the cytoplasmic secondary Argonautes, nuclear Argonautes may also be a limiting silencing resource. Furthermore, our analyses of *pgl-1* show that it is important for the enhanced RNAi phenotypes of a nonoverlapping subset of target genes. These results thus define two separate and independent limiting RNAi resource pathways, which establish *pgl-1* and *nrde-3* as two parallel activities that together are required for all detectable enhanced RNAi associated with *eri-1* and the other conserved *eri* mutants. Finally, our analysis of *trans*-generational silencing identified roles for nuclear RNAi in both the exposed parent and the unexposed progeny, which may indicate that a nuclear limited step mediates the transition from short-term RNAi processes that include PTGS of mature transcripts and longer-term RNAi processes, such as transgene silencing and *trans*-generational RNAi.

nrde-3 functions in exogenous RNAi are predominantly nuclear

NRDE-3 is a siRNA-binding protein that shuttles siRNAs between the cytoplasm and the nucleus, thus there is a possibility that *NRDE-3* could function in either compartment. However, our analyses of nuclear-restricted *nrde-2* (Guang *et al.* 2010; Burton *et al.* 2011) as well as the **NLS-nrde-3* and *SV40NLS-nrde-3* constructs provide strong evidence for the primary importance of nuclear-limited RNAi steps. Nevertheless, this does not preclude prior or subsequent non-nuclear activities in these silencing processes. For example, transgene silencing, which is completely dependent on *NRDE-3* in an *Eri* background, is partially dependent on *SID-1*, a dsRNA transporter that enables cell-to-cell spreading of RNAi silencing signals (Jose *et al.* 2009). Therefore, *NRDE-3* may either directly or indirectly use mobile silencing signals and/or *NRDE-3*-dependent processes may produce a silencing signal that can move between cells. Once the structure and origin of mobile silencing signals is known, it will be interesting to determine whether *nrde-3* is required for their biogenesis.

Interestingly, some of our analysis indicates that the **NLS-nrde-3-gfp* transgene sometimes do partially rescue the *nrde-3(gg66)* defects, especially in the contexts of weak exogenous *Rde* (Figure 1, A and J) and overexpressor-induced *Eri* (Figure 2C) phenotypes. This may be due to an incomplete removal of nuclear localization, although the lack of

Figure 7 *rrf-3(pk1426)* has distinct endogenous gene regulation and exogenous RNAi response. (A) *eri*- and *nrde-3*-dependent changes in relative mRNA levels for select *rrf-3*-regulated genes. Log2 ratio (relative to N2 = 0) of qPCR determined mRNA levels for the indicated genes from L4 larvae of the indicated genotypes. Expression of these five genes was previously reported to be down-regulated in *rrf-3(pk1426)* mutants (Asikainen et al. 2007). These genes are expressed with very different profiles among the four mutant strains tested. (B) Log2 ratio (relative to N2 = 0) of qPCR determined for *dpy-13* mRNA and pre-mRNA transcript levels in the indicated mutant strains undergoing *dpy-13(RNAi)* relative to empty vector L440 feeding. Transcript abundances are normalized to *gpd-3*. P-values are calculated by *t*-test; **P* < 0.05; ***P* < 0.01.

gfp nuclear localization suggests otherwise. This may also be due to the perhaps overexpressed transgene soaking up endogenous siRNAs—even if it is only present in the cytoplasm—so that the exogenous RNAi triggers have more access to competed-for cytoplasmic RNAi resources, hence creating an ever-so slight advantage in RNAi efficacy.

nrde-2 was initially characterized as a *nrde-3* effector (Guang et al. 2010), thus it is interesting that many *nrde*-dependent functions, such as RNAi transmission and interaction with *hrde-1*, involve *nrde-2* but not *nrde-3* (Burkhart et al. 2011; Buckley et al. 2012). Furthermore, *nrde-2* mutants have defects that *nrde-3* mutants do not, including germline mortality, suggesting that *nrde-2* responds to multiple inputs (Guang et al. 2010). Additionally, at least four other loci were identified in the *Nrde* screen, suggesting that *nrde-3* may not function exclusively via *nrde-2*. Consequently, these observations may provide an explanation for why the *nrde-2* RNAi defects are less penetrant than *nrde-3* RNAi defects (Figure 1, A and J). We therefore limit our interpretation of *nrde-2* results as an approximate confirmation of *nrde-3*'s RNAi functions within the nucleus.

***nrde-3* and *pgl-1* are required for enhanced RNAi in *eri* mutants**

Eri mutants are depleted for endogenous siRNAs, which are proposed to compete with exogenous siRNAs for silencing resources. It is this absence of competition for limiting RNAi resources that presumably accounts for enhanced exo-RNAi efficacy (Figure S3). *sago-1* has previously been proposed to be a downstream effector of the *eri* pathway, not because its absence attenuates the *Eri* phenotype, but because its overexpression relieves the competition for this limiting resource (Yigit et al. 2006). Here we show that *nrde-3* is *Rde* (Figure 1A), thus establishing a role for *NRDE-3* in the exo-RNAi pathway. We also show that *nrde-3* mutations disrupt the expression of endogenous siRNA-regulated genes (Figure

4A), confirming a role for *NRDE-3* in the endogenous RNAi pathway(s). We then show that loss of *nrde-3* attenuates the *Eri* phenotype, at least for some genes (Table 1). Finally, we show that, like *sago-1*, overexpression enhances RNAi (Figure 2, A–C). This preponderance of evidence supports a role for *nrde-3* as a downstream effector of the *eri* pathway.

A similar analysis shows that *pgl-1* is also a likely *eri* effector. Similar to *nrde-3*, *pgl-1* mutants are weakly *Rde* (Figure 5A), *pgl-1* overexpression enhances RNAi (Figure 5, B and C), *pgl-1* mutants disrupt the expression of endo-siRNA regulated genes (Figure 6), and loss of *pgl-1* attenuates the *Eri* phenotypes, at least for some genes (Figure 5, D and E). Remarkably, the simultaneous loss of both *pgl-1* and *nrde-3* broadly depletes enhanced RNAi in the *eri-1* mutant background (Figure 5, D and E). This result suggests that these two genes define the totality of *eri-1* and likely endo-siRNA competitive effector pathways.

PGL-1 expression and/or localization is expanded in the *Rb* pathway mutants, including *lin-15* and *lin-35*, that enhance RNAi and lead to efficient transgene silencing (Wang et al. 2005; Wu et al. 2012). However, *pgl-1* mutants abrogated *lin-15* and *lin-35* enhancement for the same genes *pgl-1* is important for in *eri-1* enhancement (Table S13). Furthermore, *lin-15; pgl-1* and *lin-35; pgl-1* double mutants continued to show RNAi enhancement for *nrde-3*-dependent gene targets. Thus, the importance of *pgl-1* for enhanced RNAi is not likely via the *Rb* pathway.

Attempts to correlate *pgl-1* phenotypes to previously defined RNAi mechanisms were not informative, thus failing to reveal how *pgl-1* activity contributes to gene silencing (Figure 6, Figure S5). However, our analysis clearly establishes that *pgl-1* contributes to both exogenous and endogenous RNAi (Figures 5 and 6). Like the *eri* mutants, sequencing and analysis of its small RNA profile will likely shed light on its endogenous roles and contributions to RNAi silencing.

Transitive RNAi and *nrde-3*'s contributions to RNAi

The result that transitive RNAi was not detectable in *nrde-3* mutants (Figure 3) may provide insight into the role of nuclear RNAi. The contribution of NRDE-3 to RNAi was most apparent when dsRNA is limiting (Figure 1A), thus it is reasonable to infer that *trans*-acting siRNAs are also limiting. This can be readily explained by the favorable stoichiometric ratio of siRNAs to expressed loci in the nucleus vs. exported transcripts in the cytoplasm. While a relative few siRNAs may be sufficient to silence a gene in the nucleus, they would be insufficient to silence hundreds or thousands of cytoplasmic transcripts. This implies that, while at high dsRNA dose, PTGS mechanisms are sufficient to enable *nrde-3*-independent silencing, at low dsRNA dose, the primary gene silencing response may be nuclear-based transcriptional gene silencing.

This model of complementary cytoplasmic and nuclear RNAi silencing processes also provides an attractive explanation for the enhanced silencing of both multigene families and multicopy transgenes. In both cases, partial or incomplete gene silencing will not produce a phenotype, providing an explanation for why these target genes show exceptionally strong enhanced RNAi potential. For example, because *dpy-13* (RNAi) causes a much stronger *Dpy* phenotype than does a *dpy-13* null allele, it is likely that the RNAi targets other functionally redundant collagen genes. In fact, the ingested *dpy-13* dsRNA trigger corresponds directly to a conserved collagen domain with near perfect identity with at least 30 other collagen members (Cox 1985). Thus, a limited number of siRNAs targeting this conserved region could—if efficiently imported into the nucleus—effectively silence many genes in the nucleus, but would be ineffective targeting the very abundant cytoplasmic collagen transcripts. This model does not provide an explanation for *nrde-3*-dependent enhanced operon silencing; however, only two operons of >1300 *C. elegans* operons have been shown to be sensitive to *eri*-enhanced silencing.

Endo-siRNA targeting and transgenes silencing are endogenous NRDE-3 functions

The depletion of endo-siRNAs that apparently underlies many *nrde-3*-dependent *Eri* phenotypes suggests that *nrde-3* might have a role in endo-siRNA target gene regulation. Indeed, similar to the *eri* mutants, we found that loss of *nrde-3* affects the gene expression of *eri* endo-siRNA targets (Figure 4A), supporting the hypothesis that endo-siRNAs mediate TGS in somatic cells. This is consistent with recent reports of *nrde-1*, *-2*, and *-4* functions in the germline, including transmission and maintenance of gene silencing (Buckley *et al.* 2012).

Furthermore, it may be noteworthy that *eri-6/7* endo-siRNA targets show enrichment for duplicated genes (Fischer *et al.* 2011), which suggests that RNAi targeting specific gene structures may indeed be a possible mechanism of action. An artificial analog to endogenous repetitive arrays in the *C. elegans* genome is transgenes, because simple transgenic arrays are often incorporated as repetitive elements (Praitis

et al. 2001). Our discovery of *nrde-3*'s contribution to transgene silencing in both *Eri* and non-*Eri* backgrounds further supports the broad endogenous roles *nrde-3* may play. These findings thus begin to form the funnel of broad upstream roles in endogenous and exogenous nuclear RNAi that ultimately channels into downstream effectors like the germline nuclear Ago *hrde-1* that impact fundamental biological processes such as germline maintenance.

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GENETICS

Supporting Information

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The Nuclear Argonaute NRDE-3 Contributes to Transitive RNAi in *Caenorhabditis elegans*

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SUPPORTING RESULTS AND DISCUSSION

***pgl-1*'s link to RNAi**

Few concrete details are known about the function of PGL-1 and its interaction with RNAi. Some reports have suggested that it is as a component of germline perinuclear P-granule structures which are sites of mRNA export and possibly degradation (KAWASAKI *et al.* 1998; SHETH *et al.* 2010), and others have shown that these P-granules' structure or stability depends, at least indirectly, on RNAi components including CSR-1, EGO-1, DCR-1, DRH-3, PRG-1, and RDE-4 (BESHORE *et al.* 2011; CLAYCOMB *et al.* 2009; SPIKE *et al.* 2008; UPDIKE and STROME 2009). While PGL-1's link to RNAs is genetically and biochemically well-established (HANAZAWA *et al.* 2011; SCHISA *et al.* 2001), especially for those in the germline, there are conflicting reports on its role in RNAi. One report was unable to observe any effects on somatic RNAi via 3'UTR-targeted RNAi in a transgene (SHETH *et al.* 2010), while another report suggested that germline RNAi silencing of a transgene was perturbed in a *pgl-1* mutant (ROBERT *et al.* 2005).

Perhaps the clearest link between RNAi and *pgl-1* was the observation that PGL-1 was overexpressed in the soma of an *rb* mutant, which is enhanced for RNAi (WANG *et al.* 2005). However, this enhanced RNAi was attributed more to the overall germline-to-soma transformation in an *rb* mutant, and *pgl-1* was simply used as a marker for demonstrating the presence of germline components. Indeed, a follow-up report showed that along with *pgl-1*, many germline components, including many involved in RNAi, were overexpressed in an *rb* mutant's soma to induce enhanced RNAi (WU *et al.* 2012).

Possible mechanisms of *pgl-1*'s contribution to enhanced RNAi

Our observations that a *pgl-1* mutant is weakly defective for RNAi against both germline and somatic targets in both Eri and non-Eri backgrounds (Fig. 5A, D) leads us to believe that *pgl-1* is indeed involved in RNAi. These RNAi assays were performed via standard feeding of L3 mothers and scoring in the next generation; we were unable to observe a difference between N2 wild type and *pgl-1* mutants' *dpy-11(RNAi)* responses in same-generation RNAi assays via feeding from a bleached embryo (25±2% versus 21±4% penetrance, n>50 in each of the triplicates). Therefore, *pgl-1*'s contribution to exogenous RNAi is likely through the germline. Because of the overwhelming number of germline components associated with P-granules (UPDIKE and STROME 2010), it is challenging to find a mechanism of how *pgl-1* contributes to general exogenous RNAi.

However, our findings that *pgl-1* contributes to *eri* mutants' enhanced RNAi in a complementary fashion to *nrde-3* and that *pgl-1* impacts some of *rb* mutant's enhanced RNAi responses (Table S13) allowed us a significantly narrower guided search for possible mechanisms. We therefore examined roles analogous to *nrde-3*'s contribution to the *eri* pathway and developmentally-related roles that *rb* genes control (which have RNAi implications) as the bases of a candidate search for a possible *pgl-1* RNAi mechanism(s).

***pgl-1* does not contribute to transgene silencing**

Enhanced RNAi mutants show increased spontaneous transgene silencing (Fig. 4E, H). Since we observed that *nrde-3* loss alleviates some of this spontaneous transgene silencing (Fig. 4G, H), we wondered if *pgl-1* loss does the same. We compared the extent of silencing of the ubiquitously expressed *sur-5p::gfp* transgene in *eri-1(mg366)* single mutant to the silencing in the *eri-1(mg366);nrde-3(gg66)* and *eri-1(mg366);pgl-1(bn101)* double mutants. We found that *pgl-1* was not required for *eri-1*-induced silencing (Fig. S5A-C). Therefore, it seems that *pgl-1* does not contribute to enhanced RNAi via facilitating the silencing of repetitive transgene arrays.

***pgl-1* does not contribute to *NRDE-3* nuclear localization**

Although *nrde-3* and *pgl-1* seem to act in parallel pathways due to their contribution to the Eri response against non-overlapping sets of gene targets (Fig. 5D-E), we nevertheless wondered if *pgl-1* loss had any impact on *nrde-3* nuclear localization due to PGL-1's perinuclear location. In wild type backgrounds, *nrde-3::gfp* localizes to the nucleus, most prominently observed in the seam cells (Fig. 5SD). In a *pgl-1(bn101)* background, this localization still occurs (Fig. 5SE). Therefore, it seems that *pgl-1* loss does not impact enhanced RNAi via perturbing *nrde-3* nuclear localization.

***dcap-1*, *mes-4*, and *smg-7* loss do not affect enhanced RNAi the way *pgl-1* loss does**

Due to the motifs present in PGL-1, one mechanistic possibility is that it plays a structural role in maintaining P-granules (KAWASAKI *et al.* 1998), whose components include the RNAi effector(s) responsible for contributing to enhanced RNAi. We therefore selected three candidates, *dcap-1*, *mes-4*, and *smg-7*, who interacts with PGL-1 within P-granules (UPDIKE and STROME 2010) and have previously been known to have RNAi-associated phenotypes (DOMEIER *et al.* 2000; DUDLEY *et al.* 2002; SQUIRRELL *et al.* 2006), to examine their loss' effects on enhanced RNAi. Because DCAP-1 is part of the mRNA decapping complex, MES-4 is important for germline maintenance and RNA metabolism, and SMG-7 is part of the nonsense mediated decay complex, they all have mechanistic potential for eliminating mRNAs. The *eri-1(mg366);dcap-1(ok2139)*, *eri-1(mg366);mes-4(ok2326)*, and the *eri-1(mg366);smg-7(r1197)* double mutants all did not behave like the *eri-1(mg366);pgl-1(bn101)* mutant on *unc-73(RNAi)* (Fig. 5SF); loss of *dcap-1*, *mes-4*, and *smg-7* essentially had no effect on the *eri-1* enhanced RNAi response. Therefore, it seems that PGL-1 does not contribute to enhanced RNAi via facilitating DCAP-1, MES-4, or SMG-7 mechanisms.

***pgl-1* loss does not impact *lin-3* growth pathway**

The *rb* gene family normally represses gene expression; their mutants are enhanced for RNAi because there is an induction of germline expressed genes in the soma (WANG *et al.* 2005; WU *et al.* 2012). One result of this unrepressed expression is inappropriate induction of growth; in the *Rb* mutants, there's a multi-vulva (Muv) phenotype because of unrepressed epidermal growth induction (Fig. 5SG) (SAFFER *et al.* 2011). To determine whether *pgl-1* functions in the growth pathway, we looked for the repression of the Muv phenotype in *lin-15ab(n765)* and EGF growth factor (*lin-3*) receptor constitutive mutant *let-23(sa62)* backgrounds (Fig. 5SI). While the *lin-15ab(n765);pgl-1(bn101)* double mutant was no longer Muv (Fig. 5SH), the *let-23(sa62);pgl-1(bn101)* double mutant still was (Fig. 5SJ). Therefore, it seems that *pgl-1* functions to repress in the *rb* pathway rather than in a general growth response pathway.

***pgl-1* knockdown does not affect *daf-16::gfp* nuclear localization**

Starved worms are more responsive to RNAi (ZHUANG and HUNTER 2012) and *rb* mutants have a perturbed *daf-16* starvation response (KIRIENKO *et al.* 2008). To test if *pgl-1* acts to enhance RNAi via the starvation response, we tested for *daf-16::gfp* nuclear localization which occurs in the absence of food and is abnormally high in *lin-35(n745)* mutants regardless of food status (KIRIENKO *et al.* 2008). We found that knocking down *pgl-1*, which induced a temperature-sensitive sterility phenotype much like *pgl-1* loss does (Fig. 5SL), had no effect on the *daf-16::gfp* nuclear localization in either wild type or *lin-35(n745)* backgrounds (Fig. 5SK). Therefore, it seems that *pgl-1* loss does not perturb the *daf-16* starvation response pathway as a mechanism of enhancing RNAi.

***pgl-1* is not overexpressed in *eri-1* and *rrf-3* mutants**

Although not previously reported, it could be possible that the *eri* mutants, like the *rb* mutants, misexpress some components of the germline, including *pgl-1*, to cause their enhanced RNAi phenotype. qPCR of 15°C hour 100 post-hatching adult *eri-1(mg366)*, *rrf-3(pk1426)*, and *lin-35(n745)* worms for their expression levels of *pgl-1* and *mex-3* were normalized to N2 wild type expression (**Fig. S5M**). Compared to the “amount” of germline approximated by *mex-3* expression, *lin-35(n745)* mutants have overexpression of *pgl-1*, consistent with previous reports (WANG *et al.* 2005). *eri-1* or *rrf-3* loss does not seem to perturb *pgl-1* expression. Therefore, it seems *pgl-1* overexpression is unlikely to be a mechanism of *eri*-class enhanced RNAi.

***pgl-1* mutant small RNA deep sequencing likely to yield broader perspectives on its roles**

While we were unable to attribute any of the aforementioned common and/or *rb*-associated RNAi mechanisms to *pgl-1*, our results do show an effect on endo-RNAi in *pgl-1* mutants (**Fig. 6**). A recent report has suggested that perinuclear structures similar to P-granules are important for siRNA amplification due to RRF-1 recruitment in both exogenous and endogenous contexts (PHILLIPS *et al.* 2012). Therefore, future small RNA deep sequencing should yield rich results for the many roles that *pgl-1* likely plays.

Non-allelic background effects of *rrf-3(pk1426)*

Amongst the *eri* mutants, *rrf-3(pk1426)* has frequently been used as a reference allele for enhanced RNAi. In fact, it was the first Eri reported (SIMMER *et al.* 2002), first Eri implicated in endogenous gene regulation (GENT *et al.* 2009; PAVELEC *et al.* 2009), amongst the first Eri deep-sequenced for its endogenous small RNA profile (GENT *et al.* 2010), and is phenotypically the most robust (ZHUANG and HUNTER 2011). However, this allele has had two reported discrepancies. First, although it is phenotypically almost identical to *eri-1(mg366)*, two different groups reported that its gene expression profile is drastically different from *eri-1(mg366)* (ASIKAINEN *et al.* 2007; LEE *et al.* 2006). Second, biochemical analysis indicates that the *rrf-3(pk1426)* mutant fails to form a RRF-3/ERI-5/DCR-1 endo-RNAi protein complex that is formed in the *rrf-3(mg373)* missense mutant (THIVIERGE *et al.* 2011).

Our results suggest that there's no simplistic model which could explain all the discrepancies in the *rrf-3(pk1426)* background. It is not a simple background Rde, because a rescued strain is still partially Eri (**Table 2**) and *rrf-3(pk1426)* exogenous RNAi seems more robust (**Fig. 7B**). It is not a simple background Eri, because a *rrf-3(pk1426);nrde-3(gg66)* double mutant is weaker in its RNAi responses than the *rrf-3(mg373);nrde-3(gg66)* and *rrf-3(pk2042);nrde-3(gg66)* double mutants (**Table 2**). Furthermore, our observations that *rrf-3(pk1426/mg373);nrde-3(gg66)* trans-heterozygotes behave mostly like *rrf-3(pk1426);nrde-3(gg66)* do not suggest simple recessive background effect(s) (**Table 2**).

Fortunately, it seems like most of the previously reported analysis of *rrf-3(pk1426)* is still valid. For instance, endo-siRNA targets found in the *rrf-3(pk1426)* background (GENT *et al.* 2010) seem to be confirmed in the *rrf-3(mg373)* background as well (**Fig. 4A**), which makes us comfortable in using that same reference dataset for determining the *pgl-1* mutant's effects on these same endo-siRNA targets (**Fig. 6**). With the three exceptions we described (**Table 2; Fig. 7**), *rrf-3(pk1426)* seems to behave the same as all the other Eri mutants in all the rest of our analysis. Previously reported reference datasets of *rrf-3(pk1426)*, however, should still be used with caution without second-allele verification.

SUPPORTING MATERIALS

In addition to the strains listed in the **Materials and Methods** and **Table S11**, the following strains were also used: (HC788) *daf-16::GFP (zls356)*, (HC794) *nrde-3(gg66); ggls01[nrde-3p::FLAG3x-gfp-nrde-3]*, (HC819) *pgl-1(bn101); ggls01[nrde-3p::FLAG3x-gfp-nrde-3]*, (HC845) *let-23(sa62); pgl-1(bn101)*, (HC846) *eri-1(mg366); pgl-1(bn101); nrls20[sur-5::gfp]*, (HC867) *eri-1(mg366); smg-7(r1197)*, (HC868) *eri-1(mg366); dcap-1(ok2139)*, (HC869) *eri-1(mg366); mes-4(ok2326) / nT1(qls51)*, (PS1839) *let-23(sa62)*, (WY546) *lin-35(n745); daf-16::GFP (zls356)*.

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Figure S1 *nrde-3(gg64)* is Rde to *dpy-11(RNAi)* and *nrde-3(gg66)* is rescued by *nrde-3-gfp*. **(A-B)** Representative images of **(A)** wild type (N2) and **(B)** *nrde-3(gg66)* progeny from L3 hermaphrodites grown on *dpy-11(RNAi)* RNAi bacteria at OD_{600nm} 1.0. N2 worms were more sensitive to *dpy-11(RNAi)* by feeding than *nrde-3(gg66)* worms were. Penetrance differences between the strains are shown in **Fig. 1A** and **Table S2**. **(C-D)** Representative images of **(C)** N2 wild type and **(D)** *nrde-3(gg64)* progeny from L3 hermaphrodites grown on *dpy-11(RNAi)* RNAi bacteria at OD_{600nm} 1.0. N2 worms were more sensitive to *dpy-11(RNAi)* by feeding than *nrde-3(gg66)* worms were. Penetrance differences between the strains are shown in **Table S3**. **(E-G)** Representative images of **(E)** N2 wild type, **(F)** *nrde-3(gg66)*, and **(G)** *nrde-3(gg66); nrde-3-gfp* progeny from L3 hermaphrodites grown on *dpy-11(RNAi)* RNAi bacteria at OD_{600nm} 1.0. Fewer *nrde-3(gg66)* animals are dumpy compared to N2 and *nrde-3(gg66); nrde-3-gfp* animals treated with the same dosage of dsRNA. **(H)** The penetrance of *dpy-11(RNAi)*'s expected phenotype described in **(E-G)** are indicated.

Figure S2 *nrde-2(gg91)* is less sensitive to *dpy-11(RNAi)* than *N2*. Representative images of *N2* and *nrde-2(gg91)* from L3 hermaphrodites grown on *dpy-11(RNAi)* RNAi bacteria at OD_{600nm} 2.0 ((A) & (B)) and at OD_{600nm} 4.0 ((D) & (E)). Fewer *nrde-2(gg91)* animals are dumpy compared to *N2* animals treated with the same dosage of dsRNA, although the difference is markedly larger at OD_{600nm} 2.0. The penetrance of *dpy-11(RNAi)*'s expected phenotype is shown in Fig. 1A and Table S2.

Figure S3 Model of competing small RNA pathways producing an Eri phenotype. Exogenous RNAi (exo-RNAi) and endogenous RNAi (endo-RNAi) pathways compete for limiting common resources, which limits the efficacy of the experimentally measurable, or exogenous, RNAi pathway. Mutations that disrupt the production or stability of endo-siRNAi (right) results in increased access to these limiting resources, thereby enhancing the exo-RNAi pathway. The identity of these limiting resources is largely speculative.

Figure S4 *nrde-3*-dependent enhanced *dpy-13(RNAi)* and *ifc-2(RNAi)* phenotypes. **(A-D)** Representative images of **(A)** N2, **(B)** *nrde-3(gg66)*, **(C)** *eri-1(mg366)*, and **(D)** *eri-1(mg366);nrde-3(gg66)* progeny from L3 hermaphrodites grown on *dpy-13(RNAi)* RNAi bacteria at OD_{600nm} 1.5. N2 animals exhibited very mild dumpiness, *nrde-3(gg66)* animals were unaffected, *eri-1(mg366)* animals exhibited grotesque dumpiness, and *eri-1(mg366);nrde-3(gg66)* animals exhibited very mild dumpiness. Scores from **Table 1** are indicated on the micrographs. Penetrances are shown in **Table S9**. **(E-H)** Representative images of **(E)** N2, **(F)** *nrde-3(gg66)*, **(G)** *eri-1(mg366)*, and **(H)** *eri-1(mg366);nrde-3(gg66)* progeny from L3 hermaphrodites grown on *ifc-2(RNAi)* RNAi bacteria at OD_{600nm} 3.5. N2 animals exhibited mild emaciation and posterior paralysis, *nrde-3(gg66)* animals were unaffected, *eri-1(mg366)* animals exhibited grotesque emaciation and paralysis, and *eri-1(mg366);nrde-3(gg66)* animals exhibited mild emaciation and posterior paralysis. Scores from **Table 1** are indicated on the micrographs. Penetrances are shown in **Table S9**.

Figure S5 *pgl-1* does not contribute to common RNAi-related processes. **(A-C)** Representative micrographs of **(A)** *eri-1(mg366)*, **(B)** *eri-1(mg366);nrde-3(gg66)*, and **(C)** *eri-1(mg366);pgl-1(bn101)* strains expressing a *sur-5p::gfp* transgene. In the *eri-1(mg366)* background, there is extensive silencing of *gfp* (red arrows) in the intestinal cells; in the *eri-1(mg366);nrde-3(gg66)* strains, there is no such extensive transgene silencing. The *eri-1(mg366);pgl-1(bn101)* strain still exhibits extensive silencing of *gfp* (red arrows) in the intestinal cells, suggesting no alleviation of spontaneous transgene silencing like *nrde-3* loss exhibits. **(D-E)** Representative micrographs of **(D)** *nrde-3(gg66)* and **(E)** *pgl-1(bn101)* strains expressing an *nrde-3-gfp* transgene. In both backgrounds, there is nuclear localization of the *gfp* in the seam cells (red arrows), suggesting *pgl-1* loss does not affect *nrde-3* nuclear localization. **(F)** The severity of RNAi phenotypes, from '-' to '+++++', based on expressivity described in **Table S1** of indicated strains for *dpy-13(RNAi)* and *unc-73(RNAi)*, with the same OD_{600nm} concentrations as **Table 1**, are scored. All scores are normalized to the strongest Eri response for a particular RNAi assay as '+++++' and no response as '-'. DCAP-1 and SMG-7 are P-granule components proposed to interact with PGL-1; *dcap-1*, *smg-7*, and *mes-4* have also all been previously implicated in

RNAi-processes, especially in the germline, where *pgl-1* is expressed. The *eri-1(mg366)* enhanced RNAi response does not hold the same pattern upon *pgl-1* loss as *dcap-1*, *mes-4*, or *smg-7* loss. **(G-I)** Representative micrographs of **(G)** *lin-15ab(n765)*, **(H)** *lin-15ab(n765);pgl-1(bn101)*, **(I)** *let-23(sa62)*, and **(J)** *let-23(sa62);pgl-1(bn101)* strains' multi-vulva (Muv) phenotype. Inappropriate induction of growth in the *rb* mutants (*lin-15ab*) can cause germline-to-soma transformations which lead to enhanced RNAi. *pgl-1* loss however only suppresses the Muv phenotype of the *rb* mutant, but not a general growth induction pathway like the EGF receptor mutant *let-23(sa62)*. **(K-L)** *rb* mutants are perturbed in their starvation response and starved worms are more RNAi-sensitive than well-fed ones. During starvation, *daf-16::gfp* localizes to the nucleus; in a *rb* mutant, this nuclear localization is increased with or without food. The knockdown of *pgl-1* had no effect on *daf-16::gfp* nuclear localization as indicated by the penetrance of the indicated strains who were placed onto *pgl-1(RNAi)* food as L3s and scored in the next generation **(K)**, even though *pgl-1(RNAi)* increased the penetrance of temperature-sterility at 25°C like *pgl-1* loss does **(L)**, suggesting a functional knockdown. *pgl-1* loss thus does not seem to impact the *daf-16* starvation response. **(M)** *rb* mutants misexpress germline genes in the soma, including *pgl-1*. qPCR of 15°C hour 122 adult worms for their expression levels of the indicated genes in the various mutant strains are shown, with expression of N2 wild type set to 1.0 for each gene. Compared to the "amount" of germline, approximated by *mex-3* expression, *lin-35(n745)* mutants have overexpression of *pgl-1*, consistent with previous reports. *eri-1* or *rff-3* loss does not seem to perturb *pgl-1* expression, suggesting *pgl-1* overexpression is unlikely to be a mechanism of *eri*-class enhanced RNAi. Transcript abundances normalized to *gpd-3*. *p*-values calculated by *t*-test; ** indicates *p*<0.01.

Table S1 Phenotypes and expressivities scored in various RNAi assays

RNAi Target	Phenotype Scored
<i>dpy-11</i>	-Super dumpy: severely dumpy animals whose length is at most 3X its width -Weak dumpy: mildly dumpy animals at most 80% the length of N2 worms *When not specified, both categories are scored as dumpy
<i>dpy-13</i>	I: no phenotype II: very slightly shorter than wild type animals (much less severe than <i>dpy-11</i> weak dumpy; see Fig. S4A) III: Severely dumpy animals whose length is at most 3X its width
<i>dpy-28</i>	Mildly dumpy animals at most 80% the length of N2 worms
<i>elt-1</i>	-Embryonic lethal: dead embryos -Developmental defect: arrested larval growth and deformed tail morphologies
<i>hmr-1</i>	Dead embryos and animals with significantly smaller in morphology and head deformities
<i>ifc-2</i>	Bent posterior body morphology that paralyzes locomotion I: no phenotype, normal growth
<i>lir-1</i>	II: arrest during L1/L2 III: embryonic lethality
<i>myo-3</i>	Paralyzed worms with rigid columnar body morphologies
<i>par-1</i>	Dead embryos
<i>qua-1</i>	Abnormally rigid body morphology and paralyzed movement I: no phenotype, normal movement
<i>unc-15</i>	II: paralyzed movement III: abnormally rigid body morphology and paralyzed movement
<i>unc-22</i>	Abnormally rigid body morphology and paralyzed movement and/or twitching upon 10mM levamisole treatment
<i>unc-73</i>	Paralysis with bulging and curled body morphology

These phenotypes are the basis for the various penetrance recordings in all figures and tables for the indicated genes targeted by feeding RNAi assays. For representative phenotypes scored for *dpy-11(RNAi)*, *dpy-13(RNAi)*, and *ifc-2(RNAi)*, see **Fig. S1, S2, and S4**.

Table S2 n-values for *dpy-11(RNAi)* Rde penetrance for various strains shown in Fig. 1A, J

Assay	OD _{600nm}	Strain	Number Tested
Feed from L3 (1A)	1.0	N2	117
Feed from L3 (1A)	1.0	<i>nrde-3(gg66)</i>	119
Feed from L3 (1A)	1.0	<i>nrde-2(gg91)</i>	44
Feed from L3 (1A)	1.0	<i>nrde-3(gg66); SV40NLS-nrde-3</i>	72
Feed from L3 (1A)	1.0	<i>nrde-3(gg66); *NLS-nrde-3</i>	61
Feed from L3 (1A)	2.0	N2	98
Feed from L3 (1A)	2.0	<i>nrde-3(gg66)</i>	91
Feed from L3 (1A)	2.0	<i>nrde-2(gg91)</i>	28
Feed from L3 (1A)	2.0	<i>nrde-3(gg66); SV40NLS-nrde-3</i>	50
Feed from L3 (1A)	2.0	<i>nrde-3(gg66); *NLS-nrde-3</i>	50
Feed from L3 (1A)	3.0	N2	93
Feed from L3 (1A)	3.0	<i>nrde-3(gg66)</i>	69
Feed from L3 (1A)	3.0	<i>nrde-2(gg91)</i>	56
Feed from L3 (1A)	3.0	<i>nrde-3(gg66); SV40NLS-nrde-3</i>	42
Feed from L3 (1A)	3.0	<i>nrde-3(gg66); *NLS-nrde-3</i>	47
Feed from L3 (1A)	4.0	N2	104
Feed from L3 (1A)	4.0	<i>nrde-3(gg66)</i>	75
Feed from L3 (1A)	4.0	<i>nrde-2(gg91)</i>	30
Feed from L3 (1A)	4.0	<i>nrde-3(gg66); SV40NLS-nrde-3</i>	60
Feed from L3 (1A)	4.0	<i>nrde-3(gg66); *NLS-nrde-3</i>	37
Bleached embryo (1J)	2.0	N2	259
Bleached embryo (1J)	2.0	<i>nrde-3(gg66)</i>	203
Bleached embryo (1J)	2.0	<i>nrde-2(gg91)</i>	164
Bleached embryo (1J)	2.0	<i>nrde-3(gg66); SV40NLS-nrde-3</i>	170
Bleached embryo (1J)	2.0	<i>nrde-3(gg66); *NLS-nrde-3</i>	190
Bleached embryo (1J)	3.0	N2	255
Bleached embryo (1J)	3.0	<i>nrde-3(gg66)</i>	215
Bleached embryo (1J)	3.0	<i>nrde-2(gg91)</i>	238
Bleached embryo (1J)	3.0	<i>nrde-3(gg66); SV40NLS-nrde-3</i>	191
Bleached embryo (1J)	3.0	<i>nrde-3(gg66); *NLS-nrde-3</i>	159
Bleached embryo (1J)	4.0	N2	280
Bleached embryo (1J)	4.0	<i>nrde-3(gg66)</i>	178
Bleached embryo (1J)	4.0	<i>nrde-2(gg91)</i>	201
Bleached embryo (1J)	4.0	<i>nrde-3(gg66); SV40NLS-nrde-3</i>	238
Bleached embryo (1J)	4.0	<i>nrde-3(gg66); *NLS-nrde-3</i>	177

Table S3 Penetrance readings for *nrde-3*'s decreased RNAi response of *gg64* and *gg66* alleles to various RNAi targets

RNAi Target	Method of RNAi	N2	<i>nrde-3 (gg66)</i>	<i>nrde-3 (gg64)</i>
<i>dpy-11</i>	Feed L3 of mom	0.93 n=28	Fig. 1A	0.08 n=13
<i>par-1</i>	Feed L3 of mom	1.00 n=35	0.86 n=190	
<i>qua-1</i>	Feed L3 of mom	0.65 n=52	0.11 n=46	
<i>unc-22</i>	Feed L3 of mom	0.95 n=20	0.43 n=14	
<i>qua-1</i>	Bleached	0.69	0.47	
	embryos	n=89	n=183	

Single L3-stage larvae ("feed L3 of mom") or bleached embryos were placed on RNAi food targeting *dpy-11* (at OD_{600nm} 1.0), *par-1* (at OD_{600nm} 1.0), *qua-1* (at OD_{600nm} 1.0), and *unc-22* (at OD_{600nm} 2.0); 4 days later, the percent of progeny showing the expected phenotypes described in **Table S1** were determined for the listed strains.

Table S4 Penetrance readings of *nrde-3* contribution to trans-generational silencing shown in Fig. 1C-F

Brood count for positive RNAi transmission (Fig. 1C, 1E)																					
Strain	RNAi Target	Broods transmitting RNAi						Average	SD	p-value											
N2	dpy-11	19/20	19/20	16/20	17/19*	0.90	0.07														
nrde-3(gg66)	dpy-11	4/20	6/20	5/20	6/20*	0.26	0.05				<0.001										
eri-1(mg366)	dpy-11	16/19	8/9	7/9	19/20*	0.86	0.07														
eri-1(mg366); nrde-3(gg66)	dpy-11	4/17	0/7	3/9	7/20*	0.23	0.16				<0.001										
N2	unc-22	7/7*	6/7*	6/6*	0.95	0.08															
nrde-3(gg66)	unc-22	4/7*	3/7*	3/6*	0.50	0.07				0.002											
eri-1(mg366)	unc-22	17/20	8/10	9/10	18/20*	0.86	0.05														
eri-1(mg366); nrde-3(gg66)	unc-22	9/19	4/10	5/9	9/20*	0.47	0.06				<0.001										
Representative penetrances of in-brood RNAi phenotypes (from broods with * above) (Fig. 1D, 1F)																					
Strain	RNAi Target	Penetrance																			
N2	dpy-11	5/81	3/77	5/63	6/70	5/57	0/34	6/62	0/54	2/37	6/89	5/70	12/40	10/99	5/80	4/39	23/51	15/67	30/81	3/30	
nrde-3(gg66)	dpy-11	4/62	12/94	0/75	1/84	0/24	0/41	0/35	0/77	0/79	0/31	0/74	6/62	0/44	0/46	1/54	0/31	0/24	0/35	4/66	0/43
eri-1(mg366)	dpy-11	38/62	14/41	19/30	17/23	50/78	7/12	0/21	7/11	19/46	36/68	31/66	9/58	6/29	9/52	23/63	29/36	40/41	49/63	29/70	33/79
eri-1(mg366); nrde-3(gg66)	dpy-11	0/11	0/16	0/37	4/60	6/78	0/48	0/32	0/60	0/32	5/65	3/62	0/43	4/57	7/37	0/31	0/23	0/25	2/48	0/36	0/41
N2 (three samples; shading)	unc-22	5/39	5/40	4/48	4/51	1/37	2/41	2/35	1/24	3/28	0/46	4/33	7/40	4/32	3/25	2/21	7/50	6/29	1/19	2/26	3/39
nrde-3(gg66) (three samples; shading)	unc-22	8/36	3/30	0/24	0/31	3/25	1/31	0/32	0/30	4/36	0/40	0/36	0/11	9/33	2/38	4/28	2/29	0/18	3/36	0/38	0/44
eri-1(mg366)	unc-22	6/21	5/11	6/18	0/19	3/21	3/21	0/18	6/26	3/19	3/16	6/26	4/26	7/30	4/18	12/27	17/24	10/41	9/33	15/42	10/35
eri-1(mg366); nrde-3(gg66)	unc-22	0/28	2/35	5/28	0/45	0/35	3/48	4/36	0/26	4/32	3/34	0/51	2/29	7/40	0/25	0/31	0/14	0/18	0/18	8/26	0/15

Table S5 Penetrance readings of Dpy animals in heterozygous *nrde-3* crosses shown in Fig. 1G-I

Cross	Penetrance in response to OD _{600nm} 1.0 <i>dpy-11(RNAi)</i>										Average	SD	<i>p</i> -value
N2 hermaphrodite x <i>sur-5::gfp</i> male	60/64	29/47	49/53	29/32	26/40	42/46	42/48	49/54			0.84	0.13	
<i>nrde-3(gg66)</i> hermaphrodite x <i>sur-5::gfp</i> male	7/12	0/7	11/48	30/63	0/20	34/52	22/54	23/49	4/16	58/67	0.39	0.28	<0.001 to N2 <0.02 to <i>nrde-3(gg66)</i>
<i>nrde-3(gg66)</i> hermaphrodite x <i>nrde-3(gg66); sur-5::gfp</i> male	11/54	0/20	0/30	10/63	0/15	12/71	0/20	18/62			0.10	0.12	<0.001 to N2

Table S6 Penetrance readings of *nrde-3*-overexpression Eri phenotypes shown in Fig. 2C

<i>dpy-13(RNAi)</i>				Average	SD
N2	1/92	1/119	1/58	0.012	0.005
WT + [<i>nrde-3-gfp</i>]	69/72	66/73	59/64	0.928	0.028
WT + [<i>*NLS-nrde-3-gfp</i>]	7/79	14/79	11/99	0.126	0.046
<i>unc-15(RNAi)</i>					
N2	12/77	19/100	19/90	0.186	0.028
WT + [<i>nrde-3-gfp</i>]	29/44	23/44	46/69	0.616	0.081
WT + [<i>*NLS-nrde-3-gfp</i>]	16/66	18/82	13/68	0.218	0.026
<i>unc-73(RNAi)</i>					
N2	0/30	0/30	0/30	0	0
WT + [<i>nrde-3-gfp</i>]	42/59	39/49	32/50	0.716	0.078
WT + [<i>*NLS-nrde-3-gfp</i>]	4/39	3/61	3/94	0.061	0.037

Table S7 Penetrance readings of *sago-1*-overexpression Eri phenotypes shown in Fig. 2H

Strain	Penetrance				Average	SD
N2	14/135	3/119	2/108		0.049	0.047
<i>nrde-3(gg66)</i>	12/81	7/100	15/137		0.109	0.039
WT+[<i>sago-1-gfp</i>]	98/112	91/114	32/57		0.745	0.163
<i>nrde-3(gg66)</i> +[<i>sago-1-gfp</i>]	108/118	146/173	88/116	23/25	0.859	0.076

Table S8 All Eri mutants partially require *nrde-3* for enhanced RNAi

RNAi Target	Operon or Homologous Sequences		N2	<i>nrde-3(gg66)</i>		<i>rrf-3(mg373)</i>	<i>rrf-3(mg373); nrde-3(gg66)</i>		<i>eri-6/7(tm1917)</i>	<i>eri-6/7(tm1917); nrde-3(gg66)</i>		<i>eri-8(gg100)</i>	<i>eri-8(gg100); nrde-3(gg66)</i>
<i>lir-1</i>	Operon		+++	-		+++++	-		+++++	-		+++++	-
<i>dpy-13</i>	91 Homologs		+	-		+++++	+		+++++	+		+++++	+
<i>ifc-2</i>	3 Homologs		+	-		+++++	++		+++++	+		+++++	+
<i>hmr-1</i>	1 Homolog		+	-		+++++	+++		+++++	+++		+++++	+++
<i>unc-15</i>	0 homologs		-	-		+++++	+++		+++++	+++		+++++	++
<i>dpy-28</i>	0 homologs		-	-		+++++	++++		+++++	++++		+++++	++++
<i>unc-73</i>	0 homologs		-	-		+++++	++++		+++++	++++		+++++	++++

The severity of RNAi phenotypes as described in **Table 1** is based on penetrances measurements presented in **Table S9**. The RNAi bacteria concentrations used were the same as **Table 1**. The enhanced RNAi response targeting genes most dependent on *nrde-3* (highlighted in yellow) holds the same pattern of RNAi responses as *eri-1(mg366)* and *eri-1(mg366);nrde-3(gg66)* mutants.

Table S9 Penetrance readings for Eri mutants' partial requirement of *nrde-3* for enhanced RNAi shown in Table 1 and Table S8

RNAi Target	N2	<i>nrde-3</i> (gg66)	<i>eri-1</i> (mg366)	<i>eri-1</i> (mg366); <i>nrde-3</i> (gg66)	<i>eri-1</i> (mg366); <i>nrde-3</i> (gg66); [*NLS- <i>nrde-3</i>]	<i>eri-1</i> (mg366); <i>nrde-3</i> (gg66); [SV40NLS- <i>nrde-3</i>]	<i>rrf-3</i> (mg373)	<i>rrf-3</i> (mg373); <i>nrde-3</i> (gg66)	<i>eri-6/7</i> (tm1917)	<i>eri-6/7</i> (tm1917); <i>nrde-3</i> (gg66)	<i>eri-8</i> (gg100)	<i>eri-8</i> (gg100); <i>nrde-3</i> (gg66)
<i>lir-1</i>	I: 0.00 II: 0.94 III: 0.06 n=179	I: 1.00 II: 0.00 III: 0.00 n=133	I: 0.00 II: 0.00 III: 1.00 n=59	I: 0.89 II: 0.05 III: 0.06 n=136	I: 1.00 II: 0.00 III: 0.00 n>50	I: 0.01 II: 0.26 III: 0.73 n=305	I: 0.00 II: 0.00 III: 1.00 n=100	I: 1.00 II: 0.00 III: 0.00 n>50	I: 0.00 II: 0.01 III: 0.99 n=75	I: 0.96 II: 0.02 III: 0.02 n=121	I: 0.00 II: 0.00 III: 1.00 n>50	I: 1.00 II: 0.00 III: 0.00 n>50
<i>dpy-13</i>	I: 0.00 II: 1.00 III: 0.00 n>30	I: 1.00 II: 0.00 III: 0.00 n>30	I: 0.00 II: 0.00 III: 1.00 n>30	I: 0.00 II: 1.00 III: 0.00 n>30	I: 0.19 II: 0.81 III: 0.00 n=176	I: 0.00 II: 0.03 III: 0.97 n=90	I: 0.00 II: 0.09 III: 0.91 n=110	I: 0.00 II: 1.00 III: 0.00 n>60	I: 0.00 II: 0.00 III: 1.00 n>30	I: 0.00 II: 1.00 III: 0.00 n>30	I: 0.00 II: 0.00 III: 1.00 n>30	I: 0.00 II: 1.00 III: 0.00 n>30
<i>ifc-2</i>	0.03 n=32	0.00 n=47	0.37 n=27	0.08 n=24	0.15 n=182	0.44 n=189	0.36 n=151	0.20 n=138	0.23 n=22	0.05 n=20	0.24 n=33	0.05 n=19
<i>hmr-1</i>	0.00 n=93	0.00 n=106	1.00 n=37	0.53 n=30	0.80 n=53	1.00 n=34	0.98 n=100	0.75 n=127	1.00 n=35	0.68 n=31	1.00 n=16	0.53 n=32
<i>unc-15</i>	I: 0.90 II: 0.10 III: 0.00 n=20	I: 1.00 II: 0.00 III: 0.00 n=21	I: 0.07 II: 0.43 III: 0.50 n=14	I: 0.13 II: 0.87 III: 0.00 n=31	I: 0.23 II: 0.76 III: 0.01 n=106	I: 0.00 II: 0.36 III: 0.64 n=264	I: 0.00 II: 0.08 III: 0.92 n=131	I: 0.21 II: 0.79 III: 0.00 n=90	I: 0.12 II: 0.44 III: 0.44 n=27	I: 0.31 II: 0.69 III: 0.00 n=39	I: 0.06 II: 0.12 III: 0.82 n=52	I: 0.67 II: 0.33 III: 0.00 n=15
<i>dpy-28</i>	0.03 n=31	0.02 n=47	0.56 n=25	0.48 n=25	0.58 n=50	0.73 n=59	0.62 n=74	0.47 n=72	0.58 n=31	0.47 n=15	0.54 n=70	0.41 n=22
<i>unc-73</i>	0.00 n=68	0.00 n=48	0.97 n=29	0.77 n=35	0.95 n=104	0.82 n=245	0.94 n=158	0.81 n=93	0.96 n=25	0.72 n=43	0.93 n=57	0.81 n=43

The RNAi phenotypes whose penetrances are tabulated and their corresponding descriptions for I, II, and III are listed in **Table S1**.

Table S10 *eri-1* mutants partially require *nrde-2* and *nrde-3* for enhanced RNAi

RNAi target	Notes	N2		<i>nrde-2(gg91)</i>		<i>nrde-3(gg64)</i>		<i>eri-1(mg366)</i>		<i>eri-1(mg366); nrde-2(gg91)</i>		<i>eri-1(mg366); nrde-3(gg64)</i>	
<i>lir-1</i>	Operon	I: 0.00 II: 1.00 III: 0.00 n>50	+++	I: 1.00 II: 0.00 III: 0.00 n=106	-	I: 1.00 II: 0.00 III: 0.00 n=108	-	I: 0.00 II: 0.00 III: 1.00 n=60	+++++	I: 0.94 II: 0.00 III: 0.06 n=66	-	I: 0.94 II: 0.01 III: 0.05 n=136	-
<i>dpy-13</i>	91 Homologs	I: 0.00 II: 1.00 III: 0.00 n>30	+	I: 1.00 II: 0.00 III: 0.00 n=15	-	I: 1.00 II: 0.00 III: 0.00 n=13	-	I: 0.00 II: 0.00 III: 1.00 n=13	+++++	I: 0.00 II: 1.00 III: 0.00 n=11	+	I: 0.00 II: 1.00 III: 0.00 n=21	+
<i>ifc-2</i>	3 Homologs	0.01 n=92	-	0.10 n=251	+	0.00 n=100	-	0.40 n=30	+++++	0.09 n=160	+	0.12 n=40	+
<i>hmr-1</i>	1 Homolog	0.00 n=40	-	0.04 n=90	-	0.00 n=100	-	1.00 n=94	+++++	0.27 n=96	+++	0.24 n=105	+++
<i>unc-15</i>	0 homologs	I: 0.95 II: 0.05 III: 0.00 n=20	-	I: 1.00 II: 0.00 III: 0.00 n=109	-	I: 1.00 II: 0.00 III: 0.00 n=95	-	I: 0.06 II: 0.41 III: 0.53 n=85	+++++	I: 0.28 II: 0.72 III: 0.00 n=134	+++	I: 0.34 II: 0.66 III: 0.00 n=68	+++
<i>dpy-28</i>	0 homologs	0.03 n=90	-	0.01 n=100	-	0.04 n=112	-	0.62 n=45	+++++	0.53 n=70	++++	0.50 n=56	++++
<i>unc-73</i>	0 homologs	0.00 n>100	-	0.00 n=137	-	0.00 n=17	-	0.96 n=27	+++++	0.82 n=17	++++	0.69 n=32	++++

The severity of RNAi phenotypes, from '-' to '+++++', based on expressivity described in **Table S1** (with their corresponding descriptions for I, II, and III) and penetrances tabulated within of indicated strains for indicated RNAi gene targets are scored. All scores are normalized to the strongest Eri response for a particular RNAi assay as '+++++' and no response as '-'. The RNAi bacteria OD_{600nm} concentrations used were the same as **Table 1**. The impact on the enhanced RNAi response for *nrde-2(gg91)* and *nrde-3(gg64)* strains holds the same pattern of RNAi responses as *eri-1(mg366)* and *eri-1(mg366);nrde-3(gg66)* mutants.

Table S11 Candidate screen of nuclear-associated factors with available mutants that may contribute to *nrde-3*-independent enhanced RNAi

Candidate	<i>dpy-11(RNAi)</i>	Annotation and Notes
N2 wild type	Dpy	Almost completely penetrant
MT13544: <i>ceh-30(n4289)</i>	Dpy	Homeodomain protein
RB1447: <i>chd-3(ok1651)</i>	Mild Rde	Chromodomain protein
JT6130: <i>daf-21(p673)</i>	Dpy	Sensitive to <i>unc-22(RNAi)</i> and <i>bli-1(RNAi)</i> like WT Hsp90 chaperone
RB2589: <i>daf-3(ok3610)</i>	Dpy	SMAD transcriptional regulator
CB1386: <i>daf-5(e1386)</i>	Dpy	
CB1393: <i>daf-8(e1393)</i>	Dpy	SMAD transcriptional regulator
JJ1549: <i>efl-1(se1)</i>	Dpy	E2F transcriptional repressor
VC1217: <i>egl-27(ok1670)</i>	Dpy	MTA1 nucleosome remodeling deacetylase
VC736: <i>gfl-1(gk321)</i>	Dpy	ENL-class transcriptional factor
PFR60: <i>hpl-1(tm1624)</i>	Dpy	Heterochromatin protein 1
MT14390: <i>let-418(n3536)</i>	Dpy	CHD3 homolog nucleosome remodeling deacetylase
CB3518: <i>mab-10(e1248)</i>	Dpy	
RB1906: <i>mes-1(ok2467)</i>	Dpy	Receptor tyrosine kinase-like protein
SS222: <i>mes-3(bn21)</i>	Dpy	Polycomb chromatin repressive complex component
MT16973: <i>met-1(n4337)</i>	Mild Eri	Histone methyltransferase Mild Eri to <i>lir-1(RNAi)</i> as well
TK22: <i>mev-1(kn1)</i>	Mild Eri	Cytochrome b integral membrane protein Eri to <i>lir-1(RNAi)</i>, <i>dpy-13(RNAi)</i>, <i>unc-73(RNAi)</i>, and <i>hmr-1(RNAi)</i> as well
MT8987: <i>pag-3(n3098)</i>	Dpy	C2H2 zinc-finger protein
SS579: <i>pgl-1(bn101)</i>	Rde	RNA-binding protein with RGG box motifs See data presented within text
KN562: <i>pop-1(hu9)</i>	Mild Eri	TCF/LEF family transcription factor
VC824: <i>prmt-5(gk357)</i>	Dpy	Protein arginine Methyltransferase
VC1688: <i>sex-1(gk808)</i>	Dpy	Nuclear hormone receptor family DNA-binding protein
KC565: <i>sin-3(tm1276)</i>	Dpy	Histone deacetylase subunit
LW478: <i>sma-9(tm572)</i>	Dpy	Zinc-finger domain protein
VC1362: <i>taf-11.1(gk648)</i>	Dpy	TBP-associated transcription factor
VC1482: <i>taf-11.2(gk682)</i>	Dpy	TBP-associated transcription factor
VC915: <i>taf-4(ok1399)</i>	Rde	TATA-binding protein associated factor Rde to <i>unc-22(RNAi)</i>, <i>bli-1(RNAi)</i>, <i>lir-1(RNAi)</i>, and <i>par-1(RNAi)</i> as well
VC1502: <i>taf-7.1(gk696)</i>	Dpy	TATA-binding protein associated factor
VC2203: <i>taf-9(ok2871)</i>	Dpy	TBP-associated transcription factor

VC764: <i>tag-235(ok1265)</i>	Dpy	Histone Acetyltransferase
JC1970: <i>tbx-2(ut180)</i>	Dpy	T-box transcription factor
CB262: <i>unc-37(e262)</i>	Dpy	Transducin-like WD-repeat protein
SK86: <i>zag-1(zd86)</i>	Dpy	ZFH class homeodomain protein
RB774: <i>zfp-1(ok554)</i>	Dpy	Leucin-zipper containing protein

The indicated strains were placed onto OD_{600nm} 1.0 *dpy-11(RNAi)* food as L3s and scored in the next generation. Any Rde phenotypes were followed up with additional RNAi tests as described.

Table S12 Penetrance of Rde phenotypes of *pgl-1(bn101)*, *pgl-1(bn102)*, and *pgl-1(ct131)* mutants shown in Fig. 5A

Strain	RNAi Food	Phenotype	Penetrance										Avg	SD		
N2	<i>lir-1</i> OD _{600nm} 3.5	L1/L2 arrest	146/151		154/166		85/91		126/130		100/103		71/72		0.96	0.02
<i>pgl-1(bn101)</i>			37/126		39/124		18/94		52/169		27/74		19/61		0.30	0.06
<i>pgl-1(bn102)</i>			37/109		21/98		31/104		83/171		39/73		55/116		0.39	0.13
<i>pgl-1(ct131)</i>			19/49	9/21	64/90	56/126	44/76	37/62	63/94	55/117	53/93	0.54	0.11			
N2	<i>par-1</i> OD _{600nm} 2.0	Embryonic lethality	167/180		170/192		157/158		162/167		133/144		138/157		0.93	0.05
<i>pgl-1(bn101)</i>			31/129	62/122	31/170	71/141	70/237	79/195	34/95	8/47	0.33	0.13				
<i>pgl-1(bn102)</i>			22/110		14/102		34/139		58/175		85/207		0.26	0.11		
<i>pgl-1(ct131)</i>			16/31	66/118	99/128	54/115	115/165	77/100	120/155	86/135	23/50	0.63	0.13			
N2	<i>dpy-11</i> OD _{600nm} 4.0	Very dumpy worms	73/80		89/91		114/119		87/88		80/83		92/94		0.96	0.03
<i>pgl-1(bn101)</i>			2/48		8/92		2/84		7/112		3/38		3/55		0.06	0.02
<i>pgl-1(bn102)</i>			3/59		4/40		2/52		5/79		1/47		3/64		0.05	0.03
<i>pgl-1(ct131)</i>			5/33	6/42	3/13	5/47	3/26	11/32	5/41	4/38	0.16	0.08				
N2	<i>unc-22</i> OD _{600nm} 3.0	Paralyzed sticks	88/92		51/59		50/62		55/62		56/69		0.87	0.06		
<i>pgl-1(bn101)</i>			11/47	9/46	6/35	11/29	10/28	10/24	9/17	9/22	0.34	0.12				
<i>pgl-1(bn102)</i>			4/13	12/25	19/28	30/38	5/26	16/51	9/35	0.43	0.23					
<i>pgl-1(ct131)</i>			2/7	10/21	21/40	5/13	5/13	14/32	5/12	5/11	0.42	0.07				

Table S13 Penetrance readings for Eri mutants' partial requirement of *pgl-1* for enhanced RNAi

RNAi Target	N2	<i>pgl-1</i> (<i>bn101</i>)	<i>eri-1</i> (<i>mg366</i>)	<i>eri-1</i> (<i>mg366</i>); <i>pgl-1</i> (<i>bn101</i>)	<i>eri-1</i> (<i>mg366</i>); <i>pgl-1</i> (<i>bn102</i>)	<i>eri-1</i> (<i>mg366</i>); <i>pgl-1</i> (<i>bn101</i>); <i>nrde-3</i> (<i>gg66</i>)	<i>rrf-3</i> (<i>pk1426</i>)	<i>rrf-3</i> (<i>pk1426</i>); <i>pgl-1</i> (<i>bn101</i>)	<i>eri-6/7</i> (<i>tm1917</i>)	<i>eri-6/7</i> (<i>tm1917</i>); <i>pgl-1</i> (<i>bn101</i>)	<i>eri-8</i> (<i>tm1860</i>)	<i>eri-8</i> (<i>tm1860</i>); <i>pgl-1</i> (<i>bn101</i>)	<i>lin-15ab</i> (<i>n765</i>)	<i>lin-15ab</i> (<i>n765</i>); <i>nrde-3</i> (<i>gg66</i>)	<i>lin-15ab</i> (<i>n765</i>); <i>pgl-1</i> (<i>bn101</i>)	<i>lin-35</i> (<i>n745</i>)	<i>lin-35</i> (<i>n745</i>); <i>nrde-3</i> (<i>gg66</i>)	<i>lin-35</i> (<i>n745</i>); <i>pgl-1</i> (<i>bn101</i>)
<i>lir-1</i>	I: 0.02 II: 0.98 III: 0.00 n=83	I: 0.67 II: 0.22 III: 0.11 n=164	I: 0.00 II: 0.00 III: 1.00 n=89	I: 0.15 II: 0.69 III: 0.16 n=355	I: 0.05 II: 0.50 III: 0.45 n=312	I: 1.00 II: 0.00 III: 0.00 n=90	I: 0.00 II: 0.00 III: 1.00 n=84	I: 0.22 II: 0.70 III: 0.08 n=80	I: 0.00 II: 0.00 III: 1.00 n=75	I: 0.04 II: 0.61 III: 0.35 n=23	I: 0.00 II: 0.00 III: 1.00 n=97	I: 0.08 II: 0.92 III: 0.00 n=153	I: 0.00 II: 0.00 III: 1.00 n=106	I: 0.13 II: 0.87 III: 0.00 n=55	I: 0.09 II: 0.91 III: 0.00 n=138	I: 0.00 II: 0.00 III: 1.00 n=57	I: 0.08 II: 0.78 III: 0.14 n=88	I: 0.25 II: 0.56 III: 0.19 n=109
<i>dpy-13</i>	I: 0.93 II: 0.07 III: 0.00 n=121	I: 1.00 II: 0.00 III: 0.00 n=141	I: 0.00 II: 0.01 III: 0.99 n=70	I: 0.15 II: 0.15 III: 0.70 n=88	I: 0.00 II: 0.19 III: 0.81 n=63	I: 1.00 II: 0.00 III: 0.00 n=40	I: 0.00 II: 0.00 III: 1.00 n=156	I: 0.07 II: 0.05 III: 0.88 n=57	I: 0.00 II: 0.00 III: 1.00 n=64	I: 0.16 II: 0.27 III: 0.57 n=37	I: 0.00 II: 0.00 III: 1.00 n=170	I: 0.21 II: 0.05 III: 0.74 n=58	I: 0.00 II: 0.23 III: 0.67 n=97	I: 0.00 II: 0.20 III: 0.80 n=99	I: 0.08 II: 0.22 III: 0.70 n=53	I: 0.00 II: 0.30 III: 0.70 n=101	I: 0.00 II: 0.21 III: 0.79 n=63	I: 0.00 II: 0.36 III: 0.64 n=33
<i>ifc-2</i>	0.09 n=137	0.06 n=188	0.51 n=81	0.23 n=228	0.32 n=82	0.05 n=153	0.76 n=134	0.43 n=60	0.50 n=56	0.33 n=27	0.51 n=71	0.43 n=60	0.40 n=45	0.40 n=182	0.28 n=64	0.33 n=153	0.21 n=124	0.32 n=76
<i>unc-15</i>	I: 0.94 II: 0.06 III: 0.00 n=106	I: 0.99 II: 0.01 III: 0.00 n=146	I: 0.00 II: 0.12 III: 0.88 n=67	I: 0.67 II: 0.23 III: 0.00 n=270	I: 0.41 II: 0.59 III: 0.00 n=95	I: 0.94 II: 0.06 III: 0.00 n=103	I: 0.00 II: 0.03 III: 0.94 n=80	I: 0.69 II: 0.31 III: 0.00 n=51	I: 0.00 II: 0.04 III: 0.96 n=114	I: 0.72 II: 0.28 III: 0.00 n=18	I: 0.00 II: 0.17 III: 0.83 n=70	I: 0.28 II: 0.72 III: 0.00 n=46	I: 0.30 II: 0.70 III: 0.00 n=63	I: 0.30 II: 0.70 III: 0.00 n=40	I: 0.35 II: 0.65 III: 0.00 n=88	I: 0.33 II: 0.67 III: 0.00 n=110	I: 0.50 II: 0.50 III: 0.00 n=52	I: 0.73 II: 0.27 III: 0.00 n=44
<i>hmr-1</i>	I: 0.94 II: 0.06 III: 0.00 n=72	I: 0.89 II: 0.05 III: 0.06 n=125	I: 0.00 II: 0.00 III: 1.00 n=34	I: 1.00 II: 0.00 III: 0.00 n=100	I: 0.73 II: 0.03 III: 0.24 n=186	I: 1.00 II: 0.00 III: 0.00 n=116	I: 0.00 II: 0.00 III: 1.00 n=61	I: 1.00 II: 0.00 III: 0.00 n=158	I: 0.00 II: 0.00 III: 1.00 n=121	I: 0.89 II: 0.11 III: 0.00 n=54	I: 0.00 II: 0.00 III: 1.00 n=136	I: 1.00 II: 0.00 III: 0.00 n=76	I: 0.11 II: 0.23 III: 0.66 n=184	I: 0.62 II: 0.32 III: 0.06 n=117	I: 1.00 II: 0.00 III: 0.00 n=116	I: 0.23 II: 0.11 III: 0.66 n=62	I: 0.66 II: 0.22 III: 0.12 n=94	I: 0.66 II: 0.32 III: 0.02 n=91
<i>dpy-28</i>	0.00 n=14	0.00 n=60	0.79 n=43	0.00 n=140	0.02 n=123	0.00 n=100	0.67 n=21	0.00 n=58	0.70 n=67	0.00 n=17	0.69 n=36	0.00 n=68	0.65 n=34	0.74 n=74	0.00 n=65	0.68 n=31	0.64 n=64	0.22 n=68
<i>unc-73</i>	0.00 n=38	0.01 n=205	0.96 n=45	0.00 n=100	0.01 n=142	0.00 n=40	1.00 n=122	0.00 n=59	1.00 n=96	0.00 n=40	0.94 n=136	0.07 n=121	0.77 n=61	0.69 n=152	0.00 n=96	0.36 n=142	0.34 n=74	0.05 n=81

The RNAi phenotypes whose penetrances are tabulated and their corresponding descriptions for I, II, and III are listed in **Table S1**. The RNAi bacteria OD_{600nm} concentrations used were the same as **Table 1**. The *eri*-class enhanced RNAi response against gene targets most dependent on *pgl-1* holds the same pattern of RNAi responses as *eri-1(mg366)* and *eri-1(mg366);pgl-1(bn101)* for *rrf-3(pk1426)*, *eri-6/7(tm1917)*, *eri-8(tm1860)*, and their doubles with *pgl-1(bn101)*. The impact on the enhanced RNAi response for *pgl-1(bn102)* holds the same pattern of RNAi responses as *pgl-1(bn101)* mutants. The *eri-1(mg366);nrde-3(gg66);pgl-1(bn101)* strain is completely wild type in its response to all tested RNAi targets. The *rb*-class (*lin-15ab(n765)* and *lin-35(n745)*) enhanced RNAi response against gene targets do not depend on *nrde-3(gg66)*, but against gene targets most dependent on *pgl-1* in the *eri*-class, the *lin-15ab(n765);pgl-1(bn101)* and *lin-35(n745);pgl-1(bn101)* mutants hold the same pattern of RNAi responses as *eri-1(mg366);pgl-1(bn101)*.

Table S14 Penetrance readings for *rff-3(pk1426)*'s unique dependence on *nrde-3* for enhanced RNAi

RNAi Target	N2	<i>nrde-3</i> (<i>gg66</i>)	<i>rff-3</i> (<i>mg373</i>)	<i>rff-3</i> (<i>mg373</i>); <i>nrde-3</i> (<i>gg66</i>)	<i>rff-3</i> (<i>pk2042</i>)	<i>rff-3</i> (<i>pk2042</i>); <i>nrde-3</i> (<i>gg66</i>)	<i>rff-3</i> (<i>pk1426</i>)	<i>rff-3</i> (<i>pk1426</i>); <i>nrde-3</i> (<i>gg66</i>)	<i>rff-3</i> (<i>mg373/pk1426</i>) het	<i>rff-3</i> (<i>mg373/pk1426</i>) ; <i>nrde-3</i> (<i>gg66</i>) het	NL2099: <i>rff-3</i> (<i>pk1426</i>) + [WT <i>rff-3</i>]
<i>lir-1</i>	I: 0.00 II: 0.94 III: 0.06 n=179	I: 1.00 II: 0.00 III: 0.00 n=133	I: 0.00 II: 0.00 III: 1.00 n=100	I: 1.00 II: 0.00 III: 0.00 n>50	I: 0.00 II: 0.00 III: 1.00 n=219	I: 1.00 II: 0.00 III: 0.00 n=131	I: 0.00 II: 0.00 III: 1.00 n=16	I: 0.96 II: 0.04 III: 0.00 n=28	I: 0.00 II: 0.00 III: 1.00 n=30	I: 1.00 II: 0.00 III: 0.00 n=54	I: 0.00 II: 1.00 III: 0.00 n=12
<i>dpy-13</i>	I: 0.00 II: 1.00 III: 0.00 n>30	I: 1.00 II: 0.00 III: 0.00 n>30	I: 0.00 II: 0.09 III: 0.91 n=110	I: 0.00 II: 1.00 III: 0.00 n>60	I: 0.00 II: 0.08 III: 0.92 n=331	I: 0.88 II: 0.12 III: 0.00 n=189	I: 0.00 II: 0.00 III: 1.00 n>30	I: 1.00 II: 0.00 III: 0.00 n>30	I: 0.00 II: 0.11 III: 0.89 n=19	I: 0.90 II: 0.10 III: 0.00 n=71	I: 0.00 II: 1.00 III: 0.00 n=14
<i>ifc-2</i>	0.03 n=32	0.00 n=47	0.36 n=151	0.20 n=138	0.41 n=336	0.17 n=210	0.38 n=38	0.08 n=25	0.33 n=12	0.06 n=54	0.00 n=17
<i>hmr-1</i>	0.00 n=93	0.00 n=106	0.98 n=100	0.75 n=127	1.00 n=221	0.71 n=77	1.00 n=22	0.00 n=23	1.00 n=14	0.13 n=48	0.80 n=15
<i>unc-15</i>	I: 0.90 II: 0.10 III: 0.00 n=20	I: 1.00 II: 0.00 III: 0.00 n=21	I: 0.00 II: 0.08 III: 0.92 n=131	I: 0.21 II: 0.79 III: 0.00 n=90	I: 0.00 II: 0.05 III: 0.95 n=192	I: 0.44 II: 0.56 III: 0.00 n=183	I: 0.00 II: 0.17 III: 0.83 n=36	I: 1.00 II: 0.00 III: 0.00 n=22	I: 0.00 II: 0.25 III: 0.75 n=12	I: 0.96 II: 0.04 III: 0.00 n=74	I: 0.35 II: 0.21 III: 0.44 n=29
<i>dpy-28</i>	0.03 n=31	0.02 n=47	0.62 n=74	0.47 n=72	0.73 n=123	0.53 n=107	0.65 n=48	0.00 n=54	0.62 n=13	0.07 n=104	0.42 n=24
<i>unc-73</i>	0.00 n=68	0.00 n=48	0.94 n=158	0.81 n=93	0.98 n=253	0.93 n=206	1.00 n=29	0.00 n=38	0.58 n=12	0.12 n=121	0.74 n=19
25°C sterile	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No

The RNAi phenotypes whose penetrances are tabulated and their corresponding descriptions for I, II, and III are listed in **Table S1**. All *rff-3* alleles are temperature sensitive sterile at 25°C, even in an *nrde-3(gg66)* background. The rescue construct restores viability at 25°C. A transgenic rescue using *rff-3* with its native UTRs of the NL2099 strain containing the *pk1426* allele does not fully restore the wild type RNAi response against gene targets most dependent on *pgl-1*. The trans-heterozygous strains were mated using *rff-3(pk1426)* or *rff-3(pk1426);nrde-3(gg66)* hermaphrodites and *rff-3(mg373);sur-5::gfp* or *rff-3(mg373);nrde-3(gg66);sur-5::gfp* males for green cross progeny.