The nuclear Argonaute NRDE-3 contributes to transitive RNAi in *Caenorhabditis elegans*

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ABSTRACT

The *Caenorhabditis elegans* nuclear RNAi 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 over-expressing 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 non-overlapping 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 mis-express 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.
INTRODUCTION

Genetic analysis of RNA interference (RNAi) in *C. 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; Tabara et al. 2002). Because these gene products target mature 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 (Burkhart et al. 2011; Burton et al. 2011; Guang et al. 2010; Guang et al. 2008). 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 (Burton et al. 2011; Guang et al. 2010). This RNA-directed epigenetic mechanism also enables multi-generational 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) (Duchaine et al. 2006; Fischer et al. 2008; Kennedy et al. 2004; Pavelec et al. 2009; Simmer et al. 2002). Genes complementary to endo-siRNAs are mis-regulated 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 peri-nuclear protein PGL-1 as important for the enhanced RNAi phenotypes of a non-overlapping 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 multi-copy 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);vpl9, (HC764) eri-1(mg366);nrde-3(gg66);vpl9, (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);ggl01(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); vpl9, (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);vpl9, (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); vpl9, (HC860) nrde-3(gg66); nel11, (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) vpl9 (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-
(bn101); neIs5[Y43B11::pgl-1::gfp + pRF4(rol-6(su1006))] (WM120) Mago; neIs11(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°C 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 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 (Fig. 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 vpl9 (unc-119(+) + elt-1::GFP) insertion is <2cM 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 hour) mid-L4 worms from five NG large plates grown at 15°C for 91 hours were pooled, washed extensively (M9) and allowed to swim for 20 minutes 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/µL.

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 µL of the 20 µL RT reaction in a 50 µL QuantiTect SYBR Green (Qiagen) reaction with nested PCR primers. The PCR reaction cycles were: 15 minutes 95 degrees, 15 seconds 94 degrees, 30 seconds 52 degrees, 1 minute 72 degrees, read, cycle to step 2 for 45 cycles, using an Eppendorf Mastercycler Realplex4 and Noiseband quantification. Subsequent
analysis was performed using a ΔΔCT approach (LIVAK and SCHMITTGEN 2001), using \textit{gpd-3} RNA levels for normalization (WELKER \textit{et al.} 2010), as previously described (ZHANG and HUNTER 2012).

**Cross Progeny Assays:** Males were marked by the \textit{sur-5::gfp} transgene while hermaphrodites were unmarked. Only green cross progeny were scored in all assays.

**Transgene Silencing Assay:** Hatch-synchronized (within 1 hour) young adult worms grown at 20°C for 53 hours were imaged, at the same magnification and exposure for all strains indicated in Fig. 4. Outlined individual worms were analyzed using ImageJ (National Institutes of Health) by first “subtract background” and then “measure” functions for determining \textit{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. emb. lethal, dev. arrest, or WT) are represented by sums of all trials; breakdown of individual trials are available upon request. All error bars indicate standard deviation. \textit{p}-values calculated by \textit{t}-test; * indicates \textit{p}<0.05 and ** indicates \textit{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 (Fig. 1A; Table S1, S2, 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, (Fig. S1; Table S3) and showed that an nrde-3-gfp transgene construct completely rescued the RNAi defects (Fig. 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 (Fig. 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 (BURTON et al. 2011; GUANG et al. 2010). 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 (Fig. 1A; Fig. 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) (Fig. 1A). These results show that nuclear localized activities broadly contribute to RNAi triggered by exogenous dsRNA.

nrde-3 contributes to inter- and intra-generational 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 for phenotypes on that same plate. Therefore, the requirement for nrde function for efficient silencing (Fig. 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 \textit{nrde-3} is important for transmission of silencing from dsRNA-exposed mothers to non-exposed progeny. Specifically, we grew wild-type and \textit{nrde-3(gg66)} worms from hatching to the fourth larval stage (L4) on either \textit{dpy-11} or \textit{unc-22} dsRNA expressing bacteria. We then transferred the RNAi-affected Dpy or Unc L4 worms to control bacteria and scored their non-exposed progeny for Dpy-11 or Unc-22 phenotypes (Fig. 1B). We found that the fraction of broods inheriting RNAi silencing was two-to-four-fold higher in the wild-type background (Fig. 1C, E; Table S4). Furthermore, the penetrance of Dpy or Unc within each RNAi-inheriting brood was also higher in the wild-type background (Fig. 1D, 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 \textit{nrde-3(gg66)} mothers could restore the penetrance of the inherited silencing. Specifically, we crossed dsRNA-exposed \textit{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 (Fig. 1G), while in \textit{nrde-3(gg66)} by \textit{nrde-3(gg66)} negative control crosses, almost none of the mothers transmitted the phenotype (Fig. 1I). However, in only one out of 10 crosses did \textit{nrde-3(gg66)} mothers robustly (>80% penetrant) transmit the Dpy-11 RNAi phenotype to their wild-type cross progeny, while most \textit{nrde-3(gg66)} mothers only partially transmitted the Dpy-11 RNAi phenotype (Fig. 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 (**Fig. 1J; Tables S2, 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 (**Fig. S1E, G**). Because *C. elegans* multi-copy transgenes are often over-expressed (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)* (**Fig. 2A-C; Table S6**), as well as to *unc-15(RNAi)* and *unc-73(RNAi)* (**Fig. 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 (Fig. 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 over-induced enhanced RNAi in muscle cells (Fig. 2D-G). We observed that the *nrde-3(gg66); myo-3p::gfp-sago-1* strain remained Eri (Fig. 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 (Fig. 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 (Fig. 2C). Third, *nrde-3* was identified as a mutant that abolished the *eri-1*-dependent phenotype.
of pan-operon silencing (GUANG et al. 2008). Fourth, nrde-3 is auxiliary to RNAi silencing: the Rde phenotype is only apparent when dsRNA trigger is limiting (Fig. 1A).

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, rrf-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; Tables S8, 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; Tables S1, S8, S9; Fig. 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; Fig. 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 \textit{nrde-3} dependent nuclear RNAi processes may preferentially silence multiple related genes.

\textbf{nrde-3 contributes to transitive RNAi}

Intriguingly, a mechanism that could explain the \textit{nrde-3}-dependent enhanced RNAi targeting sequence-related multi-gene families is transitive RNAi. Transitive RNAi is the observation that RNAi silencing specificity can spread \textit{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 \textit{in trans} (such as homologous transcripts), which is essential for the potency of RNAi in \textit{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 \textit{C. elegans} using transgenes that artificially fuse separate genes, resulting in secondary siRNAs that independently target both sequences (Alder et al. 2003; Hannon 2002).

To determine whether \textit{nrde-3} can contribute to transitive RNAi, we used a strain with an \textit{elt-1-gfp} fusion transgene that when exposed to \textit{gfp} dsRNA causes \textit{elt-1}-related developmental defects (Fig. 3A) (Alder et al. 2003; Smith et al. 2005). Specifically, we exposed wild-type, \textit{nrde-3}(gg66), and \textit{eri-1}(mg366) strains that express the \textit{elt-1-gfp} transgene to \textit{gfp} dsRNA and scored the progeny for \textit{elt-1}-related developmental defects. In a wild-type background, feeding \textit{gfp} dsRNA to worms expressing the \textit{elt-1-gfp} fusion causes mild levels of developmental deformities and embryonic lethality (Fig. 3B, E), while in an \textit{eri-1}(mg366)
background, gfp(RNAi) causes near complete elt-1-related embryonic lethality (Fig. 3C, 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 (Fig. 3D, 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 (Fig. 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 (Fig. 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 (Fig. 4A). To our knowledge, this is the first report of an endogenous
function for \textit{nrde-3}, as previous reports on \textit{nrde} roles in germline maintenance implicated only \textit{nrde-1}, \textit{nrde-2}, and \textit{nrde-4}. Consistent with previous reports, we observed that \textit{eri-1} and \textit{rrf-3} mutants had overlapping but incompletely shared effects on endogenous gene expression (Fig. 4A), (Asikainen et al. 2007; Lee et al. 2006).

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

\textbf{\textit{pgl-1} acts in parallel to \textit{nrde-3}}

Mutants that disrupt core RNAi functions, such as \textit{rde-1} and \textit{dcr-1}, have near 100 percent Rde penetrance (Tabara et al. 1999; Tabara et al. 2002), while the \textit{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 post-core 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 (Fig. 5A-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 5D, 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 5D, 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: rrf-3, eri-6/7, and ergo-1/eri-8 (Table S13). Finally, because PGL-1 is
over-expressed 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 (Fig. 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 (Fig. 6A-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 (Supplementary Results and Discussion; Fig. S5). However, despite our lack of mechanistic insight, our results suggest that *pgl-1* is important for endo-siRNA regulated gene expression via a process that may compete with exogenous RNAi.

**rrf-3(pk1426) is likely associated with a non-allelic 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 (Asikainen et al. 2007; Lee et al. 2006) showed a significantly different expression profile in an rrf-3(mg373) background (Fig. 7A). Previous gene expression profile analyses also show little overlap between rrf-3(pk1426) and eri-1(mg366) (Asikainen et al. 2007; Lee et al. 2006). 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 (Fig. 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 (Supplementary Results and Discussion).

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. lin-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 semi-dominant effect, the *rrf-3* trans-heterozygote strain was, at a very reduced level, Eri for some *pgl-1*-dependent targets. In summary, our results indicate that an *rrf-3(pk1426)* associated background effect, which may act via a *pgl-1*-dependent pathway, accounts for the unusual properties associated with the *rrf-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 multi-gene families and multi-copy 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 non-overlapping 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* (BURTON et al. 2011; GUANG et al. 2010) 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 (Fig. 1A, J) and overexpressor-induced Eri (Fig. 2C) phenotypes. This may be due to an incomplete removal of nuclear localization, although the lack of gfp nuclear localization suggests otherwise. This may also be due to the perhaps overexpressed transgene soaking up endogenous siRNAs – even if it’s only present in the cytoplasm – so that the exogenous RNAi triggers have more access to competed-for cytoplasmic RNAi resources, hence creating an ever 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* (BUCKLEY et al. 2012; BURKHART et al. 2011). 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 (Fig. 1A, 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 (Fig. 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 over-expression relieves the competition for this limiting resource (Yigit et al. 2006). Here we show that *nrde-3* is Rde (Fig. 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 (Fig. 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*, over-expression enhances RNAi (Fig. 2A-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 (Fig. 5A), *pgl-1* overexpression enhances RNAi (Fig. 5B-C), *pgl-1* mutants disrupt the expression of endo-siRNA regulated genes (Fig. 6), and loss of *pgl-1* attenuates the Eri phenotypes, at least for some genes (Fig. 5D-E). Remarkably, the simultaneous loss of both *pgl-1* and *nrde-3* broadly depletes enhanced RNAi in the *eri-1* mutant background (Fig. 5D-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 (*Fig. 6, S5*). However, our analysis clearly establishes that *pgl-1* contributes to both exogenous and endogenous RNAi (*Fig. 5, 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 (*Fig. 3*) may provide insight into the role of nuclear RNAi. The contribution of NRDE-3 to RNAi was most apparent when dsRNA is limiting (*Fig. 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 versus 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 multi-gene families and multi-copy 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 \textit{dpy-13(RNAi)} causes a much stronger Dpy phenotype than does a \textit{dpy-13} null allele, it is likely that the RNAi targets other functionally redundant collagen genes. In fact, the ingested \textit{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 \textit{nrde-3}-dependent enhanced operon silencing; however, only two operons out of over 1300 \textit{C. elegans} operons have been shown to be sensitive to \textit{eri}-enhanced silencing.

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

The depletion of endo-siRNAs that apparently underlies many \textit{nrde-3}-dependent Eri phenotypes suggests that \textit{nrde-3} might have a role in endo-siRNA target gene regulation. Indeed, similar to the \textit{eri} mutants, we found that loss of \textit{nrde-3} affects the gene expression of \textit{eri} endo-siRNA targets (Fig. 4A), supporting the hypothesis that endo-siRNAs mediate TGS in somatic cells. This is consistent with recent reports of \textit{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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REFERENCES


**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 larva grown on either *dpy-11* or *unc-22* dsRNA expressing bacteria were transferred to non-RNAi bacteria. The non-exposed 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 non-exposed 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. 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 calculated by *t*-test; * indicates *p*<0.05 and ** indicates *p*<0.01.

**Figure 2** nrde-3 overexpression causes enhanced RNAi independent of *sago-1*

(A-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 is shown. (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 is shown. 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 calculated by *t*-test; * indicates *p*<0.05 and ** indicates *p*<0.01.

**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 is shown.

**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 calculated by *t*-test; *indicates *p*<0.05; ** indicates *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-
l(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 hour adult (20 °C) worms. For each genotype the average whole body fluorescence (0.1 second exposure) of 10 worms is shown. Error bars represent standard deviation. p-values 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).

**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 is shown. 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 S1. p-values calculated by t-test; ** indicates p<0.01. (B-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 is shown. 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 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 calculated by t-test; * indicates p<0.05 and ** indicates p<0.01.

**Figure 7** rrf-3(pk1426) has distinct endogenous gene regulation and exogenous RNAi response

(A) eri- and nrde3-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 amongst 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 calculated by \( t \)-test; *indicates \( p<0.05 \); ** indicates \( p<0.01 \).
Figure 1

**A**

![Graph showing OD readings for different concentrations.](image)

- OD 1.0
- OD 2.0
- OD 3.0
- OD 4.0

Legend:
- Super Dpy
- Weak Dpy
- WT

**B**

Worms grow up on dpy-11(RNAi) or anc-22(RNAi) plates ➔ Strongly Dpy or Unc 4s transferred to individual OP50 plates ➔ Determine fraction of broods that produce any Dpy or Unc progeny.

**C**

![Graph showing fraction of broods transmitting dpy-11(RNAi).](image)

**D**

![Graph showing fraction of total broods counted.](image)

**E**

![Graph showing fraction of broods transmitting anc-22(RNAi).](image)

**F**

![Graph showing fraction of total broods counted.](image)

**G**

N2 hermaphrodite x sur-5:egfp male

- Fraction of total broods counted (n=8)

**H**

nrde-3(gg66) hermaphrodite x sur-5:egfp male

- Fraction of total broods counted (n=10)

**I**

nrde-3(gg66) hermaphrodite x nrde-3(gg66);sur-5:egfp male

- Fraction of total broods counted (n=8)

**J**

Penetration of Dpy Animals

- OD 680nm of dpy-11(RNAi)

Legend:
- N2
- nrde-3(gg66)
- nrde-3(gg66);ST40XLS-nrde-3
- nrde-3(gg66);+;ST40XLS-nrde-3
Figure 3

(A) Secondary siRNAs

(1) Feed gfp dsRNA

(2) Score for elt-1 knockdown

Endogenous elt-1 targets

B C D

Wildtype eri-1(mg366) nrde-3 (gg66)

E

Fraction with elt-1-related defects

n=514 n=818 n=291 n=439 n=508 n=585

N2 md-3(gg66) eri-1(mg366) md-3(gg66) eri-1(mg366), md-3(gg66) md-3(gg66) + [5NLSmd-3] md-3(gg66) + [5NLSmd-3]

Emb. Lethal

Dev. Defect

WT
Figure 5

D

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<tr>
<td><em>hem-1</em></td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>unc-15</em></td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>dpy-28</em></td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>unc-73</em></td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

E

<table>
<thead>
<tr>
<th>N2</th>
<th>eri-1(mg366)</th>
<th>eri-1(mg366); nrde-3(gg66)</th>
<th>eri-1(mg366); pgl-1(bn101)</th>
<th>eri-1(mg366); nrde-3(gg66); pgl-1(bn101)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>dpy-13(RNAi)</em></td>
<td>*</td>
<td>****</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><em>unc-15(RNAi)</em></td>
<td>****</td>
<td>*</td>
<td>***</td>
<td>*</td>
</tr>
<tr>
<td><em>unc-73(RNAi)</em></td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>
Figure 7

A

B

log_2 fold change relative to N2(0)

ref-3(pk1426)  ref-3(mg373)  eri-1(mg366)  nrde-3(gg66)  dpy-13 mature mRNA  dpy-13 pre-mRNA

**  **  

-6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6
Table 1 *eri-1* mutant partially requires *nrde-3* for enhanced RNAi

<table>
<thead>
<tr>
<th>RNAi Target</th>
<th>Operon or Homologous Sequences</th>
<th>N2</th>
<th><em>nrde-3 (gg66)</em></th>
<th><em>eri-1 (mg366)</em></th>
<th><em>eri-1 (mg366); nrde-3 (gg66)</em></th>
<th>*eri-1 (mg366); nrde-3 (gg66); [<em>NLS-nrde-3]</em></th>
<th><em>eri-1 (mg366); nrde-3 (gg66); [SV40-NLS-nrde-3]</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>lir-1</em></td>
<td>Operon</td>
<td>+ +</td>
<td>-</td>
<td>+ + + + +</td>
<td>-</td>
<td>-</td>
<td>+ + + + +</td>
</tr>
<tr>
<td><em>dpy-13</em></td>
<td>91 Homologs</td>
<td>+</td>
<td>-</td>
<td>+ + + + +</td>
<td>+</td>
<td>+</td>
<td>+ + + + +</td>
</tr>
<tr>
<td><em>ifc-2</em></td>
<td>3 Homologs</td>
<td>+</td>
<td>-</td>
<td>+ + + + +</td>
<td>+</td>
<td>+</td>
<td>+ + + + +</td>
</tr>
<tr>
<td><em>unc-15</em></td>
<td>0 Homologs</td>
<td>-</td>
<td>-</td>
<td>+ + + + +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ + + + +</td>
</tr>
<tr>
<td><em>hmr-1</em></td>
<td>1 homolog</td>
<td>+</td>
<td>-</td>
<td>+ + + + +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ + + + +</td>
</tr>
<tr>
<td><em>dpy-28</em></td>
<td>0 homologs</td>
<td>-</td>
<td>-</td>
<td>+ + + + +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ + + + +</td>
</tr>
<tr>
<td><em>unc-73</em></td>
<td>0 homologs</td>
<td>-</td>
<td>-</td>
<td>+ + + + +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ + + + +</td>
</tr>
</tbody>
</table>

The severity of RNAi phenotypes, from ‘-’ to ‘+ + + + +’, based on expressivity described in Table S1 (illustrated for select mutants in Fig. 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 (highlighted in yellow) is most dependent on *nrde-3*. The number of homologous sequences was determined by the number 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.*
**Table 2 rrf-3(pk1426) has unique dependence on nrde-3 for enhanced RNAi**

<table>
<thead>
<tr>
<th>RNAi Target</th>
<th>N2</th>
<th>nrde-3 (gg66)</th>
<th>rrf-3 (mg373)</th>
<th>rrf-3 (mg373); nrde-3 (gg66)</th>
<th>rrf-3 (pk2042); nrde-3 (gg66)</th>
<th>rrf-3 (pk1426); nrde-3 (gg66)</th>
<th>rrf-3 (pk1426) + [WT rrf-3]</th>
<th>rrf-3 (mg373/pk1426) het</th>
<th>rrf-3 (mg373/pk1426); nrde-3 (gg66) het</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>lir-1</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>dpy-13</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>ifc-2</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>hmr-1</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>unc-15</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>dpy-28</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>unc-73</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The severity of RNAi phenotypes as described in **Table 1** is based on penetrances measurements presented in **Table S14**. 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 the other Eri mutants, while the enhanced RNAi response targeting genes most dependent on *pgl-1* (highlighted in green) holds the same pattern of RNAi responses as the other Eri mutants for the *mg373* and *pk2042* alleles, but not the *pk1426* allele.