Acute and inherited piRNA-mediated silencing in a rde-3 ribonucleotidyltransferase mutant

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Abstract

We recently developed a piRNA-based silencing assay (piRNAi) to study small-RNA mediated epigenetic silencing: acute gene silencing is induced by synthetic piRNAs expressed from extra-chromosomal array and transgenerational inheritance can be quantified after array loss. The assay allows inheritance assays by injecting piRNAs directly into mutant animals and targeting endogenous genes (e.g., him-5 and him-8) with obvious phenotypes (increased male frequency). Here we demonstrate the piRNAi assay by quantifying acute and inherited silencing in the ribonucleotidyltransferase rde-3 (ne3370) mutant. In the absence of rde-3, acute silencing was reduced but still detectable, whereas inherited silencing was abolished.

Figure 1. Testing acute and inherited silencing in rde-3 mutants.

A. Schematic of synthetic piRNAi construct. B. Synthetic piRNAs target sites in him-5. C. Quantification of males frequency after piRNAi against him-5 in N2 wild type and rde-3(ne3370) animals; N2: N=3, rde-3: N=3 (him-5 piRNAi and neg. control); Statistics: Mann-Whitney one-tailed test, N2 vs control (P = 0.05), rde-3 vs control (P = 0.05), N2 vs rde-3 (P = 0.05). D. Inherited him-5 piRNAi silencing in N2 and rde-3 animals; N = 3 all conditions. Negative control: non-targeting piRNAs. The bar above the graph indicates the presence ("yes"), mixed generation ("mixed"), and absence ("no") of a Pmyo-2::mCherry marked extra-chromosomal array expressing synthetic piRNAs targeting him-5. Each data point is a biologically
C. elegans is a convenient model for studying small RNA-mediated inherited silencing due to the animal's short generation time (three days) and the ability to identify molecular pathways in genetic screens (Burton et al., 2011; Buckley et al., 2012; Spracklin et al., 2017). Epigenetic silencing of an endogenous gene is often done by targeting a temperature-sensitive gain-of-function allele of oma-1(zu405) with dsRNA, and silencing persists for up to three generations (Alcazar et al., 2008). For a visual read-out, single-copy transgenes with GFP expression in the germline (Zeiser et al., 2011; Frøkjær-Jensen et al., 2012; Nance and Frøkjær-Jensen, 2019) have been engineered to contain endogenous piRNA binding sites in the 3' UTR (Ashe et al., 2012; Lee et al., 2012; Shirayama et al., 2012; Bagijn et al., 2012). For transgenes, piRNA-induced silencing persists longer and sometimes indefinitely. Genetic factors required for small RNA-mediated inherited silencing have primarily been identified by crossing silenced piRNA gfp sensor strains into mutant genetic backgrounds (Ashe et al., 2012; Lee et al., 2012; Shirayama et al., 2012; Luteijn et al., 2012). However, introducing mutations by genetic crosses raises several concerns. First, there are several examples of mating causing changes in epigenetic inheritance. For example, the lack of transgene pairing during meiosis after a cross can lead to permanent transgene silencing via PRG-1-dependent mechanisms (Leopold et al., 2015), and mating can induce multigenerational silencing inherited for over 300 generations (Devanapally et al., 2021). Moreover, Dodson and Kennedy (2019) characterized a transgenerational disconnect between the genotype and phenotype (sensitivity to exogenous RNAi) of meg-3/4 mutants for more than seven generations after a genetic cross. Second, crosses frequently require molecular genotyping, which makes it cumbersome to perform many biological replicates. Third, there are some concerns about using transgenes as a proxy for endogenous gene silencing. For example, most piRNA sensor strains include synthetic piRNA binding sites in the 3' UTR (Ashe et al., 2012; Lee et al., 2012; Shirayama et al., 2012; Bagijn et al., 2012), but endogenous genes are resistant to piRNA silencing when targeting their 3' UTRs (Priyadarshini et al., 2022; Wu et al., 2022). Moreover, transgene insertion site (Frøkjær-Jensen et al., 2014), non-coding DNA structures (Frøkjær-Jensen et al., 2016), coding sequence (Fielmich et al., 2018; Aljohani et al., 2020), and transgene structure (El Mouridi et al., 2022) can influence epigenetic silencing. These observations suggest that transgenes may not fully recapitulate the balance between silencing foreign DNA and protecting endogenous gene expression (Frøkjær-Jensen, 2019). Finally, distinguishing between silencing initiation and maintenance phases is complicated using genetic crosses. Experiments require crossing mutant alleles to sensor strains, de-repress silencing, and outcrossing mutations to monitor de novo establishment of silencing (Shirayama et al., 2012).

We recently developed a method called piRNA interference (piRNAi) that can efficiently silence both transgenes and endogenous genes by expressing synthetic piRNAs from arrays generated by injection (Priyadarshini et al., 2022; Gajic et al., 2022). Using piRNAi, we identified two endogenous targets, him-5 and him-8, that inherit silencing for three and six generations, respectively (Priyadarshini et al., 2022). him-5 (Meneely et al., 2012) and him-8 (Phillips et al., 2005) mutants are generally healthy but have a similar loss-of-function phenotype that is easy to score (~35% males in the population). We reasoned that piRNA-mediated silencing of him-5 or him-8 might be useful as a tool to directly test the role of gene mutations in initiating and maintaining inherited silencing. Here, we show that piRNAi can be used to test acute and inherited silencing in rde-3, a gene also known as mut-2 (Davis et al., 2022).

rde-3 is required for Tc1 transposon silencing in the germline (Collins et al., 1987) and RNA interference (RNAi) (Chen et al., 2005). In vitro, RDE-3 has ribonucleotidyltransferase activity (Preston et al., 2019) and, in vivo, rde-3 is required for the addition of non-templated poly (UG) tails to the 3' end of mRNAs targeted by RNAi and repressed transposons (Shukla et al., 2020). pUGylated mRNAs are templates for RNA-dependent RNA polymerases (RdRPs), resulting in small RNA amplification and inherited silencing (Shukla et al., 2020). RDE-3 is required to maintain the silencing of piRNA transgene sensors (Lee et al., 2012; Shirayama et al., 2012; Bagijn et al., 2012). However, the role of rde-3 in initiating silencing is unclear; re-introducing RDE-3 led to rapid re-silencing of a gfp::cdk-1 transgene, but variable and incomplete re-silencing of a gfp::csr-1 transgene (Shirayama et al., 2012). Also, rde-3 mutants are insensitive to the injection of dsRNA targeting unc-22 but are sensitive to dsRNA expressed from transgenes (Chen et al., 2005). These conflicting results could be caused by differences between transgenes, the effects of mating, or the levels of the primary silencing dsRNA. We, therefore, decided to use piRNAi to test the role of RDE-3 in the initiation and maintenance of silencing of an endogenous gene. We targeted him-5 with six synthetic guide piRNAs (sg-piRNAs) (Fig. 1A-B) in wild-type (N2) animals and rde-3(ne3370) mutants. rde-3 is a mutant strain and is relatively unhealthy, with a small brood size and infrequently produces males. To account for an elevated male frequency in the mutant population, we generated transgenic rde-3 animals with non-targeting sg-piRNAs as a control. Targeting him-5 with piRNAi resulted in an increased frequency of males in N2 animals but a significantly lower male frequency in rde-3 animals (30 ± 1.9% vs 7.6 ± 1.3%, P = 0.05, mean ± SEM) (Fig. 1C). However, male frequency in rde-3...
animals was significantly increased compared to negative controls (7.6 ± 1.3% vs 0.2 ± 0.2%, P = 0.05, mean ± SEM). We tested the role of RDE-3 in maintaining silencing by losing the piRNAi trigger (the piRNAi arrays with a Pmyo-2::mCherry fluorescent marker) and scoring male frequency in the following generations. In wild-type animals, the male frequency remains elevated for at least three generations after the primary piRNAs targeting him-5 are lost (Fig. 1D), consistent with prior observations (Priyadarshini et al., 2022). In contrast, we could not detect an inherited elevation of male frequency in rde-3 mutants (Fig. 1D). The initial frequency of males was relatively low in rde-3 animals, which limits our ability to make strong conclusions. However, our results support a model where primary piRNAs can post-transcriptionally silence a target transcript (him-5 mRNA) at reduced efficiency, but rde-3 is required for small RNA amplification and transcriptional silencing. These results support the observations by Chen et al. (2005) that persistently high somatic expression of dsRNA targeting unc-22 from a plasmid causes a phenotype. In contrast, a single transient injection of in vitro transcribed dsRNA is inefficient. Presumably, RDE-3 amplifies the primary trigger by generating him-5 pUG RNA templates for RdRP-mediated 22G amplification; these secondary RNAs are subsequently used to set up transcriptional silencing via repressive chromatin marks deposited by the hrde-1 dependent nuclear RNAi pathway.

More generally, we demonstrate that piRNAi can be used as a tool to directly test genetic factors required for acute and inherited silencing of endogenous genes. Elevated male frequency (induced by targeting him-5 or him-8) is easy to score in various genetic backgrounds and allows distinguishing between silencing initiation and maintenance of endogenous genes.

Methods

Transgenesis. Transgenic animals with piRNAi extrachromosomal arrays were generated according to standard injection protocols (Mello et al., 1991). The injection mix for all experiments consisted of ~15-20 ng/µl of synthetic dsDNA piRNA fragments (Twist Bioscience), 12.5 ng/µl of a plasmid encoding hygromycin resistance (pCFJ782), and 2 ng/µl of a fluorescent co-injection marker Pmyo-2::mCherry (pCFJ90). The total concentration of the injection mix was adjusted to 100 ng/µl with a 1kb DNA ladder (1 kb Plus DNA Ladder, catalog no. 10787018, Life Technologies). This mix was injected into young adult hermaphrodite animals and allowed to recover on standard NGM plates seed with OP50 bacteria. 36-48 hours post-injection, 500 µl of 4 mg/ml stock of Hygromycin solution (Gold Biotechnology, catalog no. H-270-1) was topically added to the bacterial lawn of injection plates to select for transgenic (F1) progeny. A single healthy transgenic F2 adult was picked from each plate to generate a clonal strain, and pharyngeal mCherry fluorescence was visually confirmed.

Quantification of male frequency. Quantification of male frequency was performed as previously reported by (Priyadarshini et al., 2022). Briefly, six virgin L4 hermaphrodites were picked to freshly seeded NGM plates with hygromycin selection to select for the piRNAi array. The frequency of males was determined using a dissection microscope and by visual inspection of 100 adult animals on plates incubated on ice for 30 minutes to immobilize animals.

Inherited silencing assay. Six virgin L4 animals were picked to non-selective NGM plates to obtain a mixed progeny population with and without the piRNAi array. Males were not quantified in this mixed population; however, L4 animals carrying the sg-piRNAs were propagated in parallel, and their progeny were scored for males (G0). In the following generation, non-transgenic L4 animals were carefully picked from the mixed population based on the absence of pharyngeal mCherry expression (a marker for the piRNAi array). The progeny of these animals was quantified for male frequency (G1). Male frequency was quantified in all following generations by picking L4s until the male frequency was below 1%.

Data quantification and statistics. Independently generated transgenic animals were treated as biological replicates. piRNA-mediated silencing is stochastic (i.e., most strains show robust silencing, but some strains are not silenced at all), and the data do not follow a normal distribution. We performed statistical tests using one-side parametric Mann-Whitney tests to account for this.

Software. Statistical analysis was performed with GraphPad Prism (v 9.4.1), figures were generated with Adobe Illustrator (v 26.4.1), and the manuscript was written with Microsoft Word (v 16.63.1).

Reagents

List of strains, plasmids, and piRNAi fragments used in this study.

Strains

N2 Standard wildtype strain (Brenner 1974)
WM286 rde-3(ne3370) I

Plasmids

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piRNAi fragments

T288 5’- (six targeting piRNAs in upper-case):

cgcgtcgacgcgtagtcacaaactacataaaaaaggtgaaacattgcgtagggtagataatagttctgattttgcgtttgaatgacatcctctgtttcatgatctctgctctgtcttcatgcacagagctttactatctttgttctgtcttctttttgcgtgtttttcttcatgtctaccttttttttcctttgtgtttttcttttcttttctttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
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