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RDE-1 slicer activity is required only for passenger-strand cleavage during RNAi in *Caenorhabditis elegans*

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RNA interference (RNAi) is a process in which double-stranded RNA is cleaved into small interfering RNAs (siRNAs) that induce the destruction of homologous single-stranded mRNAs. Argonaute proteins are essential components of this silencing process; they bind siRNAs directly and can cleave RNA targets using a conserved RNase H motif. In *Caenorhabditis elegans*, the Argonaute protein RDE-1 has a central role in RNAi. In animals lacking RDE-1, the introduction of double-stranded RNA does not trigger any detectable level of RNAi. Here we show that RNase H activity of RDE-1 is required only for efficient removal of the passenger strand of the siRNA duplex and not for triggering the silencing response at the target-mRNA level. These results uncouple the role of the RDE-1 RNase H activity in small RNA maturation from its role in target-mRNA silencing *in vivo*.

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In the process of RNAi, long double-stranded RNA (dsRNA) induces the destruction of homologous single-stranded mRNAs[1]. The long dsRNA is processed into small RNA duplexes by a Dicer protein family member[2,3]. The resulting siRNAs are subsequently bound by Argonaute proteins, which are essential components of most silencing processes involving small RNAs[4,5]. In RNAi, Argonaute proteins not only bind siRNAs directly but can also cleave target RNA[6–11]. The latter is achieved through RNase H, or 'slicer' activity within the PIWI domain, which is a common feature of Argonaute proteins[7,12–18]. Three conserved amino acids, two aspartates and a histidine, have been identified as catalytic residues of the RNase H activity and form the so-called DDH motif[10,19]. RNase H–mediated target cleavage is essential for RNAi in mammals and flies, and mutation of any residue within the RNase H catalytic triad leads to the loss of cleavage activity and silencing[7,17,20,21]. In many organisms, the RNase H activity also has a function in siRNA maturation. Following processing by Dicer, the siRNA is loaded in duplex form into the Argonaute protein. The passenger strand has to be subsequently removed to make the guide strand accessible for the target. The RNase H motif of Argonaute proteins has been shown to be important for passenger-strand cleavage, a process required for removal of this strand[18,21–27]. In cases where RNase H–mediated cleavage is prevented by mismatches within the duplex, as is true for many microRNAs (miRNAs), or upon amino acid substitutions in the catalytic triad of the DDH motif of the Argonaute protein, an unclear mechanism removes the passenger strand[25].

In *C. elegans*, several members of the Argonaute protein family are required to achieve efficient RNAi. RDE-1 binds primary siRNAs derived from the double-stranded trigger RNA. This primary Argonaute complex triggers an amplification machinery containing an RNA-directed RNA polymerase to generate secondary siRNAs[28–30]. The secondary siRNAs are bound by a set of redundant secondary Argonaute proteins (SAGOs), which are also involved in target degradation[31]. Of the Argonaute proteins implicated in RNAi, only RDE-1 and CSR-1 seem to have a catalytically active RNase H domain. Many other Argonaute proteins in *C. elegans* also carry the DDH motif, implying that other pathways require slicer activity as well[31] (**Supplementary Fig. 1** online).

In vitro analysis demonstrated that siRNA-mediated mRNA-cleavage activity in *C. elegans* extracts is mediated mainly by CSR-1 and not by RDE-1 (ref. 32). We therefore set out to analyze the role of the DDH motif in RDE-1, using an *in vivo* approach in which we complemented an *rde-1*–defective strain with wild-type and mutant versions of *rde-1*. We find that RDE-1 with mutations in the DDH motif is defective in passenger-strand turnover. These defects can be bypassed by providing miRNA-like RNAi triggers. The integrity of the catalytic triad becomes totally dispensable for effective RNAi in these cases. The functional DDH motif in RDE-1 is thus only required for siRNA maturation, but not for target-mRNA cleavage.

RESULTS

DDH motif mutants are only partially RNAi deficient

In RDE-1, the *C. elegans* Argonaute protein that binds primary siRNAs, the residues of the DDH motif are present at conserved positions, suggesting that the protein carries RNase H activity (**Supplementary Fig. 1**). To analyze the importance of an intact DDH motif in RDE-1 for RNAi, we replaced the conserved residues Asp718, Asp801 and His974 with alanines in a hemagglutinin epitope (HA)-tagged version of RDE-1. The resulting mutant DDH motifs are abbreviated DDA (H974A), DAH (D801A), ADH (D718A) and AAA (D718A, D801A, H974A). We tested the ability of the wild-type and

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mutant versions to establish RNAi *in vivo* in a strain carrying a nonsense mutation in the endogenous *rde-1* gene and expressing a single primary siRNA (**Fig. 1a**). This single siRNA, termed 22siRNA, targets the mRNA of the endogenous *unc-22* gene and induces a twitching phenotype in an *rde-1*–dependent manner[30].

No phenotype was observed in the absence of a rescuing version of RDE-1, proving that RDE-1 is absolutely required for RNAi, and its functions cannot be replaced by other Argonaute proteins (**Fig. 1b**). Wild-type HA–RDE-1 readily rescues the *rde-1* stop mutation, leading to *unc-22* silencing and a severe twitching phenotype. Mutation of the DDH residues within HA–RDE-1 results in a decrease in silencing, ranging from a mild decrease in the case of HA–RDE-1 DAH to a complete loss of silencing in the cases of HA–RDE-1 ADH and HA–RDE-1 AAA (**Fig. 1b**). In human AGO2, *Drosophila melanogaster* AGO2, *Neurospora crassa* QDE-2 and *Schizosccharomyces pombe* Ago1, single amino acid changes of any residue within the RNase H motif led to a complete loss of target-cleavage activity and silencing[7,16,18,19,23]. In contrast to these findings, we show that HA–RDE-1 DAH and HA–RDE-1 DDA mutants are still RNAi proficient, although at a lower level than wild-type HA–RDE-1 (**Fig. 1b**). This implies that the RDE-1 DDH motif is not required for target silencing once it has been loaded with a single-stranded siRNA.

Passenger-strand turnover is deficient in DDH motif mutants

To test whether the mutations in the RDE-1 DDH motif cause deficiencies in passenger-strand turnover, we replaced the fully matching 22siRNA duplex with miRNA-like siRNA duplexes containing a 1-nucleotide (nt) or 3-nt mismatch in the passenger strand (ps mm10, ps mm9-11, ps mm14 and ps mm17; **Fig. 1a**). The passenger strands of these siRNAs cannot be cleaved by RDE-1 because of the mismatch but should instead be removed by an alternative mechanism, possibly analogous to the removal of miRNA* strands from miRNA duplexes[25]. The mismatched siRNA duplexes restored silencing by HA–RDE-1 ADH, some up to wild-type RDE-1 levels, suggesting that the prime defect of RDE-1 ADH lies in passenger-strand removal (**Fig. 1b**,**c**).

Consistent with the phenotypic observation of the defects in passenger-strand turnover, we detected both strands of the 22siRNA in lines expressing the HA–RDE-1 DDH mutants by northern blotting (**Fig. 2a**). In HA–RDE-1 wild type–expressing nematodes, and when the 22siRNA was replaced with an siRNA containing mismatches in the duplex (ps mm10, ps mm9-11), only the guide strand was detected, a result that is in accordance with the observation of active silencing (**Fig. 2a**). Note that, although the ps mm10 construct produces low levels of mature siRNA that are hard to detect in total-RNA preparations, isolation of small RNAs bound to HA–RDE-1 clearly contain the mature guide strand derived from this construct. The reason behind this low expression may either be lower transcription rates from the transgene or lower stability of the precursor. Quantification experiments showed that the passenger strand is downregulated by a factor of more than 100 compared to the guide strand in these lines (**Supplementary Fig. 2** online).

In RDE-1 AAA mutant nematodes, both strands of the 22siRNA cofractionate with the HA–RDE-1 protein in FPLC analyses (**Supplementary Fig. 3** online). To show that RDE-1 DDH mutants indeed bind the siRNA duplex, we immunoprecipitated the hemagglutinin-tagged versions of RDE-1 and detected both strands of the 22siRNA by denaturing and native northern blotting (**Fig. 2b**,**c** and **Supplementary Fig. 4** online).

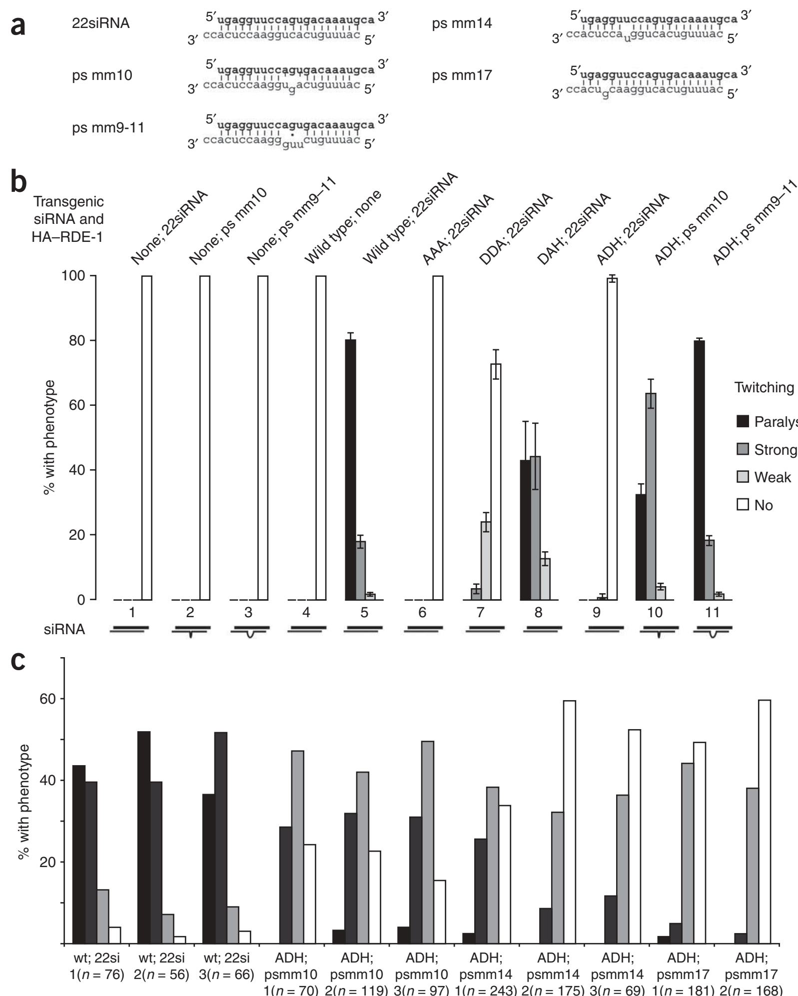

Figure 1 Mismatched siRNA duplexes bypass mutations in the RDE-1 DDH motif. (**a**) Structure and sequence of the transgenic small RNA duplexes used in this study. The guide strand is shown in bold. ps mm14 and ps mm17 constructs induce mismatches across the seed sequence of the mature siRNA. (**b**) RDE-1 DDH mutants show various degrees of reduced silencing. The phenotype of 100 adult nematodes from each transgenic line was assessed in three independent trials, and a score was given for penetrance. Phenotypical categories are paralyzed (strongest, black), strong twitching (dark gray), weak twitching (light gray) and nontwitching (white). All lines contain a stop mutation in the endogenous *rde-1* gene. The guide strand in the cartoon representation of the siRNA duplex is in bold. Error bars indicate s.d. Arithmetic mean and s.d. of each category are also given in **Supplementary Table 1** online. (**c**) ps mm14 and ps mm17 transgenic lines induce *unc-22* RNAi. The efficiency of these constructs is, however, less than the ps mm10 and ps mm9-11 constructs. Several independent transgenic lines were tested, as indicated with numbers 1–3. Details are the same as in **Figure 1b**.

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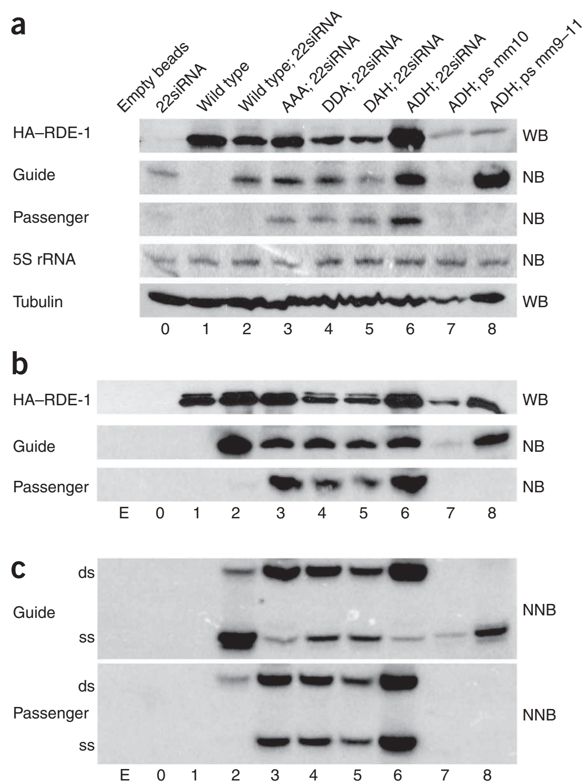

Figure 2 In RDE-1 DDH mutants, the passenger strand accumulates and remains associated with the guide strand and RDE-1. (**a**) Hemagglutinin (HA)-tagged wild-type and mutant versions of RDE-1 (HA–RDE-1) and guide and passenger strands of transgenic small RNAs (22siRNA, ps mm10, ps mm9-11) were detected in total nematode extract or RNA by western blotting (WB) or northern blotting (NB), respectively. 5S ribosomal RNA and α-tubulin were detected by NB and WB, respectively, and used as loading controls. (**b**,**c**) HA–RDE-1 wild-type and mutant versions were immunoprecipitated and detected by western blotting. RNA was isolated from immunoprecipitated protein complexes and siRNA guide and passenger strands were detected by denaturing NB (**b**) or native NB (NNB, **c**). Empty beads were used as a control (lane E).

Only the guide strand was found to be bound to wild-type HA–RDE-1 (**Fig. 2b**,**c**, lane 2, and **Supplementary Fig. 4**). Both strands of the 22siRNA duplex co-immunoprecipitated with the DDH mutant versions and were mostly present in duplex form on nondenaturing gels (**Fig. 2b**,**c** and **Supplementary Fig. 4**). The siRNA duplexes detected were present *in vivo* and not formed during RNA preparation (**Supplementary Fig. 5** online). When the 22siRNA was replaced with an siRNA containing mismatches in the duplex, only the guide strand was bound to HA–RDE-1 ADH (**Fig. 2b**,**c** and **Supplementary Fig. 4**). The levels of mismatched siRNAs in the immunoprecipitation were lower than the 22siRNA because small RNAs expressed from mismatched precursors feed mainly into the miRNA pathway, and only minor amounts are bound by RDE-1 (ref. 33). Placing mismatches away from the center has been shown to have little effect on loading preference into different pathways[33], in contrast to what is observed in *D. melanogaster*[34]. The populations of siRNAs derived from mismatched precursors (ps mm10, ps mm9-11) that are bound by ALG-1 and ALG-2 have, however, no effect on RNAi, as these lines are completely RNAi deficient in an *rde-1* mutant background (**Fig. 1b**).

Thus, the defects in passenger-strand turnover in the RDE-1 DDH mutants can be bypassed with siRNA duplexes containing mismatches, as the passenger strand of these duplexes is removed by an RNase H–independent mechanism.

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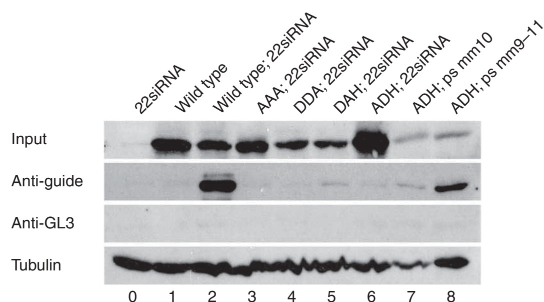

Figure 3 Passenger-strand turnover is required for target recognition. HA–RDE-1–small RNA complexes were captured with biotinylated targets complementary to the small RNAs and pulled down using streptavidin beads. HA–RDE-1 wild-type and DDH mutant versions were detected by western blotting. Capture oligonucleotides are antisense to and fully matching an unrelated luciferase GL3 sequence or the 22siRNA. α-tubulin was detected by western blotting and used as loading control.

RDE-1–target interaction is impaired in DDH motif mutants

To assess whether RDE-1 DDH mutant–bound siRNAs are single stranded and capable of interacting with single-stranded targets, we performed Argonaute protein–capture assays with the hemagglutinin-tagged versions of RDE-1 (**Fig. 3**). Nematode extracts were incubated with a biotinylated target complementary to the siRNAs, and HA–RDE-1-target complexes were pulled down. Wild-type HA–RDE-1 bound efficiently to the target and precipitated readily using this method (**Fig. 3**). In contrast, precipitation of the HA–RDE-1 DDH mutants was prevented, as we detected relatively little Argonaute protein compared to the input (**Fig. 3**). Target recognition was somewhat restored when HA–RDE-1 ADH was provided with mismatched siRNA duplexes (**Fig. 3**). These results support the finding that the silencing defects in the RDE-1 DDH mutants are caused by passenger strand–turnover defects that interfere with guide-strand accessibility for the target.

DISCUSSION

RDE-1 slices the passenger strand

In fly and mammalian RNAi, target cleavage by the (primary) Argonaute protein is sufficient for silencing[7,17]. Here we have shown that the slicer function of RDE-1 is used for passenger-strand turnover and is not required for target-mRNA cleavage. These results were obtained by expressing wild-type or mutant versions of the RDE-1 protein in an otherwise *rde-1* mutant background. This background is totally RNAi defective in the absence of rescuing RDE-1 protein, ensuring that all the phenotypic effects observed can be attributed to the re-introduced versions of RDE-1. As a consequence, expression levels of RDE-1 differ in the various lines obtained. However, we have analyzed at least two independent lines per construct and have not observed a correlation between the expression level of RDE-1 and its rescuing activity (F.A.S., R.F.K. and T.S., unpublished results). This indicates that the required amount of RDE-1 to obtain rescuing activity is relatively low, at least below that obtained in our lowest-expressing lines.

Together with previously published findings[30,33,35,36], our results show that the main role of RDE-1 in RNAi is three-fold: (i) recognition of potential siRNAs based on base-pairing properties of precursor

RNAs; (ii) siRNA maturation; and (iii) flagging mRNAs for recognition by an RNA-dependent RNA polymerase (RdRP) complex. Under this scenario, efficient target-mRNA cleavage might even interfere with RdRP initiation and secondary siRNA generation, and there may therefore be evolutionary pressure against efficient RDE-1 slicer activity towards its targets. The fact that we have previously cloned secondary siRNAs that span the presumed cleavage site on 22siRNA-targeted *unc-22* mRNA, which is only possible if secondary siRNAs are generated on noncleaved templates, supports this hypothesis[30]. In addition, target slicer activity in total *C. elegans* extracts is mainly attributed to an Argonaute protein other than RDE-1 (ref. 32); however, as RDE-1 preferentially loads with perfectly matching siRNA duplexes[33], RNase H activity remains an essential feature for RDE-1 to eliminate the passenger strand so that the guide strand becomes available for target-mRNA selection. To more directly study this reaction, an *in vitro* system will need to be developed. Unfortunately, initial attempts at this in the context of the present study have not been successful. Notably, a similar explanation for the retainment of the inefficient catalytic activity of the fly AGO1 protein has been proposed[37], suggesting that an exclusive requirement of the RNase H domain for passenger-strand removal may be a common feature among Argonaute proteins.

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RDE-1 loading through mismatched duplexes

In our experiments, we bypassed catalytically inactive RDE-1 protein by introducing mismatched siRNA duplexes into cells. As differences in the efficiency between these various constructs may be introduced at any step between the *in vivo* transcription of the transgene through to eventual mRNA degradation, it is hard to derive strong conclusions about the mechanism of passenger-strand removal in RDE-1 specifically. However, we do observe a trend in which mismatches introduced in the central region of the siRNA duplex are more effective in triggering RNAi than those introduced near the ends of the duplex in the context of our *rde-1* mutants. This is true also for constructs in which the pairing of the seed sequence of the mature siRNA is impaired. siRNAs containing 3-nt mismatches do not induce RNAi at all (not shown), and 1-nt mismatches at the seed are less effective than those in the center. This could reflect a strong preference of RDE-1 to bind perfectly base-paired siRNA duplexes, and the seed region especially could be an important factor in that selection process. Hence, although passenger-strand removal should in theory be more effective when the seed is mispaired, the net effect in the case of RDE-1 would be a loss of duplex binding leading to a less effective RNAi response.

RNA degradation during RNAi in *C. elegans*

The finding that RDE-1 is a limited slicer and has no role in target-RNA turnover leaves open the question of how target RNA is degraded. In flies and mammals, the strong slicer activity in the equivalent Ago2 is sufficient to downregulate target-RNA levels. *Caenorhabditis elegans*, however, requires an amplification machinery for efficient RNAi. The secondary siRNAs generated in this amplification step are bound by the secondary Argonaute proteins and can silence *in trans* to degrade mRNA molecules that are not complementary to primary siRNAs[28–31]. Both the secondary Argonaute proteins and the production of secondary siRNAs are required for silencing, implying that target degradation occurs mainly via secondary Argonaute protein complexes[29,31,32]. Notably, many secondary Argonaute proteins lack the conserved DDH motif, suggesting that a significant fraction of the secondary siRNAs may silence their targets by a mechanism other than RNase H–mediated cleavage[31]. The exact mechanism by which these cleavage-incompetent secondary Argonaute complexes induce target degradation remains to be investigated.

METHODS

Plasmids, nematode strains and transgenic lines. The alleles used in this study were *rde-1*(pk3301), 22siRNA(*pkIs2289*)[30], ps mm10(*pkIs2450*), ps mm9-11(*pkIs2446*)[32] and unc-119 (*ed3*). Mutations in the DDH motif of HA–RDE-1 were introduced into the vector pHIT-1 (ref. 34) using the QuickChange site-directed mutagenesis kit (Stratagene). Transgenic small RNAs (22siRNA, ps mm10, ps mm9-11) have been described[30,32]. We generated transgenic lines carrying an HA–RDE-1 mutant and one of the transgenic small RNAs using microinjection or standard ballistic transformation. Newly generated alleles for this study were *rde-1–HA(pkIs2449)*, *rde-1*–HA D718A D801A H974A;22siRNA(*pkIs2461*), *rde-1*–HA H974A;22siRNA(*pkIs2424*), *rde-1*–HA D801A;22siRNA(*pkIs2421*), *rde-1*–HA D718A;22siRNA(*pkIs2460*), *rde-1*–HA D718A;ps mm10(*pkIs2464*), *rde-1*–HA D718A;ps mm9-11 (*pkIs2458*). The small RNAs from the various constructs target nucleotides 11925–11946 of the *unc-22* spliced sequence. Nematodes were cultured according to standard procedures, and the *unc-22* twitching phenotype was determined by eye.

Protein assays. FPLC experiments, capture assays, RNA immunoprecipitations and western blotting were performed as described[32]. The 2′-O-methyl oligonucleotide sequences used in the capture assays were 5′-UUUC-X-AUCAC-3′, X being the sequence antisense to 22siRNA, ps mm10 and ps mm9-11, or the luciferase sequence 5′-UCGAAGUACUCAGCGUAAGUU-3′. α-tubulin was detected using an anti–α-tubulin antibody (Sigma).

RNA analysis. RNA from nematode extracts, FPLC fractions and RNA immunoprecipitations was isolated using Trizol LS (Invitrogen) according to the manufacturers protocol. No trap oligonucleotide was added during the isolation of siRNA duplexes. To distinguish between duplexes formed *in vivo* and *in vitro*, siRNA duplexes were heat denatured and re-isolated using Trizol LS (**Supplementary Fig. 4**).

We carried out primer-extension reactions and denaturing northern blot analyses as described[32]. Specific DNA 18-mer sequences used for primer extensions are 5′–TGCATTTGTCACTGGAAC-3′ for the guide strand and 5′-GGTGAGGTTCCAGTGAC-3′ for the passenger strand of 22siRNA.

For nondenaturing northern blot analyses, RNA was separated on 20% (w/v) polyacrylamide Tris-borate EDTA gels at 4 °C. After running, the gels were heated to 80 °C for 15 min followed by a brief incubation on ice and standard blotting procedures. Transgenic small RNAs and 5S ribosomal RNA were detected using DNA probes.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

F.A.S., T.S. and R.F.K. designed the experiments; F.A.S., K.L.O., S.W.H. and T.S. performed the experiments; F.A.S. and R.F.K. wrote the paper.

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