Argonaute protein PIWI controls mobilization of retrotransposons in the Drosophila male germline

Alla I. Kalmykova¹, Mikhail S. Klenov¹,² and Vladimir A. Gvozdev¹,²,*

¹Institute of Molecular Genetics RAS, Kurchatov square 2, 123 182 Moscow, Russia and ²Department of Molecular Biology, Moscow State University, Moscow, Russia

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ABSTRACT

Proteins of the Argonaute family have been identified as key components of RNA interference (RNAi) pathway. RNAi-related mechanisms are implicated in the regulation of gene expression and repression of transposable elements in eukaryotes. The piwi gene encoding protein of the Drosophila Argonaute family was shown to be required for the germ stem cells maintenance. Here, we show that piwi is involved in silencing of LTR retrotransposons in testes. piwi mutations led to derepression of endogenous retrotransposon copia as well as to upregulation of the reporter gene driven by copia LTR. piwi mutation causes accumulation of retrotransponson mdg1 transcripts at the apical tip of testes, including germinal proliferative center where PIWI protein was shown to be expressed. We applied inverse PCR approach to detect the newly arisen insertions of the mdg1 retrotransponson in the progeny of individual piwi mutant males. Owing to piwi mutation a high rate of mdg1 transpositions was revealed. Thus, piwi is involved in the silencing of retrotransposons in the precursors of male gametes. Our results provide the first evidence that protein of the Argonaute family prevents retrotranspositions. It is supposed that the disturbance of RNA silencing system in germinal cells might cause transposition burst.

INTRODUCTION

Gene silencing by homologous double-stranded RNA (dsRNA) is termed RNA interference (RNAi) (1). Long dsRNA is processed by Dicer enzymes into small interfering RNAs (siRNAs) that target repression of homologous sequences (2–4). siRNAs guide sequence-specific cleavage activity of the RNA-induced silencing complex (RISC) to complementary mRNA (4–6) or induce chromatin-based events that result in transcriptional silencing (7–10). RNAi plays a role in defense against transposable elements and viruses, which can generate dsRNA (11–13). Antisense transcription and generation of hairpin structures in nascent transcripts are considered as a source of dsRNA of transposable elements in cells (14,15). Some RNAi-deficient mutants show a relief of transposon silencing in Caenorhabditis elegans (13,15–17). Mutations in genes that impaired RNAi cause an increase of retrotransposon transcript abundance in Trypanosoma (18), Drosophila (19–22) and mouse (23). Mobilization of retrotransposon was demonstrated in Chlamydomonas as a result of mutation in RNA-helicase encoding gene involved in the post-transcriptional silencing of transgenes (24). Silencing mediated by natural dsRNA has been implicated in the essential biological processes, including functioning of centromers in Schizosaccharomyces pombe (8), development of macronucleus in Tetrahymena (25) and male spermatogenesis in Drosophila melanogaster (20,26,27). In these cases, dsRNA formation is provided by the transcription of the vestiges of transposable elements indicating that mechanisms of mobile elements silencing may be extended to the regulation of host genome.

Contribution of RNAi-based regulation to the retrotransposon-derived transcripts abundance in the Drosophila germline has been demonstrated (19–22), but no direct evidence in favor of the role of RNAi machinery in the control of their transposition rate has been obtained. Genetic control of retrotransposon mobilization in Drosophila is far from being understood. Spontaneous transposition rates of retroelements are usually low (28). However, a high level of retrotransposon mobility was detected in some stocks under particular genetic conditions. Mobility of gypsy, ZAM and Idefix retrotransposons was shown to be controlled by the yet uncharacterized X-linked flamenco locus (29,30). Non-LTR retrotransposon I is mobilized in the female germline as a result of definite outcrosses. This phenomenon is known as I–R hybrid dysgenesis (31). In this case, the mechanism of retrotransposon activation is also unclear. We revealed the effect of the RNAi genes spin-E, aub and armi
on the I element expression in the female germline (22). We propose that hybrid dysgenesis associated with I element transposition burst may be caused by the impair of RNAi (22).

We investigated a role of the piwi gene in the control of retrotransposon mobilization in the male germline. piwi encodes a nucleoplasmic protein known to be required for self-renewal of stem cells in the male and female germline and the regulation of their division (32,33). Control of stem cell maintenance is the conserved developmental function of piwi and its orthologs in plants and mammals (34–38). PIWI is related to the highly conserved proteins of the Argonaute family. Argonautes are implicated in RNAi and related silencing pathways, including those that affect heterochromatin assembly and translational regulation via microRNA (miRNA)-directed pathway in different organisms [reviewed in (39–41)]. These proteins including PIWI are defined by two characteristic domains, PAZ and PIWI. PAZ domain recognizes the unique terminal structures of siRNAs (42,43) and, therefore, is essential for small RNA binding. The PIWI domain of Argonaute from the archaeabacterium Pyrococcus furiosus is similar to a ribonuclease H domain (44), suggesting the role of Argonautes in the nucleolytic activity of RISC. However, only one of the four mammalian Argonaute I subfamily members (Ago2) was associated with the cleavage of target mRNA, suggesting that the other Argonautes might operate in different forms of RNA silencing (45). Drosophila Ago1 and Ago2 regulate the assembly of miRNAs and siRNAs, respectively, into RISC (46). piwi mutation relieves the transcriptional and post-transcriptional gene silencing of the multiple-copy transgenes (47) and impedes the repression of retrotransposon gypsy in the D.melanogaster genome (48).

In this study, we demonstrate that piwi mutations cause overexpression of retrotransposons mdg1 and copia in the male germline, alteration of their expression pattern and mobilization of mdg1 retrotransposon. Our results provide the first evidence that Argonaute family protein prevents mobilization of a retrotransposon. copia and mdg1 are related to two major families of LTR retrotransposons, Ty1copia and Ty3/gypsy groups, respectively. These groups are structurally differed by the location of integrase domain (49). The Ty1/copia group is termed the Pseudoviridae, reflecting structural similarity to retroviruses. Number of full-length copies of copia is 26 and mdg1 is 13 according to the analysis of euchromatic part of the Drosophila genome (50). Spontaneous rate of transpositions is \( \sim 10^{-4} \) per element per generation for copia (51), while no mdg1 transpositions has been revealed in related stocks (28). It has been reported that conventional inbred crosses may generate spontaneous copia transpositions (52). We show that transposition burst of retrotransposon mdg1 in the Drosophila germline is a result of mutation in the piwi gene involved in the stem cell development and RNA-mediated silencing phenomena. It is supposed that RNAi-related system of silencing controls transposition rate in the germline and its disturbance might cause transposition burst.

**MATERIALS AND METHODS**

**Drosophila strains**

Strain bearing spindle-E (spn-E) mutation was ru1 st/ spn-E/ e1 ca1/TM3, Sb1 et/. piwi mutants were piwi1 (PZ insertional mutation) and piwi2 (P-rylI transposon insertion) (32,53). piwi2 mutation was balanced with the CyO, P[w^m = hsp70: GAL4] P[w^m = UAS: GFP]. Su(var)2.50, Su(var)3.9 and E[z]^6 were alleles used. P-element transformed flies carrying copiaLTR-lacZ construct on the X and second chromosomes were kindly provided by E. G. Pasyukova (54).

**In situ RNA analysis**

In situ RNA analysis was carried out according to the procedure described previously (21). DIG-labeled riboprobes were synthesized according to the manufacturer’s recommendations. PCR products carrying T7 promoter or plasmids containing the cloned fragments of retrotransposons were used as transcription templates. mdg1: 0.35 kb EcoRV–NsiI fragment from plasmid Dm688 (55) was subcloned into pBS SK− and used to obtain sense or antisense riboprobe. 1731: PCR-amplified fragment using primers (5′-AGGAATTCGAGGAGGATGACATCTGAGTGTACTACA-3′ and 5′-ATGGATCCGAGGAGGATGACATCTGAGTGTACTACA-3′) corresponding to 1138–1271 nt (GenBank accession no. X07656) was cloned into pTZ19R and used to detect sense 1731 transcripts, copia: 2.3 kb fragment from the 5′ copia LTR to the EcoRI site into pBS SK− was used to obtain a probe to detect sense or antisense copia RNA. GATE: PCR product carrying T7 promoter corresponding to 3055–3237 nt (GenBank accession no. AJ10298) was used for the detection of GATE sense RNA. PCR product carrying T7 promoter for piwi transcripts detection was amplified using primers 5′-GGCGCGGACTTATGTGAGAGCAATGGATGTCGTCG-3′ and 5′-GCCGCCGACCGTCTGAGTATAAAAATACG-3′ corresponding to 7886–8519 nt (GenBank accession no. NG000285).

**RT–PCR analysis**

RT–PCR analysis was performed according to the protocol described previously (20). First strand of cDNA was synthesized using SuperScript II reverse transcriptase (Gibco BRL) and oligo(dT) primer according to the manufacturer’s instructions. For PCR, the following primers were used: 5′-AAAC-TGGCCGCCCATATTCCG-3′ and 5′-CAAGTCCAGTTTCCAGATG-3′ corresponding to 180 752–180 967 nt fragment of constitutively expressed Adh gene (GenBank accession no. AE003410.1); 5′-GATGAGGTTTGGCCATATAAAGC-3′ and 5′-GGCCACGACATCTGAGTACTACA-3′ corresponding to 1685–1903 nt for copia (GenBank accession no. M11240); 5′-CTAAAAGGAATGGGTGCTCTAAGT-3′ and 5′-TTCAAAGGAGGAGATGTAGATATACG-3′ corresponding to 7023–7166 nt for mdg1 (GenBank accession no. X59545). Results of RT–PCR analysis was evaluated using ImageQuant5.2.

**X-gal staining**

X-gal staining of testes was performed according to the protocol described previously (20).
We investigated the effect of piwi and piwi2 using strand-specific riboprobe. in situ mdg1 was PCR amplified using mdg1-specific primers: 5’-CGATCTGAGTGAAGTCTAGTGTCAGTG-3’ and 5’-CTCTAACAGTCCAGTTAACCTTAGTTAATTATAACAG-3’. Cloning and sequencing of three products allowed us to identify the region of insertions on the X, second and third chromosomes (GenBank accession nos AC104515, AC007291 and AC010011, respectively). Three primers corresponding to the region of insertions (5’-AGGACTAAAATCCTTCTCCTGACTT-3’, 5’-AGGCGTTGAAGTTTACCATCAGTCT-3’) and primer 5’-CTTTCCTCTCCTGACTT-3’ corresponding to mdg1 LTR were used to demonstrate the presence of insertions in F1 DNA samples and their absence in parental DNAs.

RESULTS

The piwi gene is indispensable for silencing of mdg1 and copia LTR retrotransposons in testes

We investigated the effect of piwi mutations on the expression of LTR retrotransposons in testes by in situ RNA analysis using strand-specific riboprobe. piwi2 and piwi3 mutations are caused by the P-element insertions into the coding and 5’-UTRs of the piwi gene, respectively (32). The PIWI protein expression is known to be attributed to a germinal proliferative center at the apical tip of larval and adult testes containing somatic hub cells and mitotically dividing germ stem cells (32,33) (see Figure 1A). piwi transcripts were revealed by in situ RNA hybridization using piwi riboprobe in the region of germinal proliferative center of the piwi2/+ adult and piwi2/+ larval testes (Figure 1B, arrows) in accordance with the observtions reported previously (33). The absence of piwi transcripts in the larval piwi2/piwi2 and adult piwi2/piwi2 testes is closely correlated with the emergence of the mdg1 sense transcripts at the tip regions of testes (Figure 1B, arrows). At the same time, in the remaining part of testes, the level and pattern of the sense mdg1 transcripts is roughly similar in the piwi2/+ and piwi2/piwi2 males (Figure 1B and C). The mdg1 transcripts are localized in spermatocyte nuclei, near the DAPI stained compact regions of chromatin (Figure 1B, inset). Sense copia transcripts are detected in a limited number of spermatocytes in testes of piwi2/+ males (Figure 1B). copia sense transcript accumulation in testes of homozygous piwi3 males is detected in the basal part of testes where postmeiotic stages of spermatogenesis are known to have occurred (Figure 1B). No copia sense transcripts are detected at the apical tip of testes. A significant increase in copia transcript abundance was also observed in spermatocytes of larval testes of homozygous piwi2 males as compared with heterozygous piwi2/+ males (Figure 1B). Thus, the derepression of copia in piwi mutants was observed at the later stages of spermatogenesis, where PIWI protein has not been detected (33). mdg1 and copia antisense transcripts are not detected by in situ RNA hybridization (Figure 1B). RT–PCR analysis confirmed the derepression of copia in testes of piwi mutants (Figure 1C). Amounts of copia and mdg1 transcripts, normalized against adh transcripts, increases in testes of piwi2/piwi2 males by 1.9 and 1.3 times, respectively. It is not surprising that the local accumulation of mdg1 transcripts in the region of stem cells exerts no noticeable effect on their total amount evaluated by RT–PCR (Figure 1C).

LTR retrotransposons 1731 and GATE have been shown to be transcribed in primary spermatocytes (21,57), but the level of transcripts of these elements detected by in situ RNA hybridization was not affected by piwi mutation (data not shown).

It has been reported that mutations of some Drosophila RNAi genes and piwi cause mislocalization of heterochromatic proteins HP1, HP2 and a loss of histone H3 methylation in nuclei of somatic cells (58), suggesting the role of RNAi machinery in heterochromatin assembly and transcriptional gene silencing. Thus, PIWI might be proposed to be a component of chromatin silencing complex that induces transcriptional repression of transposable elements. However, no effects of mutations in genes encoding HP1 [SU(VAR)02-05] and histone H3 methyltransferases SU(VAR)3-9 and E(Z) on the expression of copia and mdg1 were revealed in testes by in situ RNA analysis (data not shown).

piwi mutation leads to the derepression of a copia LTR-lacZ fusion construct in testes

To study whether copia LTR is a target of silencing mediated by piwi, we used a construct containing full-size copia LTR fused to lacZ (copiaLTR-lacZ). copia LTR comprises the known upstream regulatory region, including 173 bp of the transcribed fragment (54) (Figure 2A). We introduced the X-chromosome containing copiaLTR-lacZ into the stock carrying piwi2 mutation. The level of reporter gene expression is significantly increased in piwi2/piwi2 testes. No staining at the apical tip of testes is revealed (Figure 2B, arrows). lacZ expression is detected in early spermatocytes as well as in the basal parts of piwi2 homozygous testes where the accumulation of endogenous copia transcripts was observed (Figures 1B and 2B). The difference in the expression patterns of endogenous copia and copiaLTR-lacZ might be related to the absence of some copia regulatory regions in the transgene as compared with full-size endogenous copies.

To obtain additional arguments in favor of a lack of copiaLTR-lacZ expression owing to disturbance of RNA silencing, we tested the effect of mutation of RNAi gene spn-E. spn-E encodes putative RNA-helicase, and was shown to be responsible for the production/stabilization of siRNA necessary to silence the Stellate repeats in testes (27). spn-E mutation causes accumulation of copia transcripts.
Expression of the copia LTR-lacZ reporter constructs located on the X or second chromosomes is significantly increased in testes of the homozygous spn-E1 males (Figure 2C). The patterns of the lacZ expression in testes of the spn-E1 homozygous males are similar to the observed distribution of lacZ expression in testes of piwi2 homozygous males. Thus, copia LTR contains regulatory sequences that confer spn-E and piwi dependence of retrotransposon repression.
**mdg1 transposes in the piwi^3 male germline**

The mdg1 overexpression in piwi mutants is observed in the germinal stem cells, suggesting PIWI protein involvement in the repression of transpositions in the precursors of male gametes. To check the mdg1 transpositions, single piwi^3 homozygous males were crossed to the individual piwi^3/+ females. A comparative inverse PCR analysis was applied to the single F1 progeny flies and their parents. Inverse PCR approach allows us to estimate the copy number of transposable elements and to detect the newly arisen insertions. Figure 3 shows the rationale of the method. The number of full-length and partial euchromatic mdg1 copies per *D.melanogaster* genome amounts to 25 copies according to the Release 3 of euchromatic genome sequence (50). We detected ~35 PCR bands per genome and this surplus number of copies may be contributed by the heterochromatic part of the genome. Our estimation of the number of mdg1 copies corresponds well with the results obtained by using Southern-blot analysis (59). Differences between the patterns of PCR bands detected in individual parental DNAs reflect the polymorphisms of mdg1 sites peculiar to the piwi and balancer chromosomes as well as to the presence of the Y-specific bands in male DNA (Figure 4A and B). The new bands in F1 DNA samples that are non-detected in parental DNAs demonstrate the occurrence of transposition events (Figure 4A). We failed to reveal newly appeared PCR bands in the progeny of piwi^3/+ males (Figure 4B), while the number of new insertions in the progeny of the piwi^3/piwi^3 males amounts to 19 per 101 flies analyzed (Table 1). To confirm the emergence of the new bands as the result of transpositions, three new bands detected in three individuals were cloned and sequenced. The results of sequencing allowed us to attribute the mdg1 insertions in three F1 individuals to the regions of unique genomic DNA on the X, second and third chromosomes. PCR analysis of DNA from F1 and parental flies using primers specific to the insertion sites and mdg1 LTR demonstrates the occurrence of insertions in F1 samples and ‘empty sites’ in the parental DNAs (Figure 4C). The average frequency of mdg1 transposition in testes of piwi^3 mutants amounts to 0.19 transpositions per generation. However, this value may be underestimated as a result of low resolution of high-molecular weight PCR bands. The new PCR bands of the same size were observed in F1 individuals. At least some of these bands may correspond to identical mdg1 insertions, indicating the presence of the germ cell clusters carrying identical sites of insertions and suggesting that transpositions occur at the premeiotic stages of spermatogenesis.

**Figure 2.** Overexpression of the copiaLTR-lacZ construct in piwi^2 and spn-E^1 testes. (A) The copiaLTR-lacZ construct comprises full-size copia LTR fused to the lacZ reporter. (B) X-gal staining of testes from piwi^2 males carrying the copiaLTR-lacZ construct on the X-chromosome. (C) X-gal staining of testes from spn-E^1 males carrying the copiaLTR-lacZ construct on the X (upper panels) and second chromosomes (lower panels). A level of lacZ expression is greatly increased in the homozygous piwi/piwi and spn-E/spn-E testes as compared with heterozygous piwi^+/+ and spn-E/+ testes. Arrows indicate the apical tips of testes, where β-galactosidase activity is not detected.

**Figure 3.** Detection of the retrotransposon insertions by inverse PCR analysis. Gray boxes represent transposable elements; restriction sites are indicated by vertical arrows and horizontal arrows designate primers. Restriction–ligation of DNA samples followed by PCR using retrotransposon-specific primers revealed PCR products corresponding to insertions of retrotransposon.
Figure 4. Mobilization of retrotransposon mdg1 in testes of piwi mutants. Genomic DNA from individual flies was digested, ligated and PCR amplified using mdg1-specific primers. Genomic distribution of mdg1 copies in DNAs from individual F1 flies are shown to the left and to the right. Pattern of PCR bands of parents are in the middle (m, males; f, females; −/−, piwi+/piwi−; and +/+ , piwi+/+). (A) Parents are piwi+/piwi− males and piwi+/+ females. Mobilization of mdg1 is demonstrated by the new bands revealed in progenitor DNAs (indicated by arrows), which are not detected in parental DNAs. (B) Parents are piwi+/+ males and piwi+/+ females. No new bands differing from parental DNAs were detected in F1 DNA. (C) Detection of mdg1 insertion sites. PCR analysis of DNA from F1 (1, 2 and 3) and parental flies (m and f) using specific primers corresponding to the insertion sites in the X, second and third chromosomes, respectively, and the mdg1 LTR primer. Appearance of insertions in F1 samples (bands) and ‘empty sites’ in parental DNAs are shown.

Table 1. Estimation of the mdg1 mobility in testes of piwi−/piwi− (−/−) and piwi+/+ (+/+ ) males

| Male genotype | Number of analyzed F1 individuals | Number of new PCR bands |
|---------------|----------------------------------|-------------------------|
| −/−           | 50                               | 9                       |
| −/+           | 51                               | 10                      |
| +/+           | 35                               | 0                       |
| +/−           | 16                               | 0                       |

**DISCUSSION**

We presented evidence that the piwi gene, coding a member of the Argonaute protein family, is required for the silencing of the retrotransposons in the *Drosophila* male germline. piwi mutation causes the derepression of retrotransposon mdg1 in the germinal proliferative center and, as a result, relieves transpositions of this mobile element. This observation is the first evidence that mutation in a single gene triggers retrotransposition in the *Drosophila* germline. We suggest that activation of retrotransposon caused by piwi mutation occurs as a result of the RNAi machinery disturbance. Strong evidence in favor of *Drosophila* PIWI as a component of RNAi is not obtained, but it has been reported that piwi mutation causes a relief of transgene repeats silencing and diminishing transgene-related siRNA (47). We failed to detect *copia* and mdg1 antisense transcripts by *in situ* RNA hybridization suggesting the low level of these transcripts in testes. Antisense transcripts corresponding to LTR sequence of *copia* were revealed in ovaries (M. S. Klenov, unpublished data). siRNAs corresponding to transposable elements including *copia* have been detected in *Drosophila* testes (60) supporting the participation of RNAi-related mechanism in retrotransposon silencing.

Here, we demonstrated that lacZ expression driven by the *copia* LTR is upregulated in testes of RNAi mutants. piwi and spn-E mutations have similar effect on a reporter gene expression, suggesting the participation of both genes in the same silencing pathway. Thus, LTR is considered as a target of silencing machinery. The role of LTRs in RNAi-mediated regulation of retrotransposons and adjacent host genes in yeast has been emphasized (9). The most abundant repeat-associated siRNAs were related to the LTR of multicopy retrotransposon *roo* in *D.melanogaster* (60).

Presence of PIWI protein in embryos is restricted to the presumptive gonad. In adults, PIWI is required in somatic signaling cells to maintain germ line stem cells (33). Our results show the role of piwi in silencing of the mdg1 retrotransposon in the germinal proliferative cells and *copia* at the late spermatocytes and postmeiotic stages where PIWI protein is not detected. Thus, PIWI presumably affects silencing mechanisms that are operated at the later stages of spermatogenesis. Effect of piwi may be indirect and mediated through the other targeted genes operating on chromatin level. Actually, the role of PIWI, in concert with HP1 and histone H3 methylation, in the epigenetically inherited silencing has been reported previously (58). However, we revealed no effect of HP1 and SU(VAR)3-9 and E(Z) histone H3 methylases mutations on the expression of mdg1 and *copia* retrotransposons in testes. Further experiments will elucidate the mechanism of PIWI-mediated retrotransposon silencing.

Transcript abundance of retrotransposons 1731 and GATE, which are active in primary spermatocytes (21, 57) is not affected by piwi mutation. It is significant that retrotransposon transcript abundance may not correlate with retrotransposition frequency that is dependent on the DNA copy production and immediate insertion. It has been shown that mutations of RNAi genes in *C.elegans*, including putative RNA-helicase gene, have resulted only in the activation of the selected DNA transposons (15, 61). The similar effects of RNAi silencing affecting selective mobile elements in plant genome have been also reported previously (62). All these results argue against universal RNAi-dependent silencing of retrotransposons. Specific pattern of dsRNA formation, which triggers the RNAi pathway, might be responsible for these differences. Moreover, distinct members of Argonaute family may act in
distinct silencing pathways at different developmental stages. Ago2 is essential for siRNA-directed RNA cleavage in Drosophila embryos (46). The regulatory effect of the piwi gene may be realized upstream of the ago2 in the respective silencing mechanism in the germline. Alternatively, both piwi and ago2 products may be indispensable to organize specific RISC complex directed to retrotransposon silencing. In this case, piwi mutation may compromise the functioning of this complex.

We revealed the effect of piwi mutation on the mdg1 mobilization and the derepression of copia in the male germ-line. Involvement of the other RNAi genes (spn-E, aub and armi) have been revealed in the control of expression of different families of transposable elements in the Drosophila female germline, including the non-LTR retrotransposons Hef-A and I (22). At the same time, a growing body of evidence demonstrates a profound contribution of the RNAi mechanism to the regulation of host development. RNAi genes, including spn-E, aub and armi, were shown to be responsible for specifying the axes of developing oocyte and embryonic patterning in Drosophila (63–65). RNAi genes are proposed to prevent premature translation of the key embryonic determinant oscar via miRNA pathway (65). Thus, a dual function of RNAi-related genes implicated both in the developmental control and in the retrotransposon silencing is unveiled. We suggest that the same RNAi-related mechanism mediated by naturally occurring dsRNA via PIWI protein assistance suppresses mobile element transpositions and regulates host genes ensuring stem cell renewal in testes. Further refining studies are needed to reveal the mechanism ensuring this dual function of RNAi protein complexes.

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