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Panoramix enforces piRNA-dependent cotranscriptional silencing

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Abstract

The Piwi-interacting RNA (piRNA) pathway is a small RNA-based innate immune system that defends germ cell genomes against transposons. In *Drosophila* ovaries, the nuclear Piwi protein is required for transcriptional silencing of transposons, though the precise mechanisms by which this occurs are unknown. Here we show that the CG9754 protein is a component of Piwi complexes that functions downstream of Piwi and its binding partner, Asterix, in transcriptional silencing. Enforced tethering of CG9754 to nascent messenger RNA transcripts causes cotranscriptional silencing of the source locus and the deposition of repressive chromatin marks. We have named CG9754 “Panoramix,” and we propose that this protein could act as an adaptor, scaffolding interactions between the piRNA pathway and the general silencing machinery that it recruits to enforce transcriptional repression.

The Piwi-interacting RNA (piRNA) pathway controls transposons through a number of distinct, but likely interlinked, mechanisms. Whereas cytoplasmic Piwi proteins silence their targets posttranscriptionally through piRNA-directed cleavage and the ping-pong cycle, nuclear Piwi-piRNA complexes function at the transcriptional level (1). Piwi-directed repression of transcription is thought to be dependent on piRNA-guided recognition of

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SUPPLEMENTARY MATERIALS

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Materials and Methods

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Table S1

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nascent transposon transcripts (1, 2). Transcriptional gene silencing (TGS) correlates with the presence of histone H3 lysine 9 trimethylation (H3K9me3) marks (3–7), yet the mechanism through which Piwi binding promotes the deposition of these marks remains enigmatic. With the exception of the zinc finger protein Asterix (also known as DmGTSF1), the components of Piwi effector complexes at target loci are largely unexplored (7–9).

We systematically mined candidate genes from RNA interference (RNAi) screens for potential TGS effector proteins and identified CG9754 in three independently published screens as being critical in both the germ cells and follicle cells for transposon silencing (8, 10, 11). Loss of CG9754 had essentially no effect on the abundance or content of piRNA populations or on the nuclear localization of Piwi protein (fig. S1), suggesting that it is probably an effector component (11). CG9754 encodes a ~60-kD nuclear protein with no identifiable domains (11). The expression of CG9754 is restricted to the female gonads, as is seen for other core piRNA pathway components such as Asterix (fig. S2).

To examine global effects on transposon expression, we used RNA sequencing (RNA-seq) to measure steady-state RNA levels from ovaries with germline-specific knockdowns of either CG9754 or Piwi (Fig. 1A) (also see supplementary materials and methods). Piwi knockdown caused a sharp rise in transposon transcripts, with minimal effects on protein-coding gene expression (Fig. 1A and fig. S3A). Knockdown of CG9754 caused effects very similar to those of Piwi (Fig. 1A and fig. S3), with most transposon targets being shared (Fig. 1B). Changes in steady-state RNA levels could have resulted from alterations in either element transcription or the stability of transposon mRNAs. We used global run-on sequencing (GRO-seq) to measure nascent RNA synthesis following gene knockdown (Fig. 1C and fig. S4). Loss of either CG9754 or Piwi produced very similar profiles (Fig. 1D), suggesting that CG9754 is specifically required for transcriptional silencing of transposons targeted by Piwi.

Piwi-mediated TGS correlates with the presence of H3K9me3 marks at silenced transposons (1–6). Depletion of either CG9754 or Piwi resulted in nearly identical losses of H3K9me3 over transposons (Fig. 2, A and B, and fig. S6A). Four independent frameshift mutations of CG9754 generated via the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) system were isolated (fig. S5A) (12). We observed a consistent global up-regulation of transposable elements (fig. S5, B to E) and the corresponding loss of H3K9me3 marks in CG9754 mutant ovaries without changes in piRNA levels (figs. S1 and S6B). Similarly to other core piRNA pathway mutants, female flies lacking CG9754 were sterile. Moreover, flies double-mutant for CG9754 and Asterix showed transposon derepression comparable to that of flies with either single mutation, suggesting that both genes act in the same pathway (fig. S7). Thus, CG9754 functions along with Piwi and Asterix in the repression of transposon transcription.

Next, we asked whether the presence of CG9754 at a target locus might be sufficient to induce its silencing. Because piRNAs likely direct binding to nascent RNAs rather than to the DNA of their targets (13), we delivered CG9754 via protein-RNA interactions. We constructed a series of luciferase reporters with BoxB sites in their 3' untranslated regions (UTRs) and used them to create transgenic reporter flies (fig. S8A). BoxB sites are bound by

the λ N protein, which can also bring other components to the RNA as part of a fusion (14). We also generated flies expressing λ N proteins fused to CG9754, Asterix, nuclear Piwi, and, as a negative control, a cytoplasmic Piwi missing its nuclear localization signal (dN-Piwi). When coexpressed with the reporter, the dN-Piwi fusion failed to induce any change in luciferase expression (Fig. 2C and fig. S12). Of the remainder, only the CG9754 fusion considerably reduced luciferase activity (Fig. 2, C and D). Silencing appeared to be dosage-dependent, as the degree of repression correlated with the number of BoxB binding sites inserted into the reporter mRNA (fig. S9). Consistent with a role for CG9754 in transcriptional silencing, the abundance of the reporter mRNAs was significantly reduced upon tethering (Fig. 2D).

Although CG9754-triggered repression appeared to be independent of chromatin context (Fig. 2, C and D), integration of the reporter into genomic DNA appeared to be critical for repression. Transient cotransfection of reporter constructs into the OSS cell line, which contains an active piRNA pathway, resulted in little to no detectable silencing (Fig. 2E). In contrast, tethering *Drosophila* Ago1 (λ N-dAgo1) to the luciferase reporter mRNA in OSS cells caused substantial repression of the same reporter (Fig. 2E) (15). These results indicate that CG9754 can function properly only in the context of chromatin, likely acting at the transcriptional level, by interacting with nascent transcripts.

To test the hypothesis that λ N-CG9754 acts on nascent transcripts, we generated a reporter for which the BoxB binding sites were located within the intron of the primary transcript (fig. S8B). λ N-CG9754 maintained the ability to repress this reporter but not a similar transcript carrying BoxB sites in the antisense orientation integrated into the same genomic locus (Fig. 2F). Because the spliced, mature reporter transcripts lack the BoxB sites (fig. S10), we reason that λ N-CG9754 must be able to exert its effects by binding to unspliced precursor mRNAs. Given that splicing occurs cotranscriptionally (16), this implies that CG9754 confers its effects by interaction with the nascent transcript.

If CG9754 mediates Piwi-dependent transcriptional silencing, delivery of CG9754 alone might recapitulate hallmarks of piRNA-directed repression. Tethering of CG9754 had a highly specific effect, changing levels of only the reporter mRNA (Fig. 3A). Repression occurred at the transcriptional level, as GRO-seq indicated a loss of nascent RNA from the integrated reporter (Fig. 3B and fig. S11B). Repression by CG9754 also correlated with specific deposition of H3K9me3 marks over the reporter locus (Fig. 3, C and E, and fig. S11C). Tethering of CG9754 failed to trigger piRNA production from the reporter, as has been seen previously for some loci that become targets of the piRNA pathway (Fig. 3D) (17, 18). Of note, we observed spreading of H3K9me3 marks to other regions of the reporter gene (Fig. 3E), as described previously for regions flanking piRNA-targeted transposon insertions (4). Thus, delivering CG9754 to the nascent RNA causes repression of a locus in a manner that mimics targeting by the piRNA pathway.

We next tested whether CG9754 might be a component of Piwi complexes, as predicted by our epistasis experiments. Functional GFP-Piwi fusion proteins copurified with hemagglutinin (HA)-tagged CG9754 from OSS cells, but not with a negative-control fusion (HA-mKate2) (Fig. 4A). Conversely, Flag-tagged CG9754 was able to specifically

precipitate endogenous Piwi proteins from OSS cell lysates (Fig. 4B), confirming the interaction between these two proteins. Given its properties, we named CG9754 “Panoramix,” after the mentor who empowers the French comic book character Asterix to perform his feats of strength.

The identification of Panoramix as a key mediator of piRNA-directed TGS presented an opportunity to use the tethering assay to dissect the mechanism of transcriptional silencing. We used RNAi to deplete selected piRNA pathway genes in flies in which λ N-Panoramix was tethered to the luciferase-BoxB reporter (Fig. 4C). Knockdown of Panoramix itself weakened the repression significantly, as compared with a control knockdown (mCherry). Silencing of factors required for piRNA biogenesis (Zuc and Armi) or those that are expected to act upstream of Panoramix (Piwi and Asterix) did not significantly affect repression. Depletion of dLSD1/Su(var)3-3 and its cofactor, CoREST, which normally form a complex that removes H3K4me2 marks from promoters (19, 20), had significant effects on the ability of Panoramix to repress the reporter. Because H3K4me2 marks actively transcribed genes, it is possible that dLSD1-mediated removal of these marks is a key step in Panoramix-mediated transcriptional silencing. This raises a potential parallel with piRNA-directed silencing in mice, wherein engagement by DNMT3L, which is necessary for piRNA-induced DNA methylation, requires removal of such marks (fig. S13) (21, 22). Similarly, knockdown of HP1a caused derepression, in agreement with its role as a constitutive heterochromatin component required for transposon silencing (23) and with the observation that the presence of Panoramix is correlated with the deposition of H3K9me3 marks at target loci (Figs. 2, A and B, and 3E). The H3K9 methyltransferase Eggless/dSETDB1 and its cofactor Windel appeared to be required specifically for Panoramix-mediated silencing, as knockdown of G9a, another H3K9 methyltransferase, showed no effect on the reporter (Fig. 4C). In *eggless* mutants, we observed essentially complete derepression of the reporter, despite Panoramix tethering (Fig. 4D). In contrast, the piRNA biogenesis mutant *zuc* showed little to no effect on the repression of the reporter, as also observed in *zuc* RNAi experiments (Fig. 4C). Our data raise the possibility that Eggless could be one of the enzymes responsible for the deposition of H3K9me3 marks over silenced transposons in a Piwi-targeted fashion.

Panoramix functions downstream of Piwi and Asterix and is both necessary and sufficient to elicit transcriptional repression when bound to nascent transcripts. Panoramix represents an example in metazoans of a protein inducing cotranscriptional silencing when recruited to the nascent transcript from a locus (24). In fact, only cotranscriptional silencing can resolve the conundrum of a target being transcriptionally repressed while transcripts from that target locus are responsible for recruiting their own repressors. Orthologs of some of the general silencing factors that act with Panoramix to deposit and interpret repressive chromatin marks have also been implicated in mammalian transposon silencing, in which the pathway functions by causing heritable DNA methylation (fig. S13) (21, 24–27). Though one cannot identify a mammalian ortholog of Panoramix based on primary sequence alone, the overall conservation of the piRNA-mediated transcriptional machinery suggests that a protein with an equivalent function likely exists in mammals (fig. S13).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Knockdown of *CG9754* increases transposon expression

(A) Heat map displaying steady-state RNA levels (measured with RNA-seq) as reads per million (rpm) for the top 70 detected transposons from *nanos*-GAL4-driven knockdowns of the indicated genes. The average of two replicates is shown. (B) Comparison of steady-state RNA levels is shown as rpm mapping to the sense strands of each transposon consensus from the *nanos*-GAL4-driven knockdowns (KD) of the indicated genes. Dashed lines indicate twofold changes. (C) Heat map displaying nascent RNA levels (GRO-seq) as rpm for the top 70 detected transposons [organized exactly as described for (A)]. (D) Data are presented for nascent RNA levels measured by GRO-seq [organized as described for (B)]. In (A) to (D), red dots indicate germline-biased elements, green dots represent soma-biased elements, yellow dots denote intermediate elements targeted in both compartments, and gray dots indicate elements with no apparent designation.

Fig. 2. Tethering of CG9754 to RNA leads to cotranscriptional silencing

(A and B) Comparisons of normalized H3K9me3 densities mapping to the indicated transposons in the control knockdown versus the indicated knockdown. (Top) *HetA*; (bottom) *TART*. Yellow, red, and blue denote H3K9me3 enrichments over input in control *white* knockdown, *piwi* knockdown, and *CG9754* knockdown samples, respectively. (C) Effects of the indicated λ N fusion proteins on luciferase activity of reporters integrated into different genomic loci. Data show mean $\pm$ SD (error bars); $n = 15$ replicates; $*P = 1.41387 \times 10^{-7}$. (D) Results of reverse transcriptase quantitative polymerase chain reaction experiments, showing *rp49*-normalized RNA levels of the luciferase reporters [as described in (C); mean values $\pm$ SD from three independent experiments]. (E) Effects of the indicated λ N fusion proteins on luciferase activity of a reporter containing 10 copies of BoxB sites transiently transfected into OSS cells. Mean values $\pm$ SD from three independent experiments are shown. (F) Effects of λ N-CG9754 tethering on luciferase activity of the indicated reporters with varying positions (intron versus 3' UTR) or orientations (sense versus antisense) of the BoxB sites. Error bars indicate SD.

Fig. 3. Tethering of CG9754 to RNA recapitulates targeting by the piRNA pathway

(A) Comparison of steady-state RNA levels (measured with RNA-seq) for the absence (untethered) or presence (tethered) of λ N-CG9754. Red dot, Firefly luciferase; green dot, CG9754. (B) Data for nascent RNA levels (GRO-seq) [organized as in (A)]. (C) Same as in (A) but showing H3K9me3 reads [chromatin immunoprecipitation sequencing (ChIP-seq)]. (D) Comparison of small RNA reads (24 to 29 nucleotides) mapping uniquely to piRNA clusters and Firefly luciferase in the absence (untethered) or presence (tethered) of λ N-CG9754. Red dot, Firefly luciferase; blue dot, *flamenco*; purple dot, 42AB. (E) Normalized H3K9me3 densities mapping to the luciferase transgene in the absence (gray, untethered) or presence (red, tethered) of λ N-CG9754. A schematic of the integrated transgene is shown below. For all analyses, only reads uniquely mapping to the reporter gene were considered.

Fig. 4. Panoramix (CG9754) links the piRNA pathway to the general transcriptional silencing machinery

(A) Western blots (WB) showing coimmunoprecipitation of HA-tagged CG9754 with GFP-Piwi from OSS cells. GFP, green fluorescent protein. (B) Western blots showing coimmunoprecipitation of Flag-tagged CG9754 with endogenous Piwi from OSS cells. (C) Effects of somatic (*tj*-GAL4) knockdown of the indicated genes on luciferase activity of the reporter while tethering λ N-Panoramix. (D) Expression of the reporter under the indicated transheterozygous mutant backgrounds while tethering λ N-Panoramix. Orange bars, total protein-normalized luciferase fold changes; black bars, *rp49*-normalized RNA fold changes. (C and D) Mean values $\pm$ SD from three independent experiments are shown (for luciferase data, $n = 15$; $*P = 1.41387 \times 10^{-7}$).