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DmGTSF1 is necessary for Piwi–piRISC-mediated transcriptional transposon silencing in the Drosophila ovary

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The Piwi–piRNA (PIWI-interacting RNA) complex (Piwi–piRISC) in Drosophila ovarian somatic cells represses transposons transcriptionally to maintain genome integrity; however, the underlying mechanisms remain obscure. Here, we reveal that DmGTSF1, a Drosophila homolog of gametocyte-specific factor 1 (GTSF1) which is required for transposon silencing in mouse testes, is necessary for Piwi–piRISC to repress target transposons and neighboring genes. DmGTSF1 depletion affected neither piRNA biogenesis nor nuclear import of Piwi–piRISC. DmGTSF1 mutations caused derepression of transposons and loss of ovary follicle layers, resulting in female infertility. We suggest that DmGTSF1, a nuclear Piwi interactor, is an integral factor in Piwi–piRISC-mediated transcriptional silencing.

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Mobilization of transposable elements (TEs) can lead to natural insertion mutations that generally have negative effects on the host genome (Lynch 2007). Thus, host species have evolved control mechanisms that restrict TE activity. One such mechanism is RNAi/RNA silencing, in which small RNAs 20–30 nucleotides in length trigger multiple forms of sequence-specific gene silencing by guiding Argonaute complexes or RNA-induced silencing complexes (RISCs) to target RNAs by means of base-pairing (Ketting et al. 1999; Tabara et al. 1999; Slotkin and Martienssen 2007; Siomi and Siomi 2009). It is becoming clear that piRISCs also mediate TE silencing in the nucleus but in a Piwi cleavage-independent manner (Saito et al. 2010; Klenov et al. 2011; Wang and Elgin 2011; Sienski et al. 2012; Darricarrère et al. 2013).

Although three PIWI proteins—AGO3, Aubergine (Aub), and Piwi—are expressed in the Drosophila ovary (Brennecke et al. 2007), only Piwi is expressed in ovarian somatic cells (OSCs), such as follicle cells, where piRNA intermediates are digested by a single-stranded nucleic acid-specific endonuclease, Zucchini (Ipsaro et al. 2012; Nishimatsu et al. 2012). Zucchini products are further processed in the cytoplasmic Yb bodies into mature piRNAs (Olivieri et al. 2010; Saito et al. 2010). Piwi–piRISC formation also takes place at Yb bodies, where several piRNA biogenesis factors, including Armitage (Armi), accumulate (Olivieri et al. 2010, Saito et al. 2010). piRNAs in OSCs are not amplified because these cells lack the core factors (i.e., AGO3 and Aub). Piwi–piRISCs are imported into the nucleus, where they mediate TE repression. Nuclear silencing by Piwi–piRISCs does not require cleavage of TE transcripts but involves chromatin modification of TE loci on the genome (Saito et al. 2010; Wang and Elgin 2011; Sienski et al. 2012; Huang et al. 2013; Le Thomas et al. 2013; Rozhkov et al. 2013). However, the underlying mechanism remains completely unknown.

To identify genes required for the TE silencing mediated by Piwi–piRISCs, we used a candidate approach. An earlier study showed that mouse gametocyte-specific factor 1 (GTSF1) is required for TE silencing in the testis (Yoshimura et al. 2009). GTSF1 knockout male mice show infertility owing to a lack of both DNA methylation and subsequent TE silencing during meiosis in spermatocytes, phenocopying mice lacking Mili or Miwi2 (Yoshimura et al. 2007, 2009; Aravin et al. 2008; Kuramochi-Miyagawa et al. 2008; Pillai and Chuma 2012), both of which encode mouse PIWI proteins. In the present study, we knocked down four homologs of GTSF1 in a cultured Drosophila OSC line (Saito et al. 2009) and found that depletion of one particular homolog, CG3893, results in derepression of TEs and neighboring genes, although the level and localization of Piwi–piRISCs were unchanged. We term CG3893 as DmGTSF1 hereafter. DmGTSF1 is a nuclear protein that interacts with Piwi, and a lack of DmGTSF1 results in strong enrichment of RNA polymerase II (Pol II) and less efficient enrichment of trimethylated histone H3 Lys 9 [H3K9me3] at TE loci on the genome, suggesting that DmGTSF1 is an integral factor in Piwi–piRISC-mediated transcriptional silencing.

Results and Discussion

OSCs were transfected with individual siRNAs against DmGTSF1 and its paralogs, CG14036, CG32625, and CG34283 (Supplemental Fig. S1A), and Northern blotting and RT-qPCR were performed to examine the levels of piRNAs and mdg1 transcripts, respectively, in the transfected cells. None of the siRNAs changed the expression

Keywords: DmGTSF1; piRNA; Piwi; transposon; RNA silencing; Drosophila
levels of piRNAs, Idefix-piR-1, and/or traffic jam-piR-1 (Fig. 1A; Supplemental Fig. S1B). However, knockdown of DmGTSF1, but not of its paralogs, increased the level of mdg1 [Fig. 1B; Supplemental Fig. S1C], a long terminal repeat element, suggesting that only DmGTSF1 is required for TE silencing. Depletion of Maelstrom (Mael), a known piRNA factor (Lim and Kai 2007), led to almost identical phenotypes [Fig. 1A,B]. A recent study showed that Mael is required for transcriptional TE silencing mediated by Piwi-piRISC but not for piRNA biogenesis (Sienski et al. 2012), suggesting the existence of functional parallels between Mael and DmGTSF1. Immunofluorescence staining for Myc-DmGTSF1 in OSCs revealed that DmGTSF1 shows a nuclear localization, as does Piwi (Fig. 1C). This further supports the idea that DmGTSF1 functions in nuclear TE silencing. Depletion of HP1a derepressed mdg1 [Fig. 1B], consistent with the earlier observation that HP1a functions in TE silencing in the ovaries (Wang and Elgin 2011). The expression levels of piRNAs were unaffected by a lack of HP1a [Fig. 1A; Saito et al. 2010]. Thus, piRNA biogenesis does not require HP1a. Loss of Armis, a piRNA biogenesis factor (Olivieri et al. 2010; Saito et al. 2010), results in down-regulation of piRNAs, verifying our results.

To investigate the roles of DmGTSF1 in fertility, females of the P-element insertion line P[w+mC]cesG5V6/cGS12962 (Supplemental Fig. S2A), in which DmGTSF1 mRNA and its protein product are expressed below detection levels [Fig. 2A; Supplemental Fig. S2B], were crossed with Oregon K male flies. DmGTSF1+/- females laid no eggs [data not shown]. In contrast, females in the reciprocal cross laid eggs [data not shown]. The DmGTSF1 transcript was barely detected in testes (Graveley et al. 2011), and DmGTSF1 +/- testes appeared normal [data not shown]. Thus, DmGTSF1 is specifically required for female fertility.

DmGTSF1+/- ovaries appeared rather small and severely deformed, similar to those observed in Piwi mutants (Lin and Spradling 1997). A comparison of Aub immunostaining images with images showing DAPI-stained nuclei revealed that DmGTSF1+/- ovaries lack a follicle cell layer, which encapsulates each egg chamber in DmGTSF1+/- ovaries [Fig. 2B].

Immunostaining was also performed using antibodies against the polar follicle cell marker FasIII (Ruohola et al. 1991) and the oocyte marker protein Orb (Christerson and McKearin 1994). In DmGTSF1+/- ovaries, FasIII is detected in monolayered follicle cells in both the germarium and early stage egg chambers, but later, its expression becomes restricted to two polar follicle cells in individual egg chambers, as has been observed in wild-type ovaries (Fig. 2C; Ruohola et al. 1991). However, in DmGTSF1+/- ovaries, monolayered FasIII-positive cells were lost at early stages, rather, FasIII-positive cells were dispersed within the germarium-like structures. Mutant egg chambers at later stages contained multiple FasIII-positive cells [Fig. 2C].

Polar cell specification is likely disrupted owing to the loss of DmGTSF1.

Orb expression is normally activated during mitotic division. Then, Orb gradually accumulates in oocytes during development (Christerson and McKearin 1994). This spatiotemporal expression pattern of Orb was observed in DmGTSF1+/- ovaries (Fig. 2D). However, in DmGTSF1+/- ovaries, Orb appeared in all cells within the early cysts, and its expression was seen in multiple cells in late egg chambers [Fig. 2D]. These DmGTSF1 mutant phenotypes are very similar, if not identical, to those of piRNA pathway mutants: The number of follicle cells was also reduced in the ovaries of mutant fs(1)Yb (Sakmary et al. 2009; Olivieri et al. 2010; Saito et al. 2010). Fusion of egg chambers caused by loss of Mael and fs(1)Yb also resulted in aberrant multiplicity of Orb-positive cells in late egg chambers (Johnson et al. 1995; Saito et al. 2011). These overlapping phenotypes further suggest functional parallels between Mael and DmGTSF1.

DmGTSF1 mutant flies appear otherwise normal. This correlates well with the observation that a subset of TEs was derepressed in DmGTSF1+/- ovaries but not in the carcases of DmGTSF1-/- flies (Supplemental Fig. S2C). The RT-qPCR results confirm the requirement of DmGTSF1 for TE silencing not only in OSCs, but also in the ovaries.

To compare the levels of transcripts globally, we performed mRNA sequence [mRNA-seq] analysis of OSCs transfected with siRNAs against the known piRNA pathway genes Piwi, Mael, and Armi along with DmGTSF1 and normalized the expression levels to those in OSCs transfected with control siRNAs against EGFP. mRNA-seq samples were prepared at 48 h post-transfection, yielding 168,369,656 genome-mapped reads in total (Supplemental Table S1). Reads per kilobase per million mapped reads [RPKM] values for annotated genes were highly correlated between Piwi knockdown and control knockdown cells ($R^2 = 0.99$). In contrast, several of the 125 annotated Drosophila melanogaster TE families showed strong increases in RNA levels [Fig. 3A,B]. For example, the transcript levels for the long terminal repeat elements mdg1, gypsy, and 297 were increased by >156-fold, >32-fold, and >17-fold, respectively, upon Piwi knockdown,
in OSCs. RT-qPCR confirmed that but also DmGTSF1 function in the Piwi–piRISC pathway (Okamura et al. 2004), was detected with antibody was probed with an anti-Piwi antibody in vivo. Myc-DmGTSF1 fused C-terminally with EGFP (Myc-DmGTSF1-EGFP) was expressed in OSCs, and the complex immunoprecipitated with an anti-Myc antibody was probed with an anti-Piwi antibody. Myc-DmGTSF1-EGFP and endogenous Piwi coprecipitated (Fig. 4A) even in the presence of RNase A (Supplemental Fig. S5A).

An in vitro pull-down assay using recombinant DmGTSF1 fused to glutathione S-transferase (GST-DmGTSF1) and crude OSC lysates confirmed their association (Fig. 4B; Supplemental Fig. S5B): Piwi was detected with GST-Pimet/DmHen1, which interacts with Piwi and 2′-O-methylates piRNAs upon Piwi loading (Horwich et al. 2007; Saito et al. 2007). Under the conditions we used, Mael was not detected in the DmGTSF1 complex (Supplemental Fig. SSC), although endogenous Mael was partially localized to the nucleus (Supplemental Fig. SSSD). Moreover, neither Armi nor AGO1, another Argonaute protein and a component of the microRNA pathway in Drosophila (Okamura et al. 2004), was detected with GST-DmGTSF1 (Fig. 4B). Armi is cytoplasmic and interacts with Piwi (Olivieri et al. 2010; Saito et al. 2010), suggesting that Piwi changes protein interactors upon nuclear import.

DmGTSF1 consists of 167 amino acid residues and contains two U11-48K-like CHHC-type Zn finger motifs supporting the recent results of Sienski et al. (2012). Almost identical results were obtained upon knockdown of Armi, Mael, and DmGTSF1 (Fig. 3B, Supplemental Fig. S3B). This suggests that not only Armi [Haase et al. 2010; Olivieri et al. 2010; Saito et al. 2010] and Mael [Sienski et al. 2012] but also DmGTSF1 function in the Piwi-piRISC pathway in OSCs. RT-qPCR confirmed that mdg1, but not other TEs such as I-element and Het-A, was markedly derepressed in Piwi- and DmGTSF1-depleted cells (Supplemental Fig. S3A). Although HP1α is required for silencing of various TEs in OSCs [Fig. 3B], the TE derepression pattern was rather different in HP1α-depleted cells ($R^2 = 0.49$) [Supplemental Fig. S3B]. Depletion of DmGTSF1 does not affect the levels or cellular localization of Piwi and Mael in OSCs (Supplemental Fig. S3C,D; data not shown), confirming that the observed defective phenotypes were not due to a secondary effect of DmGTSF1 depletion. Likewise, loss of Mael and HP1α did not change the cellular localization of DmGTSF1 (Supplemental Fig. S3E).

Piwi-piRISCs influence the expression of protein-coding genes near TE insertions (Sienski et al. 2012). To assess the role of DmGTSF1 in this effect, we analyzed our mRNA-seq data for 28 genes shown in the previous study [Fig. 3C,D; Sienski et al. 2012]. As expected, the expression levels of these neighboring genes were comparable between DmGTSF1 and Piwi knockdown cells. The results for some genes [for example, bab1 and CG12477] did not coincide with those of the previous study [Sienski et al. 2012], although transposon insertions near the genes were confirmed by PCR [Supplemental Fig. S4]. This variation may be due to subtle unexpected differences in knockdown and/or cell manipulation conditions. Collectively, all of these bioinformatic data strongly support the notion that DmGTSF1 is an integral factor in the Piwi-piRNA pathway.

Both Piwi and DmGTSF1 are localized to the nucleus (Fig. 1C; Supplemental Fig. S3D). We then asked whether DmGTSF1 forms a complex with Piwi in vivo. Myc-DmGTSF1 was partially localized to the nucleus (Fig. S3C,D; data not shown), although endogenous Mael was detected in the DmGTSF1 complex (Supplemental Fig. SSC), although endogenous Mael was partially localized to the nucleus (Supplemental Fig. SSSD). Moreover, neither Armi nor AGO1, another Argonaute protein and a component of the microRNA pathway in Drosophila (Okamura et al. 2004), was detected with GST-DmGTSF1 (Fig. 4B). Armi is cytoplasmic and interacts with Piwi (Olivieri et al. 2010; Saito et al. 2010), suggesting that Piwi changes protein interactors upon nuclear import.
DmGTSF1 in Piwi-mediated silencing

would affect Piwi–piRISC targeting to TE loci on the genome. Piwi-RNAi and Mael-RNAi were carried out as control experiments (Sienski et al. 2012). Chromatin complexes were immunoprecipitated using anti-Pol II and anti-H3K9me3, from which DNA fragments were prepared and subjected to quantitative PCR (qPCR) for quantification of mdg1, gypsy, and roo elements in the complexes. Pol II accumulation to specific TEs and/or genes indicates derepression of the genomic elements (because they are transcribed by Pol II), whereas H3K9me3 accumulation indicates silencing [H3K9me3 is a silent chromatin marker]. We found that a lack of DmGTSF1 resulted in accumulation of Pol II (Fig. 5A; Supplemental Fig. S7A) and less efficient enrichment of H3K9me3 (Fig. 5B) at mdg1 and gypsy loci on the genome, although accumulation at roo loci was hardly affected. These results are consistent with the mRNA-seq data shown in Figure 3A. Thus, DmGTSF1 is required for Piwi–piRISCs to regulate target TE loci on the genome. Depletion of DmGTSF1 did not change the cellular localization of Piwi [Myc-Pwi in Supplemental Fig S3D]. Likewise, depletion of Piwi did not change the localization of DmGTSF1 [Myc-DmGTSF1 in Supplemental Fig S7B]. We propose that DmGTSF1, which is a component of nuclear Piwi–piRISCs, acts as a cofactor during Piwi-mediated transcriptional transposon silencing at the genomic sites where Piwi–piRISCs exert a silencing function.

Previous studies have shown that Piwi is imported to the nucleus only after being loaded with mature piRNAs at cytoplasmic Yb bodies in ovarian somas (Saito et al. 2010). In other words, cells use an elaborate system to avoid localization of Piwi to the nucleus when the protein is devoid of piRNAs. This “gatekeeping” regulation is crucial for control of cellular events and gonadal development because Piwi devoid of piRNAs in the nucleus would not know what to target. This study and an earlier one (Sienski et al. 2012) showed that two factors, Mael and DmGTSF1, are required for the targeting of Piwi–piRISCs to achieve efficient TE silencing in the nucleus. Currently, it is unclear why this type of nuclear regulation is required and what functions of Mael and DmGTSF1 are required. To determine this, it will be important to isolate the nuclear Piwi–piRISC as a full unit, identify the components, and unveil their functions and the relationships among them. This would shed new light on nuclear RNA silencing pathways.

Materials and methods

Knockdown, Northern blotting, Western blotting, antibody production, immunoprecipitation, immunofluorescence, RNA sequencing (RNA-seq) analysis, plasmid construction, rescue assay, GST pull-down assay, and RT-qPCR analysis

siRNA transfection, Northern blotting, Western blotting, production of anti-DmGTSF1 antibody, immunoprecipitation, immunofluorescence,
GST pull-down assay, and RT-qPCR analysis were performed as previously described (Supplemental Material; Saito et al. 2007, 2010). DNA oligonucleotides used in RT-qPCR are summarized in Supplemental Table S2. RNA-seq analysis, plasmid construction, and rescue assay are shown in the Supplemental Material.

**Drosophila strains and brooding analysis**

Yellow white (yw) and Oregon R were employed as wild-type strains. The DmGTSF1 allele used was P[w+>c/+];GSV6(GS29227;TM3; Drosophila Genomics Resource Center stock no. 204406). All stocks were maintained at 25°C. Mating tests were done in batches of test males and at least 10 virgin females per vial. The presence or absence of progeny was scored after at least 7 d. For brooding tests, virgin DmGTSF1<sup>++/−</sup> and DmGTSF1<sup>−/−</sup> females were mated to Oregon R males in yeasted apple agar plates. The females were left in the plate to continue to lay eggs until the 10th day after mating. Laid eggs on the plate were counted on the fourth, seventh, and 10th days under a microscope.

**ChIP assay**

Cells (2 × 10<sup>6</sup>) were cross-linked with 1% formaldehyde for 10 min at room temperature, and cross-linking was then stopped with 125 mM glycine for 5 min at room temperature. The DNA–protein complexes were lysed with 1% lysis buffer [50 mM HEPES-KOH at pH 7.4, 150 mM sodium chloride, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, protease inhibitors]. The cross-linked chromatin was sheared into ~200 base pairs using Covaris S2 ultrasonicators. The DNA-protein complexes were incubated with nonimmune IgG antibody, anti-Pol II antibody 8WG16 [Santa Cruz Biotechnology], or anti-H3K9me3 antibody (Abcam, 8898) immobilized on Dynabeads-Protein G for 2 h at 4°C. The beads were washed with high-salt lysis buffer [50 mM HEPES-KOH at pH 7.4, 450 mM sodium chloride, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, protease inhibitors] and wash buffer [50 mM Tris-HCl at pH 8.0, 1 mM EDTA, 250 mM lithium chloride, 0.5% NP-40, 0.5% sodium deoxycholate] followed by TE (10 mM Tris-HCl at pH 8.0, 1 mM EDTA). After reversing the cross-linking for 12–16 h at 65°C, samples were treated with RNase A for 30 min at 37°C, and then DNA was recovered. Short DNA fragments corresponding to retrotransposons were amplified using a LightCycler 480 Real-Time PCR Instrument II with SYBR Premix Ex Taq (Takara). The primers used are listed in Supplemental Table S2. Pol II ChIP sequencing (ChIP-seq) libraries for EGFP, Piwi, Mael, and DmGTSF1 knockdown OSCs were analyzed using a single lane of Illumina MiSeq. This yielded ~2.6 million to 3.4 million genome-mapped reads in each transfected sample. For computational analyses, we trimmed for adapter sequences, mapped the reads to *Drosophila* genome release dm3, and calculated reads per million genome mappers (RPM) values as described within the RNA-seq computational analysis section. Sequencing data are available through Gene Expression Omnibus (accession no. GSE47006).

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**Note added in proof**

While this manuscript was under revision, Muerdter et al. [2013] reported that DmGTSF1/CG3893 is required for TE silencing. The investigators termed DmGTSF1/CG3893 as Asterix [Muerdter et al. 2013].


