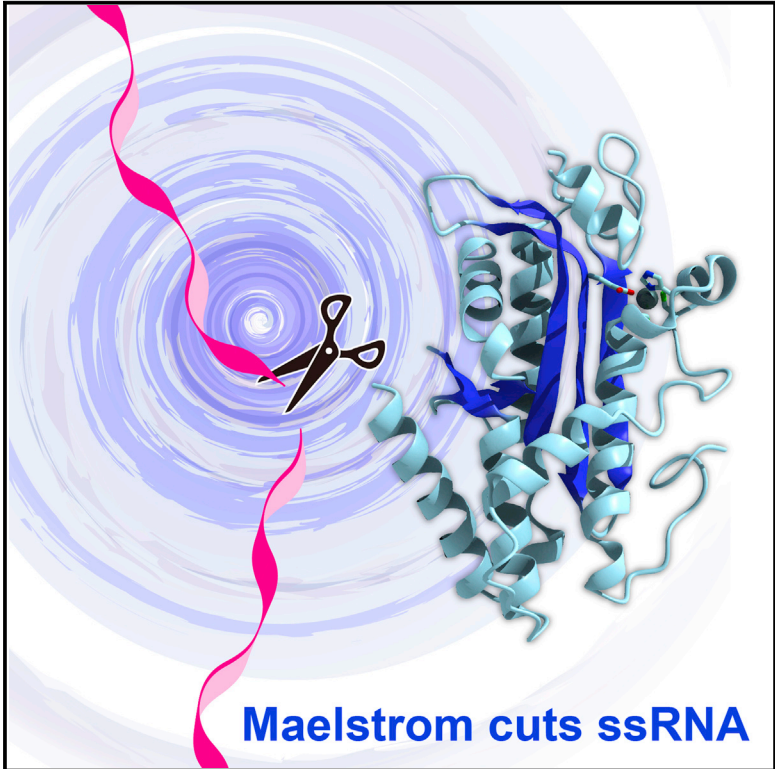


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Crystal Structure and Activity of the Endoribonuclease Domain of the piRNA Pathway Factor Maelstrom

Graphical Abstract



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In Brief

Maelstrom (Mael) is essential for transposon silencing in the piRNA pathway. Using structural and functional approaches, Matsumoto et al. show that Mael lacks the canonical nuclease active-site structure but exhibits single-stranded RNA cleavage activity, unrelated to transposon silencing, in flies.

Highlights

- Crystal structure of the MAEL domain in *Drosophila* Maelstrom is determined
- The MAEL domain has an RNase H-like fold but lacks canonical catalytic residues
- The MAEL domain shows single-strand-specific endoribonuclease activity
- The ssRNase activity of Mael is unrelated to transposon silencing

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Crystal Structure and Activity of the Endoribonuclease Domain of the piRNA Pathway Factor Maelstrom

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SUMMARY

PIWI-interacting RNAs (piRNAs) protect the genome from transposons in animal gonads. Maelstrom (Mael) is an evolutionarily conserved protein, composed of a high-mobility group (HMG) domain and a MAEL domain, and is essential for piRNA-mediated transcriptional transposon silencing in various species, such as *Drosophila* and mice. However, its structure and biochemical function have remained elusive. Here, we report the crystal structure of the MAEL domain from *Drosophila melanogaster* Mael, at 1.6 Å resolution. The structure reveals that the MAEL domain has an RNase H-like fold but lacks canonical catalytic residues conserved among RNase H-like superfamily nucleases. Our biochemical analyses reveal that the MAEL domain exhibits single-stranded RNA (ssRNA)-specific endonuclease activity. Our cell-based analyses further indicate that ssRNA cleavage activity appears dispensable for piRNA-mediated transcriptional transposon silencing in *Drosophila*. Our findings provide clues toward understanding the multiple roles of Mael in the piRNA pathway.

INTRODUCTION

Small RNA-based defense systems repress the aberrant expression of transposable elements (TEs) and thus maintain genome integrity in animal gonads (Malone and Hannon, 2009; Siomi et al., 2011). The germline-specific PIWI clade of Argonaute family proteins and the 23- to 30-nt noncoding PIWI-interacting RNAs (piRNAs) are the core of this defense system. PIWI proteins bind piRNAs to form piRNA-induced silencing complexes (piRISCs), which silence their complementary target TEs at the transcriptional or posttranscriptional level (Malone and Hannon, 2009; Siomi et al., 2011; Ishizu et al., 2012; Luteijn and Ketting,

2013). The *Drosophila* genome encodes three PIWI proteins: Piwi, Aubergine (Aub), and Argonaute3 (AGO3). The *Drosophila* ovary consists of two types of cells, somatic cells such as follicle cells, and germ cells such as nurse cells and oocytes. Piwi is localized in the nucleus in both somatic and germ cells, where it participates in the primary piRNA pathway (Czech et al., 2013; Handler et al., 2013; Olivieri et al., 2010). In contrast, Aub and AGO3 are enriched in cytoplasmic perinuclear granules called nuage in germ cells, where they participate in secondary piRNA biogenesis (Brennecke et al., 2007; Gunawardane et al., 2007; Li et al., 2009; Malone et al., 2009). In the primary piRNA pathway in *Drosophila* ovarian somatic cells, single-stranded, long piRNA precursors are transcribed from discrete genomic loci, called piRNA clusters, and are processed into mature piRNAs by the single-strand-specific endoribonuclease Zucchini (Zuc) (Ipsaro et al., 2012; Nishimasu et al., 2012). The primary piRNAs are loaded into Piwi at cytoplasmic perinuclear Yb bodies (Saito et al., 2010). piRISC then enters the nucleus and promotes repressive histone H3 lysine 9 trimethylation (H3K9me3), thereby silencing target TEs at the transcriptional level (Sienski et al., 2012; Wang and Elgin, 2011; Le Thomas et al., 2013; Rozhkov et al., 2013). In the secondary piRNA biogenesis pathway, Aub and AGO3 reciprocally cleave sense and antisense TE transcripts, respectively (Brennecke et al., 2007; Gunawardane et al., 2007). This feed-forward piRNA amplification loop, called the ping-pong cycle, enables simultaneous secondary piRNA biogenesis and TE silencing.

Maelstrom (Mael) is an evolutionarily conserved protein implicated in the piRNA pathway (Lim and Kai, 2007; Soper et al., 2008; Aravin et al., 2009; Sienski et al., 2012; Castañeda et al., 2014). In somatic cells of the fly ovary, Mael is predominantly localized in the nucleus (Sienski et al., 2012). In contrast, in germ cells of the fly ovary and mouse testis, Mael is localized in both the nucleus and cytoplasmic granules (nuage in flies, piP-bodies or chromatoid bodies in mice) (Findley et al., 2003; Costa et al., 2006; Lim and Kai, 2007; Soper et al., 2008; Aravin et al., 2009; Sato et al., 2011; Castañeda et al., 2014). Mael is also implicated in various biological processes, such as oocyte development and germline stem cell (GSC) differentiation (Clegg

et al., 1997, 2001; Pek et al., 2009, 2012; Sato et al., 2011). Mael is composed of an N-terminal HMG domain and a central MAEL domain, which was predicted to adopt an RNase H-like fold by a bioinformatics analysis (Zhang et al., 2008) (Figures 1A and S1). However, the biochemical function of Mael remains elusive.

In cultured *Drosophila* ovarian somatic cells (OSCs) (Niki et al., 2006; Saito et al., 2009), *mael* knockdown (KD) did not affect piRNA biogenesis but resulted in the derepression of TEs, indicating that Mael is essential for Piwi-mediated TE silencing (Sienski et al., 2012). Intriguingly, *mael* KD only modestly impacted the H3K9me3 patterns at the target heterochromatic loci, suggesting that Mael acts downstream of or in parallel to the Piwi-mediated H3K9me3 modification (Sienski et al., 2012). In addition, Mael has been implicated in piRNA biogenesis in germ cells of the fly ovary and mouse testis (Lim and Kai, 2007; Sienski et al., 2012; Aravin et al., 2009; Castañeda et al., 2014). Despite the crucial role of Mael in the piRNA pathway, the molecular mechanism of Mael/Piwi-mediated TE silencing remains elusive, due to the lack of structural and biochemical information.

In this study, we solved the crystal structure of the MAEL domain of *D. melanogaster* Mael. The structure revealed that the MAEL domain adopts an RNase H-like fold but lacks the canonical catalytic residues conserved among the RNase H-like superfamily of endonucleases and exonucleases. Moreover, our biochemical and biological analyses revealed that the MAEL domain has single-stranded RNA (ssRNA) cleavage activity, which appears dispensable for Mael/Piwi-mediated transcriptional TE silencing in *Drosophila* OSCs. Our findings provide clues toward understanding the multiple functions of Mael in the piRNA pathway.

RESULTS

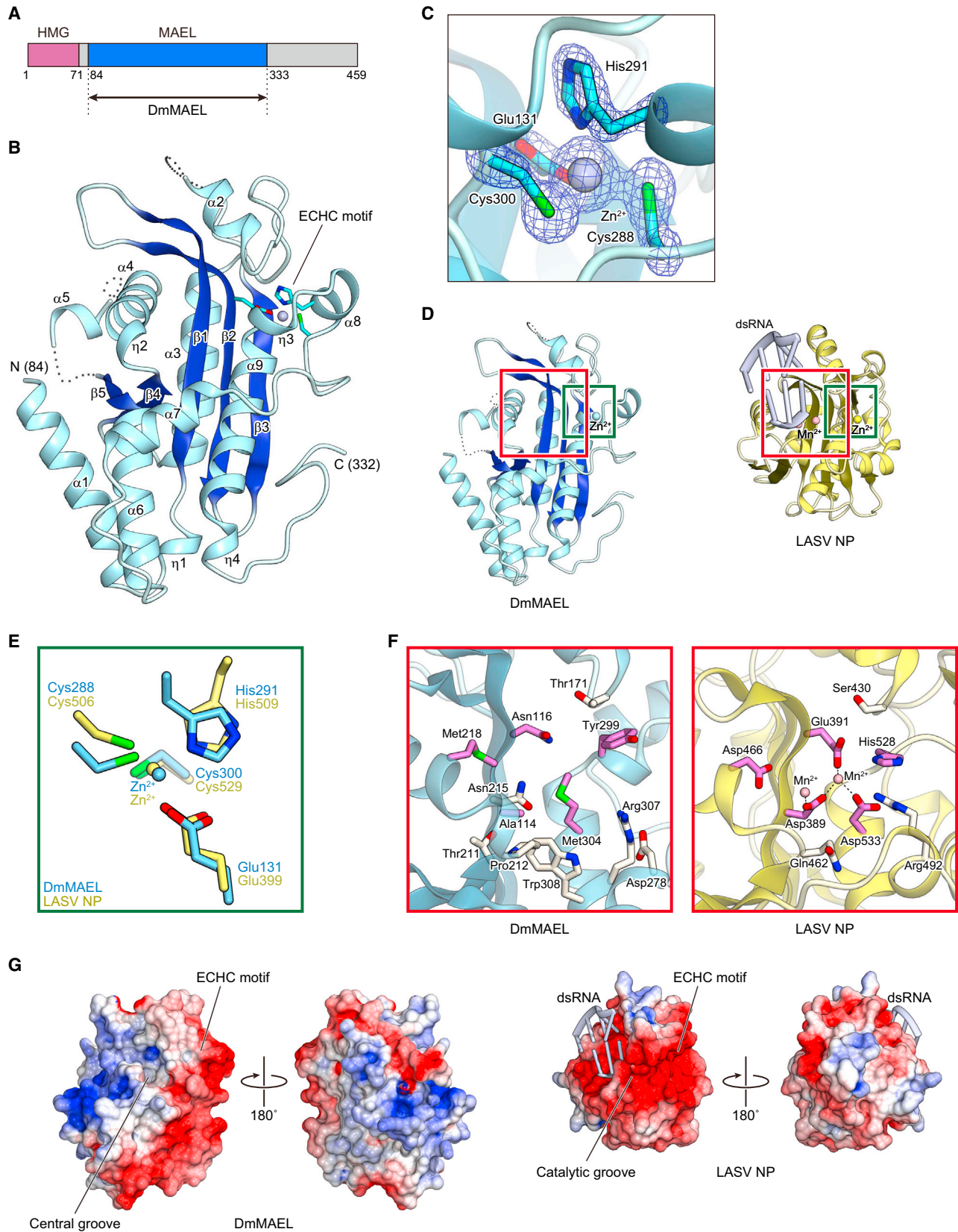
Crystal Structure of the MAEL Domain from *D. melanogaster* Mael

To gain mechanistic insights into the function of Mael, we attempted to determine the crystal structure of full-length *D. melanogaster* Mael (residues 1–459, referred to as FL-DmMael) but were hampered by its low expression levels in *Escherichia coli*. Limited trypsin proteolysis of FL-DmMael revealed that the MAEL domain (residues 84–333, referred to as DmMAEL) is a well-expressed, stable region suitable for structural analysis (Figure 1A). Furthermore, we found that the substitution of a less-conserved cysteine residue (Cys228) with serine dramatically improved the diffraction quality. X-ray fluorescence spectra of the crystal indicated that DmMAEL binds a zinc ion, consistent with a previous bioinformatics analysis suggesting that a zinc ion is coordinated by the ECHC motif, which is conserved among Mael orthologs (Zhang et al., 2008) (Figures S1 and S2). We determined the crystal structure of DmMAEL (C228S) at 1.6 Å resolution by the single-wavelength anomalous diffraction (SAD) method, using the intrinsic zinc atom (Figure 1B; Table S1). The structure revealed that DmMAEL consists of a twisted five-stranded mixed β sheet surrounded by 13 helices, with a zinc ion coordinated by Glu131, Cys288, His291, and Cys300 in the ECHC motif (Figure 1C).

A Dali search (Holm and Rosenström, 2010) revealed that DmMAEL shares structural similarity with the RNase H-like superfamily of endonucleases and exonucleases (Majorek et al., 2014), especially with the DEDDh family exonucleases, such as a Lassa virus nucleoprotein (LASV NP) (PDB 4GV9) (Jiang et al., 2013). LASV NP is a 3′–5′ exonuclease involved in the suppression of virus-induced interferon production (Martínez-Sobrido et al., 2007; Qi et al., 2010; Hastie et al., 2011; Jiang et al., 2013). Despite their low sequence identity (~13%), DmMAEL shares an RNase H-like fold, consisting of a five-stranded β sheet flanked by α helices on both sides, with LASV NP (Jiang et al., 2013) (root mean square deviation of 2.9 Å for 131 aligned C α atoms) (Figure 1D). Like DmMAEL, LASV NP contains a zinc ion coordinated by the ECHC motif, consisting of Glu399, Cys506, His509, and Cys529, which may contribute to structural stabilization or substrate binding (Qi et al., 2010; Hastie et al., 2011) (Figures 1E and S3A). In DmMAEL, the bound zinc ion may play at least a structural role, since point mutations of the ECHC motif drastically reduced the solubility of DmMAEL in vitro (data not shown). The DEDDh family exonucleases have a negatively charged catalytic groove formed by five invariant catalytic residues (Asp, Glu, Asp, Asp, and His; DEDDh motif) and cleave double-stranded RNAs (dsRNAs) through a two-metal-ion mechanism (Zuo and Deutscher, 2001). In LASV NP, the catalytic groove is formed by Asp389, Glu391, Asp466, Asp533, and His528 in the DEDDh motif and the highly conserved Ser430, Gln462, and Arg492 residues (Hastie et al., 2012; Jiang et al., 2013) (Figures 1F and S3B). The Asp389, Glu391, Asp466, Asp533, and His528 residues of LASV NP respectively correspond to the Ala114, Asn116, Met218, Met304, and Tyr299 residues of DmMAEL (Figures 1F and S3B). Consequently, the central groove of DmMAEL, which corresponds to the catalytic groove of LASV NP, is not negatively charged (Figure 1G). In addition, the residues in the central groove are not conserved among Mael orthologs (Figures S1 and S2), suggesting that the central groove is less important for the function of Mael. Taken together, the crystal structure of DmMAEL revealed that it adopts an RNase H-like fold but lacks the canonical catalytic residues conserved among RNase H-like superfamily members.

The MAEL Domain Has Single-Strand-Specific Endoribonuclease Activity

To determine whether DmMAEL is a nuclease, we measured the nuclease activity of purified DmMAEL using a 5′ ³²P-labeled 40-nt ssRNA (40AS ssRNA) as the substrate. Unexpectedly, DmMAEL cleaved the 40AS ssRNA (Figure 2A). The elution profile of purified DmMAEL correlated closely with that of the single-stranded ribonuclease (ssRNase) activity in gel-filtration chromatography (Figure 2A). Furthermore, DmMAEL cleaved the 40AS ssRNA in a dose- and time-dependent manner (Figures 2B and 2C). These results revealed that DmMAEL is a ssRNase. The ssRNase activity of DmMAEL did not require divalent metal ions, such as Mg²⁺ or Ca²⁺, and was rather inhibited in their presence (Figure 2D). These results are consistent with our structural finding that DmMAEL shares no catalytic residues with the DEDDh family members, which require divalent metal ions, such as Mg²⁺ and Mn²⁺, for substrate cleavage (Zuo and Deutscher, 2001). To determine the substrate specificity of



(legend on next page)

DmMAEL, we next measured the cleavage activity toward a series of 5' ³²P-labeled nucleic acid substrates. DmMAEL efficiently cleaved ssRNA, but neither dsRNA nor ssDNA (Figure 2E). DmMAEL cleaved circular 40AS ssRNA, indicating that DmMAEL is an endoribonuclease (Figure 2F). The cleavage pattern of the 40AS ssRNA revealed that DmMAEL preferentially cleaves ssRNA at a guanine residue (especially at successive guanine stretches) (Figure 2E). To exclude the possibility that the 40AS ssRNA adopts a secondary structure that affects the cleavage by DmMAEL, we measured the nuclease activity of DmMAEL toward 15-nt poly(A) RNA substrates with or without guanine residues, which are unlikely to adopt secondary structures. DmMAEL cleaved 15-nt poly(A) containing guanine residues, but not 15-nt poly(A), confirming that DmMAEL cleaves ssRNA at guanine residues (Figure 2G). We next compared the cleavage patterns of the 40AS ssRNA by DmMAEL and RNase T1, an endonuclease that specifically cleaves ssRNA at the 3' side of guanine residues (Pace et al., 1991). RNase T1 cleaved the 40AS ssRNA evenly at guanine residues (Figure 2H). In contrast, DmMAEL did not efficiently cleave the 40AS ssRNA at 3 nt from the 5' end (position 1) and 4 nt from the 3' end (position 6) (Figure 2H). The ssRNase activity of DmMAEL was inhibited in the presence of 25 mM NaCl, whereas that of RNase T1 remained robust in the presence of 100 mM NaCl (Figure 2I). These differences in their enzymatic properties indicated that the RNA cleavage mechanism of DmMAEL is distinct from that of RNase T1. To examine whether the nuclease activity is specific to *D. melanogaster* Mael, we measured the ssRNase activities of the purified MAEL domains from *Bombyx mori* Mael (residues 92–335, referred to as BmMAEL) and *Mus musculus* Mael (residues 83–327, referred to as MmMAEL) (Figure 2J). We found that both BmMAEL and MmMAEL cleave the 40AS ssRNA in similar manners to that of DmMAEL, although the ssRNase activity of MmMAEL was weaker than those of DmMAEL and BmMAEL (Figure 2J). Together, these biochemical data revealed that the MAEL domain is an evolutionarily conserved, single-strand-specific endoribonuclease.

Potential RNA-Binding Residues of the MAEL Domain

Since the MAEL domain lacks the canonical DEDDh motif, we tried to identify the catalytic residues of DmMAEL, based on the sequence conservation among Mael orthologs. However, multiple sequence alignments indicated that only the ECHC motif is solvent accessible and strictly conserved across the Mael ortho-

logs (Figures S1 and S2). Thus, based on the crystal structure of DmMAEL, we prepared 12 DmMAEL mutants, in which the solvent-exposed, hydrophilic residues were individually substituted with alanine (Figure 3A). All of the mutants eluted as a single monodisperse peak from the gel-filtration column (data not shown), confirming their structural integrity. We then examined the ssRNase activities of the purified mutants, using 40AS ssRNA as the substrate (Figure 3B). The K109A, K188A, N192A, and E292A mutants showed ssRNase activities comparable to that of the wild-type DmMAEL, and the K277A mutant showed moderately reduced ssRNase activity (Figure 3B). In contrast, the K140A, K199A, Q289A, D293A, D295A, D314A, and K328A mutants showed markedly reduced ssRNase activities (Figure 3B), indicating that Lys140, Lys199, Gln289, Asp293, Asp295, Asp314, and Lys328 are involved in the ssRNase activity. Although DmMAEL, BmMAEL, and MmMAEL cleaved ssRNA in a similar manner (Figure 2J), these residues (except for Asp295) are not conserved among the Mael orthologs (Figure S1). Moreover, these mutations reduced, but did not abolish, the ssRNase activity (Figure 3B), suggesting that these residues are involved in ssRNA binding, but not in catalysis. The positively charged residues, Lys140, Lys199, and Lys328, would interact with the negatively charged phosphate backbone of the ssRNA substrates. These residues are located on the opposite side of the central groove, which is equivalent to the catalytic groove of LASV NP (Figures 3C and 3D). To examine whether the central groove is involved in the ssRNase activity, we tried to prepare four additional DmMAEL mutants (N116A, M218A, Y299A, and M304A), in which the residues corresponding to the DEDDh motif were individually substituted with alanine. These four mutants were not expressed in *E. coli* as soluble proteins (data not shown), suggesting that these residues within the central groove contribute to structural integrity. In addition, the C228S mutant used for our structural analysis exhibited the ssRNase activity (Figure 3B), indicating that the C228S mutation does not have considerable impact on the structure and function of DmMAEL.

Since both DmMAEL and RNase T1 preferentially cleave ssRNA at guanine residues, we attempted to detect the structural similarity between them. RNase T1 cleaves ssRNA through a metal-ion-independent mechanism, in which a conserved histidine serves as a catalytic residue (Pace et al., 1991). Since His329 of DmMAEL is the only histidine residue conserved among DmMAEL, BmMAEL, and MmMAEL (Figure S1), we examined the RNase activity of the DmMAEL H329A mutant (Figure 3A). The H329A mutant retained

Figure 1. Crystal Structure of DmMAEL

(A) Domain structure of *D. melanogaster* Mael.

(B) Overall structure of DmMAEL. The zinc ion is shown as a gray sphere. Disordered regions (residues 156–162, 228–229, and 236–237) are shown as dashed lines.

(C) Close-up view of the ECHC motif. An $F_o - F_c$ simulated annealing omit map contoured at 3.5σ is shown as a blue mesh.

(D) Structures of DmMAEL (left) and LASV NP in complex with dsRNA (PDB ID 4GV9) (right). The zinc ions are shown as light blue and yellow spheres in DmMAEL and LASV NP, respectively. The manganese ions are shown as pink spheres. The ECHC motif and the central groove are indicated by green and red boxes, respectively.

(E) Superimposition of the ECHC motifs of DmMAEL and LASV NP.

(F) Central grooves of DmMAEL (left) and LASV NP (right). In LASV NP, the DEDDh-motif and RNA-binding residues are shown as magenta and white sticks, respectively. The bound manganese ions are shown as pink spheres, and the bound dsRNA is omitted for clarity. In DmMAEL, the equivalent residues in the central groove are shown in the same color code. Coordination bonds are shown as dashed lines.

(G) Electrostatic surface potentials of DmMAEL and LASV NP (contoured from -5 kT/e [red] to $+5$ kT/e [blue]).

See also Figures S1–S3 and Table S1.

the ssRNase activity, indicating that His329 is not involved in ssRNA cleavage (Figure 3B). Thus, despite our numerous attempts, the RNA cleavage mechanism remains to be elucidated. Nonetheless, our mutational analyses support the notion that Mael does not share an active site with either RNase T1 or the DEDDh family members.

The ssRNase Activity of Mael Appears Dispensable for Piwi/Mael-Mediated TE Silencing in *Drosophila* OSCs

To examine the contribution of the MAEL domain to Piwi/Mael-mediated TE silencing, we overexpressed DmMAEL, FL-DmMael, the four ECHC-motif single mutants (E131A, C288A, H291A, and C300A) of FL-DmMael, the ECHC-motif quadruple mutants (E131A/C288A/H291A/C300A) of FL-DmMael, and DmMAEL in *mael*-depleted OSCs and then monitored the expression levels of TEs by qRT-PCR. FL-DmMael and DmMAEL rescued the derepression of a variety of somatic TEs (*mdg1*, *297*, *blood*, *Tabor*, *gypsy*, and *ZAM*) (Figures 4A, 4B, and S4A), indicating that the MAEL domain plays a central role in Piwi/Mael-mediated TE silencing in *Drosophila* OSCs, consistent with a previous report (Sienski et al., 2012). All of the ECHC-motif mutants of FL-DmMael and DmMAEL failed to rescue TE derepression (Figures 4A and S4B), highlighting the functional significance of the ECHC motif for Piwi/Mael-mediated TE silencing.

To examine whether the ssRNase activity of DmMAEL is involved in Piwi/Mael-mediated TE silencing, we next overexpressed the seven ssRNase-deficient DmMAEL mutants (K140A, K199A, Q289A, D293A, D295A, D314A, and K328A) in *mael*-depleted OSCs and then monitored the levels of TE derepression. Like wild-type DmMAEL, all seven of the ssRNase-deficient mutants rescued TE derepression (Figures 4A and S4C). Since the ECHC-motif mutants of DmMAEL were not expressed in *E. coli* as soluble proteins, we could not examine their ssRNase activities *in vitro*. Overall, these results suggested that the ssRNA cleavage activity of Mael appears dispensable for Piwi/Mael-mediated TE silencing in *Drosophila* OSCs.

DISCUSSION

The present crystal structure revealed that DmMAEL adopts an RNase H-like fold but does not share catalytic residues

with the RNase H-like superfamily members. Unexpectedly, our biochemical analyses revealed that DmMAEL has ssRNase activity. We further showed that BmMAEL and MmMAEL also have the ssRNase activities, and we identified seven potential ssRNA-binding residues of DmMAEL. These results strongly support our surprising finding that the MAEL domain possesses the ssRNase activity, although it lacks the canonical active site conserved across the RNase H-like superfamily nucleases.

Previous studies showed that Mael participates in piRNA biogenesis in germ cells of the fly ovary and mouse testis (Lim and Kai, 2007; Sienski et al., 2012; Aravin et al., 2009; Castañeda et al., 2014). In the adult mouse testis, a ribonucleoprotein complex comprising Mael, the PIWI protein MIWI, and the Tudor-domain-containing protein TDRD6 is involved in the processing of precursor transcripts into mature pachytene piRNAs, a class of mammalian piRNAs (Castañeda et al., 2014). Notably, the nuclease activity of MIWI is not required for piRNA biogenesis (Reuter et al., 2011), and the ribonucleoprotein complex lacks a potential nuclease (Castañeda et al., 2014), suggesting that Mael may be responsible for the processing of pachytene piRNA precursors in adult mouse testis. This model is consistent with our finding that MmMAEL exhibits the ssRNase activity. Together, these observations suggested that the ssRNase activity of Mael is involved in the processing of piRNA precursors in mice.

A previous study showed that the MAEL domain plays a central role in Piwi/Mael-mediated TE silencing in *Drosophila* OSCs (Sienski et al., 2012). In OSCs, the *mael* KD has mild effects on the establishment of H3K9me3 but increases RNA polymerase II occupancy at target heterochromatic loci, thereby resulting in the derepression of TEs (Sienski et al., 2012). These observations indicated that Mael acts downstream of or in parallel to the H3K9me3 modification event. A large-scale genetic screen further indicated that, in addition to Piwi and Mael, the zinc finger domain-containing protein Gtsf1 (Dönertas et al., 2013; Ohtani et al., 2013) and several chromatin-associated factors, such as the histone deacetylase HDAC3 and the histone chaperone Asf1, are involved in the *Drosophila* somatic piRNA pathway (Handler et al., 2013; Muerdter et al., 2013). Consistent with the previous report (Sienski et al., 2012), our cell-based analysis indicated that the MAEL domain is involved in TE silencing in *Drosophila* OSCs. Our mutational analysis

Figure 2. ssRNA-Cleavage Activity of the MAEL Domain

- (A) ssRNase activity of DmMAEL. The gel-filtration chromatography elution profile of DmMAEL (top). SDS-PAGE analysis (middle) and ssRNase activity of fractions 1–8 (bottom). Each fraction (1 μ l) was incubated with the 40AS ssRNA and then analyzed by 15% denaturing PAGE.
- (B) Dose dependency of the DmMAEL ssRNase activity. DmMAEL (0.14–2.2 μ M) was incubated with the 40AS ssRNA at 26°C for 3 hr.
- (C) Time dependency of the DmMAEL ssRNase activity. DmMAEL (2.2 μ M) was incubated with the 40AS ssRNA at 26°C for 15–180 min.
- (D) Effect of divalent cations on the DmMAEL ssRNase activity.
- (E) Substrate specificities of DmMAEL. “S” and “AS” indicate the sense and antisense strands of dsRNA, respectively.
- (F) ssRNase activity of DmMAEL toward linear and circular 40AS ssRNA. Exonuclease T (ExoT) was used as a control.
- (G) ssRNase activity of DmMAEL toward 15-nt poly(A) ssRNA with and without guanine residues. The asterisk indicates the cleavage products.
- (H) Comparison of the ssRNase activities of DmMAEL with RNase T1. DmMAEL (0.14–2.2 μ M) or RNase T1 (0.5–10 units) was incubated with the 40AS ssRNA at 26°C for 3 hr. The nucleotide sequence of the substrate 40AS ssRNA is shown on the right of the gel, with guanine residues highlighted in bold. The predicted cleavage sites are indicated by red numbers.
- (I) Comparison of the ssRNase activities of DmMAEL and RNase T1 in the presence of NaCl.
- (J) ssRNase activities of DmMAEL, BmMAEL, and MmMAEL. SDS-PAGE analysis (left) and ssRNase activities (right) of purified DmMAEL, BmMAEL, and MmMAEL.

See also Table S2.

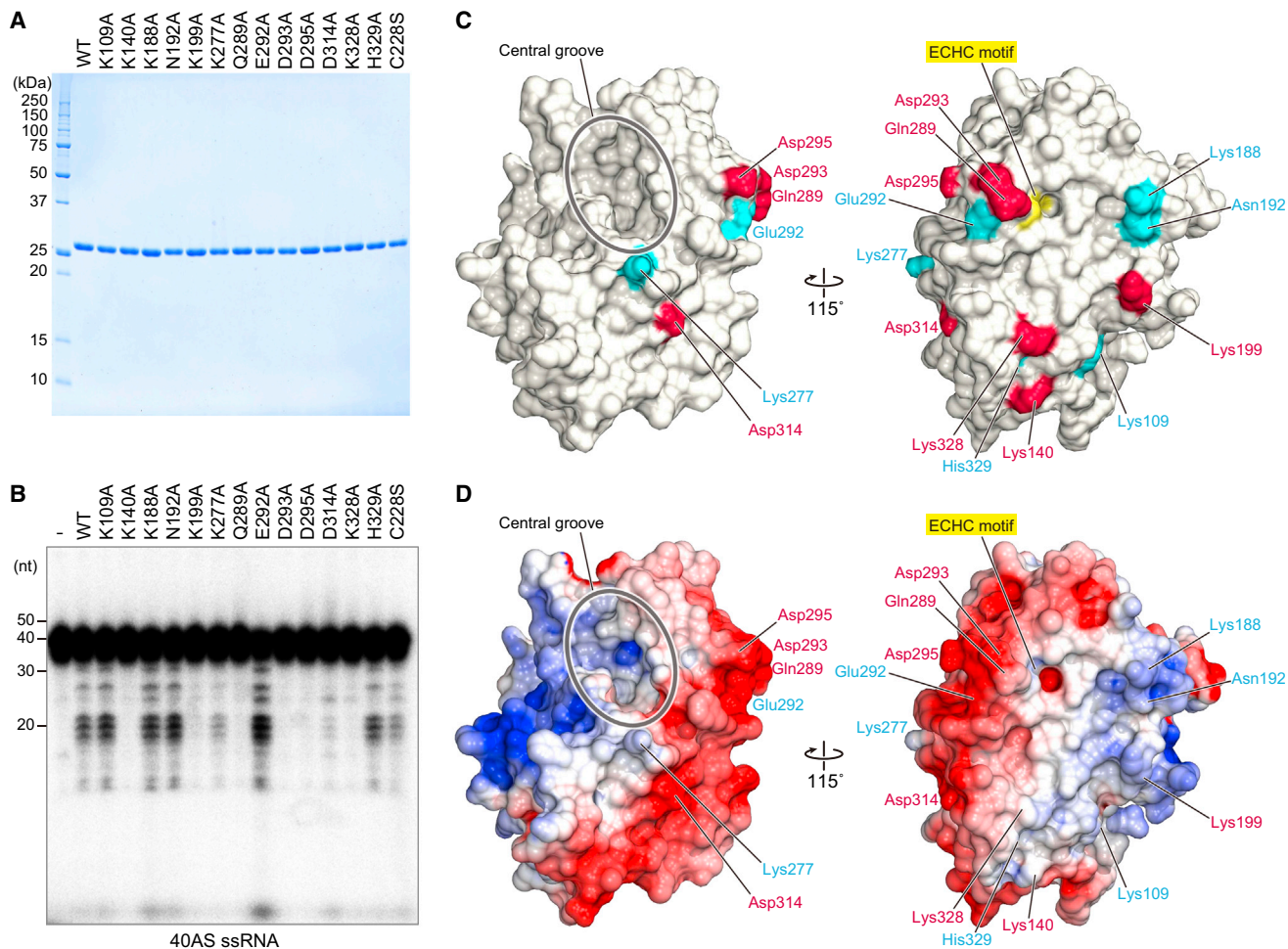


Figure 3. Potential ssRNA-Binding Residues of DmMAEL

(A) Purified wild-type and mutants of DmMAEL. WT, wild-type.

(B) ssRNase activities of purified wild-type and mutants of DmMAEL. The ssRNase activities were measured using the 40AS ssRNA as the substrate.

(C) Structural mapping of the ssRNase-deficient mutations. Residues involved in ssRNA cleavage are colored red, whereas residues not involved in ssRNA cleavage are colored cyan. The ECHC motif is colored yellow. The central groove is indicated by a gray circle.

(D) Electrostatic surface potentials of DmMAEL, viewed from the same direction as in (C) (contoured from -5 kT/e [red] to $+5$ kT/e [blue]).

further suggested that the ssRNase activity of the MAEL domain appears dispensable for TE silencing. Thus, we propose that Mael interacts with other piRNA factors via the MAEL domain and thereby participates in TE silencing in *Drosophila* OSCs.

A previous bioinformatics analysis suggested that the MAEL domain evolved from a DEDDh exonuclease by switching the catalytic residues from the DEDDh motif to the ECHC motif and that the MAEL domain may possess the nuclease activity (Zhang et al., 2008). Consistent with this, our structural and biochemical data revealed that the MAEL domain lacks the DEDDh motif but shows ssRNase activity. Given that the ECHC motif is strictly conserved among Mael orthologs (Figures S1 and S2), the ECHC motif may play a catalytic role in addition to a structural role. This idea is supported by the observation that Asp295 of DmMAEL, which is highly conserved and located close to the ECHC motif, is involved in the ssRNase activity.

If the ECHC motif participates in catalysis, then it is possible that the ssRNase activity of Mael is involved in TE silencing in *Drosophila* OSCs, since the ECHC-motif mutants failed to rescue TE derepression in our cell-based rescue experiments. All of the ssRNase-deficient DmMAEL mutants we examined in our cell-based assays retained slight ssRNase activities in vitro, which might be sufficient for TE silencing when overexpressed in OSCs. Indeed, in our previous study on Zuc, an endoribonuclease implicated in primary piRNA biogenesis, TE derepression was not rescued by the overexpression of the catalytically inactive Zuc mutant but was efficiently rescued by the overexpression of the RNA-binding-deficient Zuc mutants retaining residual ssRNase activity (Nishimasu et al., 2012). Thus, we cannot completely rule out the possibility that the ssRNase activity of Mael is required for TE silencing. To fully understand the multiple roles of Mael, an enigmatic key factor in the piRNA pathway, it will be critical to elucidate (1) its ssRNA cleavage mechanism,

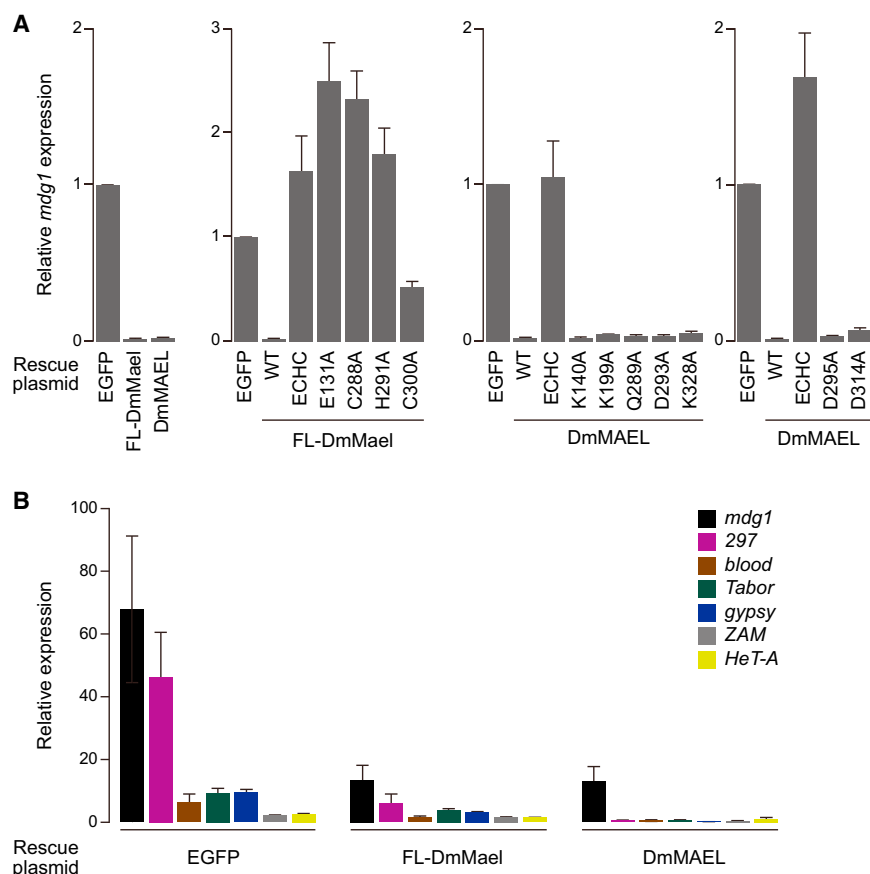


Figure 4. Effects of Mael Mutations on TE Silencing in OSCs

(A) Repression of the *mdg1* transposon by FL-DmMael, DmMAEL, the EHC-motif mutants of FL-DmMael, the EHC-motif quadruple mutant of DmMAEL, and the ssRNase-deficient mutants of DmMAEL. Myc-tagged proteins were overexpressed in *mael*-depleted *Drosophila* OSCs, and the expression levels of the *mdg1* transposon were monitored by qRT-PCR (n = 3; error bars indicate SEM). Myc-tagged EGFP was used as a control. The EHC mutant represents the E131A/C288A/H291A/C300A quadruple mutant.

(B) Repression of a subset of TEs by FL-DmMael and DmMAEL.

See also Figure S4 and Table S2.

(2) its endogenous ssRNA substrates, and (3) the physiological relevance of its ssRNase activity.

EXPERIMENTAL PROCEDURES

Detailed experimental procedures are described in Supplemental Experimental Procedures, and related sequences are shown in Table S2.

DmMAEL (residues 84–333, C228S) was expressed in *E. coli* as a His-tagged protein, and purified by chromatography on Ni-NTA Superflow (QIAGEN) and Resource Q (GE Healthcare) columns. Crystals were obtained at 20°C by the sitting-drop vapor diffusion method. X-ray diffraction data were collected on beamline BL32XU at SPring-8 (Hyogo). The crystal structure of DmMAEL was determined by the SAD method, using the intrinsic zinc atom.

For nuclease activity measurements, the wild-type and mutants of DmMAEL and BmMAEL (residues 92–335) were expressed in *E. coli* as His-tagged proteins, and the proteins were purified by chromatography on Ni-NTA Superflow, Resource Q, Resource PHE (GE Healthcare), and Superdex 200 10/300 (GE Healthcare) columns. MmMAEL (residues 83–327) was expressed in Sf9 insect cells as a His-SUMOstar-tagged protein (LifeSensors) and purified by chromatography using a similar protocol as for DmMAEL. Nuclease activity measurements were performed in buffer containing 25 mM HEPES-KOH (pH 7.4) and 5 mM DTT. Rescue experiments were performed essentially as described previously (Nishimasu et al., 2012).

ACCESSION NUMBERS

The atomic coordinates of DmMAEL have been deposited in the Protein Data Bank under accession number 4YBG. The sequencing data of *Bombyx*

mori Mael have been deposited in GenBank under accession number LC032360.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.03.030>.

AUTHOR CONTRIBUTIONS

N.M., K.S., H.S., H.N., M.C.S., and O.N. designed the experiments; N.M., R.I., and H.N. performed the structural analysis; N.M. performed the nuclease activity measurement; K.S., Y.N. and K.M. performed the cell-based analysis; N.D. performed the mass spectrometric analysis; N.M., K.S., H.N., M.C.S., and O.N. wrote the manuscript; and all authors discussed the data and the manuscript. H.N., M.C.S., and O.N. supervised all of the work.

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