The RNA helicase MOV10L1 binds piRNA precursors to initiate piRNA processing

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Piwi–piRNA (Piwi-interacting RNA) ribonucleoproteins [piRNPs] enforce retrotransposon silencing, a function critical for preserving the genome integrity of germ cells. The molecular functions of most of the factors that have been genetically implicated in primary piRNA biogenesis are still elusive. Here we show that MOV10L1 exhibits 5′-to-3′ directional RNA-unwinding activity in vitro and that a point mutation that abolishes this activity causes a failure in primary piRNA biogenesis in vivo. We demonstrate that MOV10L1 selectively binds piRNA precursor transcripts and is essential for the generation of intermediate piRNA processing fragments that are subsequently loaded to Piwi proteins. Multiple analyses suggest an intimate coupling of piRNA precursor processing with elements of local secondary structures such as G quadruplexes. Our results support a model in which MOV10L1 RNA helicase activity promotes unwinding and funneling of the single-stranded piRNA precursor transcripts to the endonuclease that catalyzes the first cleavage step of piRNA processing.

[Keywords: piRNA; Piwi; MOV10L1; Armitage; Argonaute; G quadruplex]

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Piwi-interacting RNAs [piRNAs] are a class of small RNAs [23–30 nucleotides [nt]] that bind to Piwi proteins to form piRNPs [Piwi–piRNA ribonucleoproteins], which silence selfish genetic elements in the germline of metazoans [Aravin et al. 2004, 2006; Girard et al. 2006; Grivna et al. 2006; Lau et al. 2006; Vagin et al. 2006; Kuramochi-Miyagawa et al. 2008; Ghildiyal and Zamore 2009; Siomi et al. 2011; Sienski et al. 2012; Huang et al. 2013; Rozhkov et al. 2013]. Loss of Piwi genes results in retrotransposon up-regulation, arrest of gametogenesis, and infertility [Cox et al. 1998; Harris and Macdonald 2001; Deng and Lin 2002, Kuramochi-Miyagawa et al. 2004; Carmell et al. 2007; Li et al. 2009]. In mice, Mili [Piwil2] and Miwi2 [Piwil4] loaded with retrotransposon-related piRNA precursors to establish silencing of long interspersed elements [LINE1 or L1] by DNA methylation during prenatal male gonad development [Carmell et al. 2007; Shoji et al. 2009]. Postnatally, Mili and Miwi [Piwil1] target L1 RNA for post-transcriptional silencing at the meiotic and post-meiotic stages of spermatogenesis, respectively [De Fazio et al. 2011; Reuter et al. 2011; Di Giacomo et al. 2013]. Additionally, Miwi is involved in the cytoplasmic remodeling of spermatids through the formation of the chromatoid body, and Miwi carries out a piRNA-independent stabilization of spermiogenic mRNA in round spermatids [Deng and Lin 2002; Vourekas et al. 2012].

Primary piRNAs are produced from long, single-stranded precursor transcripts in a stepwise process. The Zucchini endonuclease [Zuc, whose mouse homolog, mZuc, is also known as PLD6 or mitoPLD] [Pane et al. 2007; Huang et al. 2011; Watanabe et al. 2011; Ipsaro et al. 2012, Nishimasu et al. 2012] is most likely the nuclease that generates piRNA precursor intermediate fragments [PPIFs] that are loaded onto Piwi proteins, which stabilize their 5′ ends [Pillai and Chuma 2012; Vourekas et al. 2012]. Piwi proteins preferentially stabilize PPIFs with a 5′ U, thus generating a nucleotide bias that is inherited by the mature piRNA [Kawaoka et al. 2011; Ipsaro et al. 2012, Nishimasu et al. 2012; Vourekas et al. 2012].

High-throughput sequencing after cross-linking and immunoprecipitation [HITS-CLIP or CLIP-seq] provided in vivo evidence for PPIFs loaded to Mili and Miwi (Vourekas et al. 2015). These authors contributed equally to this work. Corresponding authors: mourelaz@uphs.upenn.edu, kezheng@njmu.edu.cn, pwang@vet.upenn.edu. Article published online ahead of print. Article and publication date are online at http://www.genesdev.org/cgi/doi/10.1101/gad.254631.114.
et al. 2012). A second processing step is the 3′-to-5′ exonucleolytic trimming of Piwi-loaded PPIFs by an enzymatic activity called Trimmer that has not yet been identified (Kawaoka et al. 2011). It is thought that 3′ trimming proceeds until it is sterically hindered by the Piwi protein, and it is functionally coupled with 2′-O methylation, a 3′ end modification of piRNAs catalyzed by Hen1 (Horwich et al. 2007; Kirino and Mourelatos 2007a,b; Saito et al. 2007; Kamminga et al. 2010; Kawaoka et al. 2011; Montgomery et al. 2012). Recently, it has been shown that a Tudor domain-containing [Tdrd] protein known as Tdrkh promotes the 3′ trimming process (Honda et al. 2013; Saxe et al. 2013). In addition to Piwi proteins and Zuc, other factors essential for piRNA processing, including GASH (germ cell protein with ankyrin repeats, sterile a motif, and leucine zipper) and Tdrd proteins (Hosokawa et al. 2007; Ma et al. 2009), are localized on the cytoplasmic surface of mitochondria in electron-dense granules known as intermitochondrial cement (ICM) in mammalian spermatocytes or more generally as nuage in Drosophila and other animals (Siomi et al. 2011; Pillai and Chuma 2012).

piRNA precursor genomic loci termed piRNA clusters, especially in Drosophila, contain sequence fragments that are antisense to retrotransposons as a form of molecular memory of past retrotransposon activity (Aravin et al. 2007; Brennecke et al. 2007). Piwi-loaded primary piRNAs derived from such clusters can target, through extensive sequence complementarity, and cleave retroelement-derived piRNA precursors and, by means of its ATP-dependent RNA helicase activity, funnels them to the endonuclease that catalyzes the first cleavage step of piRNA processing to generate piRNA intermediate fragments that are subsequently loaded to Piwi proteins.

**Results**

**MOV10L1 specifically binds piRNA precursors**

We performed MOV10L1 HITS-CLIP in testes from adult and 23-d post-partum (dpp) wild-type mice, as described previously for Mili and Miwi (Vourekas et al. 2012; Vourekas and Mourelatos 2014), without addition of exogenous nuclease to the cross-linked lysate. We also performed solid support directional (SSD) RNA-seq (Vourekas et al. 2012) of total RNA depleted of ribosomal RNA. By CLIP, we detected specific MOV10L1–RNA protein complexes (indicating direct binding of MOV10L1 to RNA) that are more pronounced in 23-dpp testes, which are enriched in pachytene spermatocytes that express high levels of MOV10L1 [Fig. 1A; Zheng et al. 2010]. We extracted RNAs and created three cDNA libraries: two from the main radioactive signal and one from a higher position [Fig. 1A,B; Supplemental Table S1]. The size distribution of the mapped reads reveals a similar size profile for all libraries [Fig. 1B]. The identity of the 5′ end nucleotide and the genomic distribution are unimodal in all three libraries across the size range of reads [Fig. 1B; Supplemental Fig. S1A]. More than 70% of MOV10L1 CLIP tags map within the previously described intergenic piRNA clusters (IPCs) [Aravin et al. 2006; Vourekas et al. 2012; Li et al. 2013], which produce the overwhelming majority of pachytene piRNAs (Fig. 1C,D). IPC coordinates can be found in Supplemental Table S1 (see also the Supplemental Material). Extremely high correlation between the three libraries and within IPCs was observed, and therefore the three libraries were considered replicates [Supplemental Fig. S1B]. Standard RNA immunoprecipitation was performed to independently verify the strong enrichment of piRNA precursor transcripts in MOV10L1 immunoprecipitation compared with control rabbit serum immunoprecipitation [Supplemental Fig. S1C,D].

The genomic distribution is strikingly similar to a typical pachytene piRNA library [Fig. 1C] even though only ~6% of total MOV10L1 CLIP tags represent mature piRNA sequences [Supplemental Table S2]. While 70% of MOV10L1 CLIP tags map within IPCs, only ~1% of RNA-seq tags from whole testis and ~4% of RNA-seq tags from purified wild-type pachytene spermatocytes map within IPCs, which again demonstrates the remarkable specificity of our CLIP experiments [Fig. 1C]. The correlation between piRNA and MOV10L1 CLIP tags mapping within piRNA clusters was very high [Supplemental Fig. S2A]. Moreover, we found a complete and reciprocal overlap at the nucleotide level between Mili-bound piRNAs (Vourekas et al. 2012) and MOV10L1 CLIP tags on all genomic elements. Specifically, even after removing putative piRNA sequences from MOV10L1 libraries, on average, 85.3% of MOV10L1 CLIP tags overlap with Mili piRNAs. The reciprocal overlap is 89.2%. Furthermore, ~66% of MOV10L1 CLIP tags overlap with Mili piRNAs that map on mRNAs; the reciprocal overlap...
is 80%. MOV10L1 CLIP tag density shows a strong enrichment within mRNA 3′ untranslated regions (UTRs) (Fig. 1E), and the abundance of MOV10L1 CLIP tags is positively correlated with piRNA abundance in these areas (Supplemental Fig. S2B), in direct accord with the observation that many genic piRNAs are preferentially accumulated on transcripts and within piRNA clusters (Vourekas et al. 2012) coverage of MOV10L1 CLIP tags reveals a remarkable preference for binding downstream from and within the 5′ end nucleotide composition (left axis) and average 5′ end nucleotide composition (right axis) for the three MOV10L1 CLIP-seq libraries. Shaded areas represent one standard deviation (SD). n = 3. (C) Genomic distribution of reads from three MOV10L1 CLIP-seq libraries and RNA-seq libraries from whole testis and isolated pachytene spermatocytes (PCTN). (D) Genome browser view of MOV10L1 CLIP tags, PCTN RNA-seq reads, and Mili piRNAs mapped within a pachytene piRNA cluster (chr15_c1266) that lies close to an unrelated gene (Arc). This view is an example of MOV10L1-binding specificity toward piRNA precursors. (E) Density of MOV10L1 CLIP tags mapped on mRNAs. Shaded areas represent one SD. n = 3. (F) Relative coverage of MOV10L1 [blue line] and Mili [red line] CLIP tags (Vourekas et al. 2012) mapping within −20/+40 bases from 5′ ends (0, X-axis) of piRNAs. For Mili, the large CLIP tag (lgClip) subpopulation was used, as it contains the PPIFs. The area typically occupied by a piRNA molecule is marked with a gray bar. Shaded areas represent one SD. n = 3.

**Figure 1.** Transcriptome-wide identification of MOV10L1 RNA targets by CLIP. (A) Western blot and autoradiography of MOV10L1–RNA complexes from CLIP. Noncross-linked and MOV10L1−/− testes served as negative controls. Separate libraries were prepared from RNA extracted from the main radioactive signal (Low, dashed line) and the higher position (High, solid line). (B) Size distribution (right axis) and average 5′ end nucleotide composition (left axis) for the three MOV10L1 CLIP-seq libraries. Shaded areas represent one standard deviation (SD). n = 3. (C) Genomic distribution of reads from three MOV10L1 CLIP-seq libraries and RNA-seq libraries from whole testis and isolated pachytene spermatocytes (PCTN). (D) Genome browser view of MOV10L1 CLIP tags, PCTN RNA-seq reads, and Mili piRNAs mapped within a pachytene piRNA cluster (chr15_c1266) that lies close to an unrelated gene (Arc). This view is an example of MOV10L1-binding specificity toward piRNA precursors. (E) Density of MOV10L1 CLIP tags mapped on mRNAs. Shaded areas represent one SD. n = 3. (F) Relative coverage of MOV10L1 [blue line] and Mili [red line] CLIP tags (Vourekas et al. 2012) mapping within −20/+40 bases from 5′ ends (0, X-axis) of piRNAs. For Mili, the large CLIP tag (lgClip) subpopulation was used, as it contains the PPIFs. The area typically occupied by a piRNA molecule is marked with a gray bar. Shaded areas represent one SD. n = 3.

Binding of MOV10L1 to piRNA precursors precedes Piwi loading

We hypothesized that the positioning of MOV10L1 CLIP tags relative to piRNAs arising from neighboring sites may provide insights into the function of MOV10L1. Instead of a random distribution, the relative nucleotide coverage of MOV10L1 CLIP tags reveals a remarkable preference for binding downstream from and within ~50 nt of both Mili and Miwi piRNA 5′ ends (Supplemental Fig. S2C), implying that MOV10L1 binds the 5′ cleaved piRNA precursor. By comparing the MOV10L1 CLIP and Mili large CLIP tag (lgClip) (Vourekas et al. 2012) coverage around 5′ ends of piRNAs, we found that the two plots show similar patterns (Fig. 1F), further supporting the above notion. Critically, the 5′ ends of MOV10L1 CLIP tags lie downstream from the 5′ ends of Mili-bound, stabilized PPIFs and piRNAs, we observed markedly lower coverage by MOV10L1 CLIP tags (compared with Mili CLIP tags) within a 9-nt distance downstream from 5′ ends of piRNAs (Fig. 1F). We verified this observation by measuring the 5′–5′ distances of MOV10L1 CLIP tags and mature piRNAs. We found a higher incidence of MOV10L1 CLIP tag 5′ ends downstream from 5′ ends of mature piRNAs and within 25 nt (Supplemental Fig. S2D). Since the 5′ ends of MOV10L1 CLIP tags are located within the piRNA body, this suggests that they represent 5′ cleaved piRNA precursors that were further attacked by a separate nucleolytic activity (Fig. 1F; Supplemental Fig. S2C). MOV10L1 CLIP tags show A/(G) nucleotide bias at the 5′ end and U/(C) bias one position upstream [which is not part of the CLIP tag], the 3′ end has a similar bias (Supplemental Fig. S2E). Mili- and Miwi-bound PPIFs have a 5′ bias for uridine (Vourekas et al. 2012). We carefully analyzed the nucleotide biases of MOV10L1 and Piwi CLIP tags using lysates with or without exogenous nucleases, as described in detail in the Supplemental Material and

MOV10L1 function in piRNA biogenesis
Supplemental Figure S2E–H. We show that the 5’ ends of Piwi-bound piRNA intermediate fragments are protected from nucleolytic activities, while the 5’ ends of MOV10L1-bound piRNA precursors are not. The sensitivity of the 5’ ends of MOV10L1 CLIP tags to nucleases indicates that the 5’ cleaved piRNA precursor, while bound by MOV10L1, is not bound and protected by a Piwi protein. Overall, the above findings indicate that MOV10L1 binds piRNA precursors close to the site of the endonucleolytic cleavage that will define the 5’ end of PPIFs before PPIFs are loaded to a Piwi protein that will protect their 5’ ends.

MOV10L1 is required for production of piRNA intermediate processing fragments

To determine whether pachytene piRNA precursors are processed in the absence of MOV10L1, we performed Mili CLIP using testes from Mov10l1<sup>−/−</sup> Neurog3-Cre<sup>(Mov10l1<sup>CKO</sup>)</sup> mice. Prenatal and early postnatal testis development is normal in these animals, but upon conditional deletion of Mov10l1 after postnatal day 7, they exhibit a deficiency in pachybyte piRNA biogenesis and post-meiotic arrest of spermatogenesis (Zheng and Wang 2012). Furthermore, to identify putative transcriptome changes, we performed RNA-seq (Vourekas et al. 2012) using total RNA extracted from wild-type and Mov10l1<sup>CKO</sup> whole testes and from highly enriched populations of pachybyte spermatocytes and round spermatids (Supplemental Table S1).

We prepared three Mili CLIP libraries from Mov10l1<sup>CKO</sup>: two from the main radioactive signal and one from larger Mili–RNA complexes (Supplemental Fig. S3A). The profiles of these libraries are dramatically different from Mili CLIP libraries from wild-type mice (Vourekas et al. 2012), and a robust piRNA population was not detected in any of the three Mov10l1<sup>CKO</sup> libraries (Supplemental Fig. S3, cf. B and C), which is in agreement with our previous report (Zheng and Wang 2012). Contrary to Mili CLIP from wild-type testes, all three Mili CLIP libraries from Mov10l1<sup>CKO</sup> have a uniform genomic distribution for smaller- and larger-sized CLIP tags, and there are fewer Mili CLIP tags mapped within precursor piRNA transcripts in the absence of MOV10L1 [Fig. 2A]. We specifically interrogated whether these libraries contained 23- to 31-nt-sized CLIP tags that had common 5’ ends with pachybyte and/or prepachybyte Mili-bound piRNAs from standard immunoprecipitation libraries (Aravin et al. 2007, Kirino et al. 2009). While a typical piRNA-enriched Mili CLIP library [Vourekas et al. 2012] from wild-type mice contains ~65% piRNA-matching sequences in this size range, Mili CLIP libraries from Mov10l1<sup>CKO</sup> have only ~10% matches while containing a similar amount of putative prepachybyte piRNAs with wild-type libraries [Supplemental Table S2]. Collectively, these data demonstrate a severe defect in piRNA processing upon conditional deletion of MOV10L1.

In addition, the enrichment of Mili CLIP tags in 3’ UTRs [Vourekas et al. 2012] is lost in the absence of MOV10L1 [Fig. 2B], consistent with a piRNA biogenesis defect. Mili CLIP tags mapped within precursor piRNA transcripts in the absence of MOV10L1 are fewer [Fig. 2A, IPC] and lack a 5’ uridine bias [Supplemental Fig. S3D], the hallmark of Piwi-stabilized piRNA intermediates. In fact, they carry the same bias as described above for 5’ ends of MOV10L1 CLIP tags and 3’ ends of Miwi-bound PPIFs [Supplemental Fig. S2E, respectively], suggesting that the 5’ ends of these RNAs were not protected by Mili and were generated after cell lysis. Additionally, Mili CLIP tag coverage relative to 5’ ends of piRNAs [Fig. 2C] in Mov10l1<sup>CKO</sup> reveals a complete loss of the characteristic binding downstream from piRNA 5’ ends, strongly suggesting that these Mili-bound transcript fragments from Miwi<sup>101</sup> mutant testes do not represent bona fide PPIFs.

To investigate these observations further, we examined RNA-seq tags mapping within piRNA clusters. The size profile of IPC reads from wild-type mice shows that piRNA processing of precursor transcripts is detectable in RNA-seq libraries [Fig. 2D] even though the total RNA is fragmented before library preparation. In contrast, the IPC read size profile in Mov10l1<sup>CKO</sup> testis is identical to that of mRNA reads in wild-type and conditional knockout genotypes, strongly suggesting the absence of piRNAs and piRNA intermediate fragments in mice lacking Mov10l1. Therefore, RNA-seq and Mili CLIP in Mov10l1<sup>CKO</sup> testis support the notion that MOV10L1 function is essential for the endonucleolytic cleavage that gives rise to intermediate piRNA fragments.

The uncovering of piRNA processing products in wild-type RNA-seq libraries has a broader implication for transcriptome analysis. Long transcripts that are processed and give rise to mature products whose stability and turnover are regulated by different mechanisms than for the precursor transcripts cannot be accurately quantified by a technique that uses short reads such as RNA-seq because the short reads cannot be unambiguously assigned to the precursor or the mature RNA. Therefore, we used quantitative RT–PCR (qRT–PCR) to verify and measure the levels of piRNA precursors, selected mRNAs, and retrotransposons. As previously shown [Zheng and Wang 2012], pachybyte piRNA precursors are increased significantly in the Mov10l1 mutant testes, while retrotransposons are only slightly increased [less than twofold] [Supplemental Fig. S3E]. Interestingly, Miwi<sup>Piwil1</sup> mRNA is modestly up-regulated, while its protein levels are notably down-regulated [Supplemental Fig. S3F]. Quantification of mRNA levels by qRT–PCR is in agreement with RNA-seq results [Supplemental Fig. S3G]. We noticed a decrease of spermiogenic mRNA levels despite virtually unchanged levels of CREM, the master spermiogenic transcriptional regulator (Blendy et al. 1996). This finding is consistent with a proposed role of Miwi in post-transcriptional stabilization of spermiogenic mRNAs (Vourekas et al. 2012). It is unclear why the Miwi protein abundance is reduced in Mov10l1<sup>CKO</sup> testis.

piRNA precursor secondary structure elements are enriched in MOV10L1 footprints

We next analyzed MOV10L1 CLIP tags to identify potential sequence-binding preferences. Intriguingly, we
discovered a statistically significant enrichment in G (~28%) and depletion of U (~20%) residues in heavily bound areas of the piRNA clusters compared with the entire cluster sequence \(P < 0.05\) [Fig. 3A]. A genome-wide nucleotide composition analysis for intergenic areas revealed that, remarkably, piRNA clusters are already significantly enriched in G residues compared with other intergenic areas, regardless of the presence of repeat elements [Fig. 3B].

These findings raised the possibility that elements of secondary structures known as G quadruplexes (G4s) [Biffi et al. 2014] may play a role in piRNA processing and prompted us to perform genome-wide prediction of G4s using strict and loose prediction algorithms [see the Supplemental Material], which we overlaid with MOV10L1 CLIP tags and piRNAs [Fig. 3C,D]. Notably, G4 elements are enriched within MOV10L1 CLIP tags, downstream from their mid-points [Fig. 3D]. Our analysis revealed a paucity of G4 density on and around 5′-ends of piRNAs from four mammalian species followed by a relative increase downstream from piRNA 5′-ends [Fig. 3E]. Additionally, Mili piRNA density within a 60-nt window upstream of G4 5′-ends is significantly higher than overall piRNA density within IPCs \(P < 10^{-4}\), paired \(t\)-test) [Fig. 3F; see also Supplemental Table S3]. Subsequently, we asked whether G4 relative positioning and frequency created a specific nucleotide “signature” around piRNA loci. Examining the genomic nucleotide composition around piRNA 5′-ends, we found a notable increase in G residues downstream from position 15 [Fig. 3G]. Strikingly, this pattern is present within human, Rhesus, mouse, and rat piRNA genomic loci [Fig. 3G] despite the absence of piRNA sequence conservation, suggesting a conserved role for G4s in mammalian piRNA precursor processing. We performed G4 prediction within piRNA cluster sequences for all four mammalian species and found significantly more G4s than would be expected by chance \(P < 10^{-4}\) [Supplemental Table S3]. The above findings suggest that G4s within piRNA precursors are not an indirect consequence of guanosine enrichment but a result of selective pressure and may have been co-opted as landmarks for piRNA biogenesis.

Additionally, we performed a secondary structure prediction of intergenic piRNA precursors using RNAfold [Hofacker et al. 1994] and plotted it relative to MOV10L1 CLIP tags and piRNAs. The base-pairing potential is remarkably increased close to the 3′ end of MOV10L1 CLIP tags, in concordance with G4 density in the same areas, and shows a local minimum around piRNA 5′ ends [Fig. 3H, I, respectively]. Collectively, these results support the notion that processing of piRNA precursor transcripts is intimately coupled with local secondary structures: 5′ ends are generated more often in areas of lesser secondary structure potential, and MOV10L1 is associated with structured regions [G4 or otherwise] of precursor transcripts.

To examine the formation of G4s within piRNA precursor transcripts in vivo, we used the highly specific BG4 antibody [Biffi et al. 2014]. BG4 antibody specifically immunoprecipitated piRNA precursors from testis lysates [Fig. 4A], especially in \(Mov10l1^{CKO}\), strongly suggesting the formation of G4s within piRNA precursor transcripts in vivo. The degree of enrichment of piRNA precursors in BG4 immunoprecipitations from \(Mov10l1^{CKO}\) lysates compared with wild type [10-fold to 75-fold] greatly exceeded the increase of their overall levels in total

![Figure 2. Mili CLIP-seq reveals a lack of PPF1 production in \(Mov10l1^{CKO}\). (A) Genomic distribution of replicate Mili CLIP libraries from wild-type (WT; blues) and \(Mov10l1^{CKO}\) (reds). Neuro3-Cre testes (\(Mov10l1^{CKO}\) lysates) in contrast to wild-type, Mili CLIP tags from \(Mov10l1^{CKO}\) exhibit a uniform genomic distribution regardless of their sizes. Error bars represent one SD. n = 3. (B) Abundance of Mili wild-type and \(Mov10l1^{CKO}\) CLIP tags (reads per kilobase per million [RPKM] mapping within mRNA untranslated and coding regions. Error bars represent one SD. n = 3. (C) Mili CLIP tag coverage within ±100 nt from piRNA 5′ ends (0, X-axis) in wild-type and \(Mov10l1^{CKO}\) testis. The percentage of total mapped tags per read size for each genomic category is plotted. Reads mapping to mRNAs or piRNA clusters (IPCs) were plotted separately. The majority of RNA-seq reads mapping to intergenic areas are piRNA-sized or smaller. Although some mRNAs, especially 3′ UTRs, are processed into piRNAs, the overwhelming majority is not; thus mRNA read size distributions are identical.

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RNA [twofold to sixfold] [Fig. 4B]. Therefore, the dramatic precursor RNA enrichment in BG4 immunoprecipitations from Mov10l1−/− testis lysates is only partially attributed to precursor transcript abundance. The increased antigenicity of piRNA precursors from Mov10l1−/− lysates in this assay indicates the increased presence of G4 structures within these transcripts in Mov10l1 mutant testis and thus a role for MOV10L1 in the resolution of these thermostable secondary structures.

MOV10L1 exhibits 5′-to-3′ RNA-unwinding activity in vitro

MOV10L1 belongs to the superfamily I (SF I) of DNA/RNA helicases [Fig. 5A; Fairman-Williams et al. 2010], and one of the closest homologs of MOV10L1 is UPF1, an RNA helicase involved in nonsense-mediated RNA decay [Supplemental Fig. S4A, Kervestin and Jacobson 2012]. Tertiary structure modeling showed extensive similarities between the helicase domains of MOV10L1 and UPF1 [Supplemental Fig. S4A–E, Supplemental Material], suggesting that MOV10L1 may use its conserved helicase core to translocate the piRNA precursor transcript, analogous to the translocation of UPF1 on mRNAs with a 5′-to-3′ directionality [Franks et al. 2010]. Recently, MOV10, a ubiquitously expressed RNA helicase that has not been implicated in the piRNA pathway, was found to possess RNA-unwinding activity and assist UPF1 in mRNA degradation [Gregersen et al. 2014]. To test whether MOV10L1 can unwind RNA in vitro, we adapted the RNA duplex-unwinding assay [Jankowsky
and Putnam 2010). We immunopurified Flag-tagged wild-type and ATP-binding-deficient point mutant MOV10L1 (K778A) from transfected 293T cells [Fig. 5B]. Lys778 is conserved among SF I RNA helicases [Supplemental Fig. S4A] and is essential for ATP binding and therefore RNA helicase activity of UPF1 [Weng et al. 1996]. Both wild-type and mutant MOV10L1 can be cross-linked efficiently and specifically to a 38-nt-long RNA bearing a 4-thio-uridine residue [Fig. 5C], indicating that the point mutation does not impair the RNA-binding capacity of MOV10L1, similar to the mutation of the homologous residue in UPF1 [Weng et al. 1996]. Wild-type MOV10L1 shows time-dependent, 5′-to-3′ [Fig. 5D, left panel] but not 3′-to-5′ [Fig. 5D, right panel] RNA-unwinding activity when using a partial RNA duplex with a single-stranded overhang, showing that MOV10L1 is indeed an RNA helicase. In the same assay, the K778A point mutant is catalytically inactive [Supplemental Fig. S5A]. These results, coupled with the CLIP analysis, indicate that MOV10L1 may play a role in directional, 5′-to-3′ translocation of the precursor transcripts and the remodeling of their secondary structures during piRNA biogenesis.

**A point mutation in the MOV10L1 RNA helicase core abolishes piRNA biogenesis**

To determine the requirement of MOV10L1 RNA helicase activity for piRNA biogenesis in vivo, we generated a Mov10l1 knock-in [Mov10l1<sup>WT</sup>] allele harboring the K778A amino acid change in the ATP-binding motif through gene targeting in embryonic stem (ES) cells [Supplemental Fig. S5B]. Heterozygous Mov10l1<sup>WT/+</sup> mice were viable and fertile, suggesting that the knock-in allele is not dominant negative. In contrast, while Mov10l1<sup>WT/WT</sup> females were fertile, Mov10l1<sup>WT/KI</sup> males were sterile and exhibited meiotic arrest at the zygote-like stage of prophase I [Fig. 5E], a phenotype similar to Mov10l1 knock-out mice [Zheng et al. 2010]. The MOV10L1<sup>K778A</sup> protein was expressed in gonocytes in embryonic day 16.5 [E16.5] Mov10l1<sup>WT/KI</sup> testis at a level comparable with MOV10L1 in Mov10l1<sup>WT/WT</sup> testis [Fig. 5F,G]. MOV10L1 localized throughout the cytoplasm in a punctate pattern in the Mov10l1<sup>WT/WT</sup> gonocytes but exhibited an abnormal polar cytoplasmic localization in the Mov10l1<sup>KI/KI</sup> gonocytes [Fig. 5G]. Miwi2 was present in the nucleus in Mov10l1<sup>KI/WT</sup> gonocytes but excluded from the nucleus in Mov10l1<sup>KI/KI</sup> gonocytes, similar to the Mili knockout phenotype [Shoji et al. 2009; Zheng et al. 2010]. Furthermore, both Mili and Miwi2 exhibited a similar polar cytoplasmic localization in Mov10l1<sup>KI/KI</sup> gonocytes, suggesting that the piRNA biogenesis pathway is perturbed by the point mutation in the MOV10L1 RNA helicase domain.

We next determined whether transposon silencing and piRNA biogenesis were affected. Immunofluorescence analysis showed that LINE1 retrotransposons were highly derepressed in Mov10l1<sup>KI/KI</sup> gonocytes [Fig. 5C]. Mili was associated with piRNAs in E16.5 Mov10l1<sup>KI/KI</sup> testes but depleted of piRNAs in E16.5 Mov10l1<sup>KI/KI</sup> testes while maintaining wild-type expression levels [Fig. 5H]. Additionally, using qRT-PCR, we detected up-regulation of piRNA precursor transcripts in E16.5 Mov10l1<sup>KI/KI</sup> testes [Fig. 5I; see also Supplemental Fig. S5C]. Taken together, these results indicate that MOV10L1 RNA helicase activity is essential for the processing of the precursor transcripts during piRNA biogenesis in vivo.

**Discussion**

Our data provide a transcriptome-wide view of primary piRNA biogenesis in unprecedented detail and describe the biochemical role of MOV10L1 in this process. We found that MOV10L1 binds to the piRNA precursor with remarkable specificity. The in vitro enzymatic assays and the generation of an ATPase point mutant knock-in mouse of MOV10L1 establish the requirement of the MOV10L1 ATP-dependent RNA helicase activity for piRNA biogenesis in vivo. In vitro systems that recapitulate primary piRNA biogenesis have not been generated, likely due to the numerous factors and activities involved and the complexity of their intricate network of interactions. Nevertheless, we were able to show that MOV10L1 exhibits a 5′-to-3′ directional RNA-unwinding activity in vitro. PPIFs were identified biochemically by Piwi HTS-CLIP [Vourkas et al. 2012] and genetically in the Tdrkh mutant mice [Saxe et al. 2013]. MOV10L1 and the endonuclease, which is most likely a mZuc dimer
(Haase et al. 2010; Ipsaro et al. 2012; Nishimasu et al. 2012; Voigt et al. 2012), are both required for the generation of PPIFs, indicating that these activities are functionally linked. We envision that MOV10L1 helicase activity is essential for proper piRNA precursor “feeding” to the endonuclease for cleavage. In this model, PPIFs are generated by consecutive cleavages of the 5′ end of the precursor, facilitated by the directional translocation of the precursor by MOV10L1 toward the endonuclease (Fig. 6). The directional translocation is supported by our in vitro duplex-unwinding assay and the pronounced downstream relative positioning of MOV10L1 CLIP tags with respect
There is substantial evidence from genetic and cell culture experiments that Zuc is essential for primary piRNA biogenesis [Pare et al. 2007; Malone et al. 2009; Haase et al. 2010; Saito et al. 2010; Watanabe et al. 2011], and it was recently shown both structurally and biochemically that Zuc is an endonuclease that cleaves ssRNA and leaves a 5′ phosphate end [Ipsaro et al. 2012; Nishimasa et al. 2012; Voigt et al. 2012]. Therefore, Zuc fits the description for the endonuclease that generates the 5′ ends of piRNA intermediates, but direct proof of this is lacking. Our results are consistent with an endonuclease that is inhibited by secondary structures and is activated by the secondary structure-resolving helicase activity of MOV10L1. In support of the possibility that Zuc endonuclease is responsible for piRNA intermediate generation in the piRNA intermediate cleavage and loading (pICL) complex (see below), we were able to detect MOV10L1 and mZuc association by coimmunoprecipitation using a heterologous expression system (Supplementary Fig. S5D).

In our model (Fig. 6), cleavage events occur downstream from previous ones and “prune” the longer precursor, creating the 5′ ends of forthcoming PPIFs and releasing inter- mediate fragments, which are bound by Piwi. The loaded Piwi breaks off from MOV10L1, and the latter may carry on with additional rounds of precursor processing. The 5′ U of PPIFs is stabilized by Piwi, and the 3′ end is trimmed and methylated, leading to generation of mature piRNA. Since MOV10L1 does not bind Piwi-stabilized PPIFs, MOV10L1 RNA-binding activity may not be directly involved in the subsequent PPIF maturation steps. To illustrate the separation of the 5′ and 3′ end maturation processes, we propose the designation of a functional pICL complex, which contains MOV10L1, Piwi, and the endonuclease (Fig. 6). Additional proteins, such as GASZ, TdRs, and chaperones, are likely components of the pICL complex [Olivieri et al. 2012; Preall et al. 2012; Xiol et al. 2012; Izumi et al. 2013]. MOV10L1 does not interact with TdRk [Saxel et al. 2013], which is required for 3′ end maturation. Experiments in Drosophila are consistent with our model; RNAs isolated from Armitage by standard immunoprecipitation conditions show characteristics similar to those of piRNA precursor fragments [Saito et al. 2010]. A significant number of Armitage-bound RNA fragments mapped within the flamenco locus, which gives rise to long piRNA precursor transcripts [Malone et al. 2009], overlapping with piRNA sequences [Saito et al. 2010]. Most of these fragments (which the investigators named piR-ILs) did not match the 5′ ends of mature piRNAs, suggesting that Armitage binds piRNA precursors. Moreover, based on the above, inhibition of endonuclease activity would result in Piwi protein free of RNA, which would be “trapped” in a complex with MOV10L1. This was indeed observed in Drosophila: Upon Zuc knockdown, Piwi was less loaded, and more Piwi could be immunoprecipitated with Armitage [Saito et al. 2010], indicating conserved function of MOV10L1 helicases in piRNA biogenesis.

How primary piRNA precursors that are 5′ capped and 3′ polyadenylated [Li et al. 2013] are selected from other
RNA polymerase II transcripts is not known. We speculate that cytoplasmic transcripts that are not translated and enter ICM/nuage can be recognized by piCL and processed into piRNAs. This could explain why the bulk of generic piRNAs are derived from 3’ UTRs and the presence in pachytene piRNA precursors of G4s and other secondary structure elements, which may function to impede translation (Arora et al. 2008; Beaudoin and Perreault 2010). Ultimately, identification of all components of the piCL complex and biochemical characterization with reconstitutions of its activities will be required to further address how piRNA precursors are selected to enter ICM/nuage, the role of G4 and other structural elements, and the precise molecular function of all piCL complex components in piRNA processing.

Materials and methods

MOV10L1 HITS-CLIP

MOV10L1 HITS-CLIP was performed essentially as for Milei and Miwi previously (Vourekas et al. 2012). The protocol was described in exhaustive detail (Vourekas and Mourelatos 2014). Testes from 23-dpp or adult mice were collected, detunicated, disrupted by mild pipetting in ice-cold HBSS, and immediately UV-irradiated three times at 254 nm (400 mJ/cm²). The cells were pelleted and washed with PBS, and the final cell pellet was flash-frozen in liquid nitrogen and kept at −80°C. UV light-treated cells (two testes) were lysed in 300 μL of 1× PMPG with protease inhibitors, 2 U/μL RNasin, and no exogenous nucleases; lysates were treated with DNase [Promega] for 5 min at 37°C and then centrifuged at 90,000 μg for 30 min at 4°C.

For each immunoprecipitation, ~5 μg of our anti-MOV10L1 rabbit polyclonal purified total IgG (Zheng et al. 2010) was bound on protein A Dynabeads in antibody-binding buffer [0.1 M Na-phosphate at pH 8, 0.1% NP-40] for 3 h at 4°C; antibody-bound beads were washed three times with 1× PMPG [1× PBS [no Mg²⁺ and no Ca²⁺], 2% Empigen]. Antibody beads were incubated with lysates (supernatant of UV-irradiated testes) for 3 h at 4°C. Low- and high-salt washes of immunoprecipitation beads were performed with 1× and 5× PMPG (5× PBS, 2% Empigen).

RNA linkers (RL3 and RL5) as well as 3’ adaptor labeling and ligation to CIP (calf intestinal phosphatase)-treated RNA CLIP tags were previously described (Vourekas and Mourelatos 2014).

Immunoprecipitation beads were eluted for 12 min at 70°C using 30 μL of 2× Novex reducing loading buffer. Samples were analyzed by NuPAGE [4%–12% gradient precast gels run with MOPS buffer]. Cross-linked RNA–protein complexes were transferred onto nitrocellulose [Invitrogen, LC2001], and the membrane was exposed to film for 1–2 h. Membrane fragments containing the main radioactive signal and fragments up to ~15 kDa higher were cut (Fig. 1B). RNA extraction, 5′ linker ligation, RT–PCR, and the second PCR step were performed with the DNA primers [DPS and DPF or DSFP3 and DSFSP5] as described previously (Vourekas and Mourelatos 2014). cDNA from two PCR steps was resolved on and extracted from 3% Metaphor 1× TAE gels stained with ethidium bromide. The size profiles of cDNA libraries prepared from the main radioactive signal and higher molecular weights were similar (Fig. 1B). DNA was extracted with QIAquick gel extraction kit and submitted for deep sequencing. The cDNA libraries were sequenced on an Illumina HiSeq 2500 at 100 cycles.

Generation of Mov10l1 knock-in mice

To generate the Mov10l1 knock-in targeting construct (Supplemental Fig. S5B), DNA fragments were amplified by high-fidelity PCR using a Mov10l1 BAC clone (RP23-269P24). Codon 778 (exon 17) was mutated from AAG [lysine] to GCC [alanine] by PCR-based mutagenesis. The neomycin selection cassette was flanked by loxp sites for Cre-mediated removal. The targeting construct was sequenced to confirm the introduced mutation. V6.5 hybrid ES cells were electroporated with linearized targeting construct (pUP109/ClaI) and cultured in the presence of 350 μg/mL G418. Five-hundred-seventy-six G418-resistant ES cell clones were screened for homologous recombination. Three targeted ES clones were obtained, and two were injected into blastocysts. The Mov10l1 knock-in allele was transmitted through the germline. To delete the neo selection cassette, Mov10l1Ki+ mice were crossed with Actb-Cre mice that express Cre ubiquitously (Lewandoski and Martin 1997). All of the studies were performed with mice without the neo cassette. The wild-type [439-base-pair [bp]] and knock-in [557-bp] alleles were assayed by PCR with the primers ACCTCCGGGACCTGAAGCCACTTG and ATCCCAA GCCCGGCTTGACAGTA. PCR primers flanked the remaining loxp site and adjacent vector sequence.

RNA immunoprecipitation using the BG4 antibody

Bacterial expression and purification of Flag/His dual-tagged BG4 antibody were performed as previously described (Biffi et al. 2014). Twenty microlitters of M2 agarose beads [Sigma] was washed three times in lysis and immunoprecipitation buffer [50 mM Tris at pH 7.5, 100 mM KCl, 0.1% Igepal CA-360 [NP-40] with EDTA-free protease inhibitors [Roche], 0.5 U/μL rRNasin] and bound with 3 μg of purified BG4 antibody in 600 μL of lysis buffer for 1.5 h at 4°C. One denaturated testis [from 3-mo-old wild-type or Mov10l1Cre+ animals] was lysed using 1200 μL of lysis buffer with a plastic pestle. The lysate was centrifuged at 16,000g for 20 min, and the supernatant was mixed with the antibody-bound M2 beads or M2 beads alone for 1.5 h at 4°C, the latter served as the negative control. RNA was extracted from the beads using Trizol, precipitated using ethanol, treated with DNase I for 1 h at 37°C, and extracted again using acid phenol. The RNA was spiked with 1 ng of in vitro transcribed Renilla luciferase mRNA. qRT–PCR was performed on a StepOnePlus system [Applied Biosystems] using the primers described above. The Renilla luciferase mRNA was used in place of an endogenous control for calculating relative mRNA quantities.

MOV10L1 protein expression and purification

The nucleotide sequence encoding the 1187 amino acids [Uniprot: Q99MV5] and the K778A mutant were cloned into the pR5 vector with an in-frame N-terminal Flag tag using Phanta Super-Fidelity DNA polymerase and ClonExpress II [Vazyme]. The pR5 constructs were used to transfect 293T cells with TurboFect transfection reagent [Thermo]. 293T cells expressing wild-type and K778A MOV10L1 were lysed by sonication in K150 lysis and immunoprecipitation buffer [50 mM HEPES at pH 7.5, 150 mM KsAc, 1 mM DTT, 0.1% NP-40 [Igepal] with EDTA-free protease inhibitors cocktail [Roche], and the lysate was centrifuged at 16,000g for 20 min. The cleared lysate was mixed with 50 μL of M2 magnetic bead slurry [Sigma], prewashed with K150, and incubated for 2 h at 4°C. Immunoprecipitation beads were washed three times with K150, once with K150 containing 250 mM NaCl, and three more times with K150. The beads were resuspended in 1 mL of K150 and kept on ice for a maximum of 2 d. Fifty microliers of bead suspension contained 1 μg of MOV10L1.

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Cross-linking of MOV10L1 with an RNA oligo bearing a 4-thio-uridine residue

The following oligo was used for cross-linking: U = 4-thio-uridine (4SU), 5′-CAACCCACCCAUAAACAUUUAGAGGCCACUCUCACGCUU-3′.

Fifty microliters of wild-type or K778A MOV10L1-bound bead slurry [1 μg of MOV10L1 protein] was washed three times with RNA-binding buffer (50 mM HEPES at pH 7.4, 100 mM KAc, 1 mM DTT, 0.01% NP-40, 0.2 mM MgCl₂) and resuspended in 25 μL of the same buffer supplied with 0.5 μL of 40 U/μL rRNasin and 1 μL of 5′-32P-labeled RNA oligo carrying 4SU residues. The reaction mixture was incubated for 20 min at 37°C with shaking and irradiated with UV [365 nm] for 30 min on ice. The beads were washed twice, and the protein–RNA complexes were eluted with reducing SDS loading buffer and analyzed on a 4%–12% Bis-Tris NuPAGE gel. The gel was dried and exposed to film overnight.

RNA duplex-unwinding assay

We used the RNA duplex-unwinding assay developed by the Jan-Kowisky laboratory, also used recently for Mov10, with some modifications [Jankowsky and Putnam 2010; Gregersen et al. 2014]. The following RNA oligos were used: duplex with 5′ end overhang (Top 5′-GCGCUUACGGGCU-3′ and Bottom 5′-AACACCAAAAAACAAAAAAGCCCGUAAAGACGC-3′) and duplex with 3′ end overhang (Top 3′-5′AGGCCGGUAAAGACGC-3′ and Bottom 3′-5′GCGUCUUACGGGCUUAAAACAAAACAAAAACAAAAACAAAAA-3′).

The top oligos were 5′ end-labeled, and all four oligos were end overhang. The duplexes were prepared in 50 mM MOPS (pH 7.5, 20 mM KAc, 0.2 mM MgCl₂, 0.01% Igepal [Nonidet P-40], and 1 mM DTT) mixed with 20 nM duplex, 1 pmol of cold top oligo, and 20 U of rRNasin in 30 μL of reaction buffer, and incubated for 10 min at 37°C with shaking in a Thermomixer (1000 rpm).

Subsequently, ATP was added at a final concentration 0.5 mM, and incubation was resumed for the indicated time. Four-microliter reaction samples were mixed with 4 μL of stop solution and kept on ice. Reaction products were analyzed by electrophoresis on a 12% acrylamide nondenaturing gel and visualized by autoradiography.

SSD RNA-seq, RNA and protein analysis, and bioinformatic analysis

SSD RNA-seq was performed as previously described [Vourekas et al. 2012] using total RNA (depleted of ribosomal RNA) isolated from wild-type and Mov1011/W– Neurog3-Cre whole adult testes or from purified pachytene spermatocytes and round spermatids obtained using the STA-PUT procedure [Bellvé et al. 1977] with modifications [Gerton and Millette 1986], qRT–PCR, histological analysis, immunofluorescence, Western blotting, pRNA immunoprecipitation, MOV10L1 and mZuc coimmunoprecipitation, MOV10L1 sequence and structure analysis, and bioinformatic analysis are described in the Supplemental Material. Deep sequencing data were deposited to Sequence Read Archive (SRA), project ID PRJNA330507.

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