TDRD5 binds piRNA precursors and selectively enhances pachytene piRNA processing in mice

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Pachytene piRNAs are the most abundant piRNAs in mammalian adult testes. They are generated from long precursor transcripts by the primary piRNA biogenesis pathway but the factors involved in pachytene piRNA precursors processing are poorly understood. Here we show that the Tudor domain-containing 5 (TDRD5) protein is essential for pachytene piRNA biogenesis in mice. Conditional inactivation of TDRD5 in mouse postnatal germ cells reveals that TDRD5 selectively regulates the production of pachytene piRNAs from abundant piRNA-producing precursors, with little effect on low-abundant piRNAs. Unexpectedly, TDRD5 is not required for the 5′ end processing of the precursors, but is crucial for promoting production of piRNAs from the other regions of the transcript. Furthermore, we show that TDRD5 is an RNA-binding protein directly associating with piRNA precursors. These observations establish TDRD5 as a piRNA biogenesis factor and reveal two genetically separable steps at the start of pachytene piRNA processing.

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Maintaining germline genome integrity and RNA homeostasis is essential for gametogenesis. During mammalian spermatogenesis, PIWI-interacting RNAs (piRNAs), which comprise a class of germ cell-specific small non-coding RNAs, play a crucial role in silencing transposons and protecting the germline genome\textsuperscript{2–7}. piRNAs also regulate spermatogenesis-associated RNAs, and are essential for the production of functional sperm\textsuperscript{8–11}. The impairment of the piRNA pathway often results in transposon upregulation, spermatogenic arrest, and male infertility\textsuperscript{12–14}. piRNAs are produced by cleavages of precursor RNAs through primary processing and secondary amplification, and exert their function through associated PIWI proteins\textsuperscript{12,15}. During mouse spermatogenesis, two distinct populations of piRNAs become associated with the PIWI proteins (MILI, MIWI, and MIWI2) at two different developmental stages. Embryonic/perinatal male germ cells produce a population of transposon sequence-rich piRNAs (TE piRNAs or prepachytene piRNAs) with a primary role in transposon suppression\textsuperscript{16–18}. TE piRNAs associate with MIWI1 and MIWI and guide transcriptional and posttranscriptional transposon silencing, respectively. The second population of piRNAs, termed pachytene piRNAs, comprises the vast majority of piRNAs in adult mouse testes\textsuperscript{2}. These piRNAs accumulate rapidly at the pachytene stage of meiosis\textsuperscript{19}. Unlike TE piRNAs, pachytene piRNAs are transposon-sequence-poor and associate with MILI and MIWI. Although a definite function in transposon regulation has not been established\textsuperscript{20}, emerging evidence indicates that pachytene piRNAs may promote spermatogenesis by regulating miRNAs and long non-coding RNAs in mouse testes\textsuperscript{2–11}. The precise biological function of pachytene piRNAs is still not well-understood\textsuperscript{21}.

Pachytene piRNAs comprise the largest and most diverse population of small non-coding RNAs in the testis with more than two million distinct piRNA species\textsuperscript{19}. These piRNAs are primarily generated from hundreds of unique genomic loci (piRNA clusters) through a primary processing pathway\textsuperscript{3,19,22}, and transcription factor A-MYB plays a critical role in driving the transcription of the bulk of pachytene piRNA precursors\textsuperscript{19}. Notably, pachytene piRNAs have thus far only been identified in mammals, but not in well-studied flies and worms. Despite this, accumulating evidence indicates that conserved piRNA biogenesis factors are expressed and active during the pachytene stage of meiosis and may contribute to pachytene piRNA biogenesis\textsuperscript{20,23–27}. Additionally, the inventory of piRNA biogenesis factors during this period is still not complete\textsuperscript{28,29}.

Tudor domain proteins play conserved roles in regulating the piRNA pathway and spermatogenesis by interacting with the PIWI proteins\textsuperscript{22,30}. Tudor domain proteins bind methylated arginines on PIWI proteins through the Tudor domain and promote the formation and localization of piRNA processing complex. The essential function of Tudor domain proteins has been implicated in distinct steps of piRNA biogenesis. Among them, TDRKH is required for pre-piRNA trimming\textsuperscript{35}. RNF17 suppresses piRNA ping-pong mechanism in meiotic cells\textsuperscript{21}. TDRD1, TDRD9, and TDRD12 are involved in ping-pong amplification and secondary piRNA production during embryonic/perinatal piRNA biogenesis\textsuperscript{31–33}.

TDRD5 is a Tudor domain protein implicated in spermatogenesis and male fertility\textsuperscript{34,35}. TDRD5 null mutations impair transposon silencing and disrupt spermiogenesis\textsuperscript{35}. However, the role of TDRD5 in piRNA biogenesis has not been established. By global deletion of Tdrd5 in mice and conditional inactivation of Tdrd5 in postnatal germ cells, we discovered a critical role for TDRD5 in piRNA biogenesis. TDRD5 directly binds piRNA precursors and is required for the production of the bulk of pachytene piRNAs during meiosis. TDRD5 exerts its role by selectively controlling the processing of a large subset of the most abundantly expressed pachytene piRNA precursors. We also provide evidence that pachytene piRNA precursor processing contains two genetically separable steps: S’ end processing and the processing of the rest of the piRNA precursors. These observations reveal previously unknown mechanistic features of pachytene piRNA biogenesis supporting spermatogenesis and male fertility.

**Results**

Reduced piRNA production in Tdrd5 null mice. Because Tdrd5 is regulated by the master piRNA transcription factor A-MYB during meiosis\textsuperscript{19}, we speculated that TDRD5 is involved in piRNA biogenesis. To test this hypothesis, we generated Tdrd5 null mice (Tdrd5\textsuperscript{KO}) using an embryonic stem cell line with targeted Tdrd5 mutation (Supplementary Fig. 1a). Western blot analysis confirmed the absence of TDRD5 proteins in Tdrd5\textsuperscript{KO} mice (Supplementary Fig. 1b). As observed before\textsuperscript{35}, TDRD5 deficiency caused spermatogenic arrest at either zygotene (severe phenotype) or round spermatid (mild phenotype) stages of spermatogenesis (Supplementary Fig. 1c and d). We next examined the total piRNA by gel electrophoresis. Testes from adult Tdrd5\textsuperscript{KO} mice with severe phenotype lacked piRNA-producing cells (pachytene spermatocytes and round spermatids) and associated piRNA production (Supplementary Fig. 1e, right). Testes from adult Tdrd5\textsuperscript{KO} mice with mild phenotypes contained pachytene spermatocytes and round spermatids, but total piRNA levels were significantly reduced as compared to the wild type (Supplementary Fig. 1e, left). This suggests that TDRD5 participates in adult piRNA biogenesis.

Loss of TDRD5 in postnatal germline impairs spermiogenesis. TDRD5 is expressed in both embryonic and meiotic male germ cells\textsuperscript{34,35}. TDRD5 null mutation affected piRNA production and spermatogenesis at both stages, complicating the conclusion for a clear effect of TDRD5 on piRNA biogenesis. To test for a direct role of TDRD5 in pachytene piRNA biogenesis, we generated Tdrd5 conditional knockout mice in which Tdrd5 becomes deleted in postnatal day 3 male germ cells by Stra8-Cre (Fig. 1a). We generated mice with a Tdrd5 knockout-first allele (Tdrd5\textsuperscript{sm1a}) and mice with a Tdrd5 conditional allele (Tdrd5\textsuperscript{fl}) via FLP recombination (Fig. 1a). In the Tdrd5\textsuperscript{fl} allele, exon 7 of Tdrd5 is flanked by two loxP sites, by combining with Stra8-Cre, we obtained Stra8-Cre\textsuperscript{+}, Tdrd5\textsuperscript{fl/+} conditional knockout mice (refer to as Tdrd5\textsuperscript{KO}) in which TDRD5 is deleted in all adult male germ cell lineages (Fig. 1a, Supplementary Fig. 2). Successful inactivation of TDRD5 in Tdrd5\textsuperscript{KO} mice was confirmed by in situ hybridization and western blotting, which revealed the absence of both TDRD5 mRNA and protein in adult Tdrd5\textsuperscript{KO} testes (Fig. 1b and c). Tdrd5\textsuperscript{KO} male mice exhibited atrophic testes with an average of 50% of wild-type control testis weight (Fig. 1d), and were infertile due to germ cell arrest at the round spermatid stage (Fig. 1e). No elongating spermatids or spermatozoa were found in Tdrd5\textsuperscript{KO} seminiferous epithelium (Fig. 1e). As a result, only round spermatid-like cells could be observed in Tdrd5\textsuperscript{KO} epididymides (Fig. 1e). In Tdrd5\textsuperscript{KO} testes, round spermatids arrested before step 5 as proacrosome granules but not acrosome caps were observed in arrested spermatids (Fig. 1f). Subsequently, arrested Tdrd5\textsuperscript{KO} round spermatids showed pronounced DNA damage after reaching seminiferous epithelium stage VIII (Fig. 1g). These results indicate that postnatal expression of TDRD5 is essential for spermiogenesis (Fig. 1h).

TDRD5 is essential for pachytene piRNA biogenesis. We next examined the effect of postnatal germ cell-specific TDRD5 loss on...
piRNA biogenesis. Radiolabeling of total RNA isolated from adult wild-type and Tdrd5cKO testes revealed that the total piRNA production was severely reduced in Tdrd5cKO testes (Fig. 2a). However, the piRNAs produced in Tdrd5cKO testes appeared to be of normal size distribution (Fig. 2a). Sequencing of small RNAs from wild-type and Tdrd5cKO total RNA revealed that two predominant populations comprised the remaining piRNAs in Tdrd5cKO testes, corresponding to 25–28 nt MILI-bound piRNAs (MILI-piRNAs) and 29–32 nt MIWI-bound piRNAs (MIWI-piRNAs) (Fig. 2b). To quantify the relative abundance of total piRNA in wild-type and Tdrd5cKO testes, we used total microRNAs (miRNAs), a widely used reference small RNA.
Fig. 2 Postnatal male germ cell-expressed TDRD5 is essential for pachytene piRNA biogenesis. a Total RNA from adult WT and Tdrd5<sup>cKO</sup> testes was end-labeled with [32P]-ATP and detected by 15% TBE urea gel and autoradiography, nt, nucleotide. b Size distribution of small RNA libraries from adult WT and Tdrd5<sup>cKO</sup> testes. Data were normalized by microRNA urea gel and autoradiography. Western blotting was performed with MILI and MIWI antibodies to show immunoprecipitation efficiency. c MILI- and MIWI-bound piRNAs from adult WT and Tdrd5<sup>cKO</sup> testes. Small RNAs were isolated from immunoprecipitated MILI and MIWI RNPs and were end-labeled with [32P]-ATP and detected by 15% TBE urea gel and autoradiography. Western blotting was performed with MILI and MIWI antibodies to show immunoprecipitation efficiency. d Nucleotide composition of first nucleotide of MILI-piRNAs and MIWI-piRNAs in adult WT and Tdrd5<sup>cKO</sup> testes. The piRNAs in Tdrd5<sup>cKO</sup> exhibited a 5′ end U bias at position 1.

TDRD5 deficiency selectively reduces cluster-derived piRNAs. We further characterized the piRNAs produced in Tdrd5<sup>cKO</sup> mice by mapping the reads to the mouse genome. In adult wild-type testes, 80% piRNAs are derived from recently defined 214 piRNA clusters as seen previously<sup>19</sup> (Fig. 3a, Supplementary Table 1). But there was a specific reduction in the percentage of 214 piRNA cluster-derived piRNAs in Tdrd5<sup>cKO</sup> testes (Fig. 3a). This contrasts with an increase in piRNA percentage from other piRNA sources including coding RNAs, non-coding RNAs, repeats, and introns. To confirm that the reduction in piRNA cluster-derived piRNAs was specific for TDRD5 deficiency, we examined the piRNA composition in Miwi<sup>cKO</sup> mice, which exhibit similar germ cell arrest and levels of overall piRNA reduction (Supplementary Fig. 4)<sup>37</sup>. The percentage of cluster-derived piRNAs in Miwi<sup>cKO</sup> was equivalent to that of wild type (Fig. 3a). This indicates that, unlike MIWI deficiency, TDRD5 deficiency selectively reduces piRNA production from piRNA clusters. When normalized to miRNA expression, the reduction in total piRNA expression was similar between TDRD5 and MIWI deficiency. piRNAs mapping to “non-cluster” regions (coding RNAs, non-coding RNAs, repeats, introns, and other) were not decreased, indicating that piRNA reduction in Tdrd5<sup>cKO</sup> mice was specific to piRNA clusters (Fig. 3b). By contrast, piRNAs derived from piRNA clusters and non-cluster regions were both reduced in Miwi<sup>cKO</sup> testes (Fig. 3b), further indicating the special role of TDRD5 to selectively control piRNA production from piRNA clusters. Analysis of MILI-piRNAs in Tdrd5<sup>cKO</sup> testes showed the same specific percentage reduction of cluster-derived piRNAs (Fig. 3c), even when MILI proteins were loaded with the similar amount of piRNAs in Tdrd5<sup>cKO</sup> testes (Fig. 2c). This also differs from Miwi<sup>cKO</sup>, in which the percentage of MILI-piRNAs from different
piRNAs from adult WT, annotation of total piRNA from sorted pachytene spermatocytes (PS) from WT and Tdrd5cKO selectively regulates top piRNA-producing clusters during the pachytene stage of male meiosis. piRNA cluster-derived, but not other source-derived piRNAs assess the effect of TDRD5 loss on piRNA production from testes normalized by miRNA. Note specifically for the production of MIWI-piRNAs as compared to the wild type (Supplementary Fig. 6c). TDRD5 selectively controls the production of piRNA cluster-derived pachytene piRNAs.

Fig. 3 TDRD5 selectively controls the production of piRNA cluster-derived pachytene piRNAs. a Genomic annotation of total piRNA from adult WT, Tdrd5cKO, and MiwiKO tests. piRNA clusters: 214 piRNA clusters defined by Li et al.19. b Relative abundance of total piRNA from adult WT, Tdrd5cKO, and MiwiKO tests normalized by miRNA. Note specific reduction of piRNA cluster-derived piRNAs in Tdrd5cKO tests. c Genomic annotation of MILI-bound piRNAs from WT and Tdrd5cKO tests. d Genomic annotation of MILI-bound piRNAs from adult WT, Tdrd5cKO, and MiwiKO tests. e Genomic annotation of total piRNA from sorted pachytene spermatocytes (PS) from WT and Tdrd5cKO tests. f Relative abundance of total piRNA from sorted pachytene spermatocytes from WT and Tdrd5cKO tests normalized by miRNA.

The origin of pachytene piRNAs was not affected (Fig. 3c). The percentage of cluster-derived piRNAs within Tdrd5cKO MIWI-piRNAs was also reduced (Fig. 3d). We next sorted and purified pachytene spermatocytes to confirm that the selective reduction in piRNA cluster-derived piRNAs occurs in this main pachytene piRNA-producing cell type. After RNA-seq of total small RNAs from wild-type and Tdrd5cKO spermatocytes, we analyzed piRNA length distribution and composition (Supplementary Fig. 6). Similar with the results from whole testes, Tdrd5cKO spermatocytes displayed a normal amount of 25–28 nt small RNAs corresponding to MILI-piRNAs, and much lower amount of 29–32 nt small RNAs corresponding to MIWI-piRNAs as compared to the wild type (Supplementary Fig. 6b). After mapping these total piRNA reads to the mouse genome, Tdrd5cKO spermatocytes displayed a specific decrease in cluster-derived piRNAs normalized by miRNAs (Fig. 3e, f). Collectively, these results indicate that TDRD5 is a key pachytene piRNA biogenesis factor required specifically for the production of piRNA cluster-derived, but not other source-derived piRNAs during the pachytene stage of male meiosis.

TDRD5 selectively regulates top piRNA-producing clusters. To assess the effect of TDRD5 loss on piRNA production from individual piRNA clusters, we analyzed piRNA reads from wild-type and Tdrd5cKO small RNA libraries mapped to each of the 214 piRNA clusters19. After normalization by miRNA counts of each library, we directly compared the number of wild-type and Tdrd5cKO piRNA reads mapped to each piRNA cluster (Fig. 4a). In wild-type testes, top 50 piRNA-producing clusters give rise to the vast majority (>90%) of 214 cluster-derived piRNAs (Fig. 4a). In Tdrd5cKO testes, piRNAs produced from these top 50 piRNA-producing clusters were uniformly reduced by an average of sevenfold. In contrast, the piRNAs mapped to low-piRNA-producing clusters were less affected by TDRD5 deficiency, particularly the 84 prepachytene and 30 hybrid piRNA clusters previously defined within these 214 piRNA clusters19 (Fig. 4a, b). This indicates that loss of TDRD5 selectively affects piRNA production from a subset of most abundantly expressed pachytene piRNA precursors. To confirm this selective effect on piRNA reduction is unique to TDRD5, we examined the pattern of piRNA reduction in individual piRNA clusters in MiwiKO testes. Unlike the effect of TDRD5 deficiency on total piRNA production, Miwi deficiency had a uniform reduction effect on both the high-piRNA-producing clusters and the low-piRNA-producing clusters (Fig. 4b). Analysis of the MILI-piRNAs from Tdrd5cKO tests and MiwiKO tests revealed a similar trend.
deficiency caused selective reduction of MILI-piRNA production from high-piRNA-producing clusters while MIWI deficiency did not globally affect MILI-piRNAs from almost all clusters (Fig. 4c). These data together indicate that, within the 214 piRNA clusters, TDRD5 deficiency selectively ablates piRNA production from a large subset of most highly expressed piRNA clusters, which correlates with A-MYB transcriptionally controlled piRNA clusters.

**Genetically separable steps in pachytene piRNA processing.** To investigate whether TDRD5 deficiency affects piRNA production uniformly within single-piRNA clusters, we analyzed Tdrd5cKO piRNA densities across the lengths of representative high-piRNA-producing clusters. As an example, the pach43 cluster (also named 17-qA3.3-27363.1) is one of the most abundantly expressed piRNA cluster. Unexpectedly, although the total amount of piRNAs produced was significantly reduced from this cluster, the 5’ ends of precursor RNAs (within ~300 bp of
transcription start site) could still produce piRNAs at levels comparable to wild-type controls. The rest of piRNA-producing regions in this precursor generated very little piRNAs (Fig. 5a). Similar results were observed from other representative piRNA clusters (Supplementary Fig. 7a). This is highly surprising and represents a special form of piRNA biogenesis defect that occurs within single clusters. The normal presence of precursor 5′ derived piRNAs and the selective depletion of piRNAs from the remainder of piRNA precursor in Tdrd5cKO mice is different from the piRNA defect in MiwiKO mice, which showed proportionally reduced piRNA density across the entire length of piRNA cluster (Fig. 5a, Supplementary Fig. 7b). We next sought to confirm this unique piRNA defect occurred in both MILI- and MIWI-bound piRNAs. Analysis of MILI-piRNAs in Tdrd5cKO mice showed the same trend as observed in total piRNAs, with piRNA production from 5′ 300 nt regions being almost unchanged, while the piRNAs from the rest length of the precursor had a significant decrease (Fig. 5b, Supplementary Fig. 8). Similar results were also observed for Tdrd5cKO MIWI-piRNAs (Fig. 5c, Supplementary Fig. 8b). To confirm that differential reduction in piRNA production from a single-piRNA cluster is common for all of the high-piRNA-producing clusters affected by TDRD5 loss, we analyzed the piRNA fold change within the 5′ end 300 nt region or across full-length of the transcript for the top 50 piRNA precursors. Although the total amount of piRNAs produced from whole precursors was reduced to an average of sevenfold, the amount of piRNAs produced from the 5′ ends of precursor RNAs within 300 nt were almost unchanged (Fig. 5d). We further analyzed piRNA densities from all 214 piRNA precursors. We divided each of the 214 piRNA precursor RNAs into 100 fragments of equal length and mapped piRNAs from wild-type and Tdrd5cKO piRNA libraries to each of the 100 fragments from each piRNA
precursor. Results indicate that the 5’ ends of precursor RNAs could still produce piRNAs at levels comparable to wild-type controls, while the rest of piRNA-producing regions in these precursors generated very little piRNAs (Fig. 5e, Supplementary Fig. 9). Together, these data reveal that pachytene piRNA production within a single precursor can be genetically separated into at least two steps: 5’ end processing, and the processing of the rest of the transcript.

**TDRD5 interacts with PIWI proteins.** We next sought to understand the potential mechanism by which TDRD5 plays its role in the piRNA pathway. The TDRD5 protein contains one Tudor domain and three LOTUS domains. The Tudor domain displays conserved binding to PIWI proteins in animal germ cells. Since TDRD5 regulates the production of both MILI-piRNAs and MIWI-piRNAs, we examined its direct association with PIWI proteins. When ectopically co-expressed with MILI or MIWI in HEK293T cells, TDRD5 was detected in both MILI and MIWI immunoprecipitates, indicating its ability to interact with PIWI proteins (Fig. 6a). To test whether the interaction is mediated by the Tudor domain, we used a series of truncated proteins of TDRD5 to examine interactions with MIWI. The Tudor domain, but not other regions of TDRD5, was interacting with PIWI proteins (Fig. 6a). These results suggest that TDRD5 could associate with piRNA precursors. To further test whether TDRD5 directly binds piRNA precursors, we performed high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation (HITS-CLIP or CLIP-seq) in testes from adult wild-type mice (Fig. 7, Supplementary Fig. 11). We first detected TDRD5-specific RNA complexes by CLIP and autoradiography (Fig. 7a). MILI-CLIP was used as a control to represent a known piRNA pathway RNA-binding protein that directly binds RNA (Fig. 7a). CLIP results indicate that, like MILI, TDRD5 directly binds RNA. We next constructed CLIP-seq libraries using RNA isolated from TDRD5-CLIP and MILI-CLIP complexes and performed deep sequencing. Length distribution of TDRD5 CLIP reads displayed a broader length range as compared to MILI-CLIP reads, which primarily contained mature piRNAs of 25–28 nt in length (Fig. 7b, c). TDRD5-CLIP reads contained a predominant A at the first nucleotide position (Fig. 7d), indicative of a signature of mature piRNAs (Fig. 7e). We performed UV cross-linking immunoprecipitation of TDRD5 in wild-type and Tdrd5 KO testes and examined the expression levels of several piRNA precursors by RT-PCR. The piRNA precursor RNAs were specifically associated with TDRD5 immunoprecipitates from wild-type testes, but not detected in the IgG control immunoprecipitates from wild-type testes or TDRD5 immunoprecipitates from Tdrd5 KO testes (Supplementary Fig. 10). These results suggest that TDRD5 could associate with piRNA precursors. To test further whether TDRD5 directly binds piRNA precursors, we performed high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation (HITS-CLIP or CLIP-seq) in testes from adult wild-type mice (Fig. 7, Supplementary Fig. 11). We first detected TDRD5-specific RNA complexes by CLIP and autoradiography (Fig. 7a). MILI-CLIP was used as a control to represent a known piRNA pathway RNA-binding protein that directly binds RNA (Fig. 7a). 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TDRD5-CLIP reads contained a predominant A at the first nucleotide position (Fig. 7d), indicative of a signature of mature piRNAs (Fig. 7e). We further analyzed the genomic origin of TDRD5-CLIP and MILI-CLIP reads (Fig. 7f). Over 40% of TDRD5-CLIP reads were mapped to 214 piRNA clusters, consistent with its critical role in piRNA precursor processing. Within the 214 piRNA clusters, the
mapped TDRD5-CLIP and MILI-CLIP reads displayed a linear correlation with the amount of piRNA production from each cluster (Fig. 7g). To explore whether TDRD5 has a preference in binding to certain regions within individual piRNA precursor RNAs, we analyzed the densities of TDRD5-CLIP and MILI-CLIP reads across the lengths of representative high-piRNA-producing clusters. The TDRD5-CLIP read densities at each position were highly correlated with MILI-CLIP read densities, indicating that TDRD5 could bind to any region within the precursor, including ~300 bp of transcription start site, lacking a clear preference in binding to any specific regions in each precursor (Supplementary Fig. 12, Fig. 7h). Together, these data indicate that TDRD5 is an RNA-binding protein that associates with piRNA precursors along their entire lengths. Thus, TDRD5 could regulate piRNA biogenesis through direct association with piRNA precursors.

piRNA precursors are not accumulated in Tdrd5<sup>KO</sup> testes. MOV10L1, an RNA helicase that binds and unwinds piRNA precursors, is required for the production of the entire population of pachytene piRNAs in mice. Conditional ablation of MOV10L1 in postnatal male germ cells causes a complete

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**Fig. 7** TDRD5 directly binds piRNA precursors. a Autoradiography (left) and western blot (right) of TDRD5-RNA and MILI-RNA complexes from CLIPs. IgG served as negative control. Red lines indicate the corresponding RNA regions that were extracted from the membrane. HC, Ig heavy chain; LC, Ig light chain. b Size distribution of RNA reads from TDRD5-CLIP libraries. n = 3; Error bars represent s.e.m. c Size distribution of RNA reads from MILI-CLIP library. d Nucleotide composition of TDRD5-CLIP reads. n = 3; Error bars represent s.e.m. e Nucleotide composition of MILI-CLIP reads. f Genomic annotation of TDRD5-CLIP reads and MILI-CLIP reads. TDRD5-CLIP libraries, n = 3; Error bars represent s.e.m. g Scatter plot of piRNA reads mapped to 214 individual piRNA clusters from TDRD5-CLIP and MILI-CLIP libraries. Pearson correlation ($R^2$) is shown. h Density plots of total piRNA reads and TDRD5-CLIP reads mapped to each of the 100 positions proportionally divided in all 214 piRNA clusters. Each of the 214 piRNA clusters is equally divided into 100 sequence fragments. The total piRNA reads or TDRD5-CLIP reads were mapped to each fragment of each piRNA cluster and total mapped reads for each position were added up for all 214 clusters. Pearson correlation ($R^2$) is shown.
blockade of piRNA precursor processing, resulting in loss of mature piRNAs and corresponding piRNA precursor accumulation\textsuperscript{20,44}. To investigate whether loss of TDRD5 has any effect on piRNA precursor abundance, we analyzed piRNA precursor levels in wild-type and Tdrd5\textsuperscript{cKO} tests (Fig. 8). piRNA precursor expression levels in Tdrd5\textsuperscript{cKO} tests were not different from the wild type. This contrasts with the significantly elevated piRNA precursor levels in Mov10l1\textsuperscript{cKO} tests (Fig. 8). The difference in piRNA precursor accumulation suggests distinct roles for TDRD5 and MOV10L1 in pachytene piRNA precursor processing. It is likely that loss of TDRD5 results in the dissociation of piRNA precursors from the piRNA processing complex, which leads to subsequent degradation.

Discussion

Our data reveal an essential role for Tudor domain protein TDRD5 in piRNA biogenesis in mice. Rather than controlling the production of the whole piRNA population, TDRD5 selectively regulates the piRNA production from a subset of the most abundant piRNA clusters during meiosis. We also discovered that TDRD5 deficiency genetically uncouples pachytene piRNA precursor 5′ end processing from the remainder the precursor. These two discoveries place TDRD5 as a unique protein among known piRNA biogenesis factors and provide insight into the mechanism of mammalian pachytene piRNA biogenesis.

After transcription, pachytene piRNA precursors are believed to be transported from the nucleus to enter intermitochondrial cement (IMC) in cytoplasm for processing. Our results reveal a function of TDRD5 downstream of piRNA precursor recruitment to IMC and upstream of piRNA loading, trimming and maturation. This is supported by the observation that there was relative normal production of precursor 5′ end-derived piRNAs from TDRD5-regulated single piRNA precursors, and that late steps of piRNA biogenesis were not apparently impaired in piRNAs produced in Tdrd5\textsuperscript{cKO} tests. This places the function of TDRD5 at the start of piRNA processing after precursor entry into piRNA processing complex (PPC). Given the localization of TDRD5 at IMC\textsuperscript{35} and its interaction with PIWI proteins, we propose here TDRD5 is a critical component of the pachytene PPC that regulates a large subset of the most abundantly expressed piRNA precursors funneled through IMC (Supplementary Fig. 13). We propose that the entire pachytene piRNA precursors are classified into TDRD5-regulated and TDRD5-independent sub-populations that are differentially processed. TDRD5-regulated piRNA precursors comprise most of the top piRNA-producing precursors transcribed from intergenic piRNA clusters. Regulated by TDRD5, these precursors account for the most abundant piRNA species produced in wild-type tests.

Other piRNA precursors emanating from other low-piRNA producing loci are processed independently of TDRD5, accounting for their insensitivity to TDRD5 deficiency. For TDRD5-regulated precursors, despite of the failure in processing and drastic reduction in mature piRNAs, we did not observe corresponding piRNA precursor accumulation. This contrasts with MOV10L1 deficiency, in which unprocessed piRNA precursors are abundantly accumulated\textsuperscript{20}. It is likely that unprocessed piRNA precursors in Tdrd5\textsuperscript{cKO} tests are degraded, which suggests a role for TDRD5 in piRNA precursor stabilization. This is consistent with our CLIP-seq data demonstrating the direct association of TDRD5 with piRNA precursors. Diminished piRNA production may account for the observed defects in IMC and chromatoid body in TDRD5 deficient germ cells\textsuperscript{35}.

Interestingly, despite the severe reduction in piRNA production, individual top piRNA-producing clusters in Tdrd5\textsuperscript{cKO} still generated significant amount of piRNAs, and the ranking of piRNA amounts produced by each cluster is essentially not changed. This indicates that TDRD5 loss could not reverse the existing advantages that the top piRNA-producing precursors have for being selected and processed by the PPC. This in turn suggests that other unknown protein factor(s) are responsible for the initial selection of piRNA precursors to enter the PPC. We envision that TDRD5 provides an essential layer of selection after piRNA precursors enter the PPC, that is, to further retain/stabilize and facilitate top piRNA-producing precursors for highly efficient processing through direct TDRD5-piRNA precursor interactions.

An important aspect of piRNA biogenesis discovered in this study is that, within a single TDRD5-dependent piRNA cluster, the apparently normal production of piRNAs mapping to the very 5′ end of the piRNA cluster and the diminished production of piRNAs mapping to the rest regions of the cluster. This indicates that the pachytene piRNA precursor processing is genetically separable. The uncoupling of precursor processing within individual clusters was observed in both MILI-piRNAs and MIWI-piRNAs. Why the processing of precursor 5′ end does not require TDRD5 is not known, but it is clear that piRNA precursor recruitment to the PPC continues in the absence of TDRD5. Our TDRD5 CLIP-seq results indicate that TDRD5 directly binds piRNA precursors evenly across their entire lengths with no preferential recognition of specific hot spots, and thereby stabilizes precursors for processing by the PPC critical enzymes MOV10L1 and MitoPLD. Conceivably, the loss of TDRD5 could destabilize precursor retention at the PPC, thereby leading to RNA loss and eventual degradation after precursor 5′ processing. Although we cannot rule out that TDRD5 loss may also affect the processivity of the cleavage enzyme MitoPLD, the direct associate of TDRD5 with piRNA precursors and the absence of piRNA
precursor accumulation upon TDRD5 loss are most consistent with a direct role for TDRD5 in piRNA precursor retention/ stabilization. Thus, we propose TDRD5 as a core component of the PPC that functions downstream piRNA precursor recruitment to stabilize and enhance precursor processing during piRNA biogenesis.

Methods

Ethics statement. All the animal procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University. All experiments with mice were conducted ethically according to the Guide for the Care and Use of Laboratory Animals and institutional guidelines.

Mouse strains. A Tdrd5 gene targeted embryonic stem (ES) cell clone, Tdrd5fl/fl, was acquired from European Mouse Mutant Cell Repository. Tdrd5fl/fl is a knock-out first allele, which allows the subsequent generation of a conditional Tdrd5lox/lox allele with exon 7 flanked by loxP sites (Supplementary Fig. 1a). To generate Tdrd5lox/lox chimeric mice, ES cells were expanded and injected into C57BL/6J blastocysts. Chimeric males were bred with C57BL/6J females to generate heterozygous Tdrd5tm1a/loxP animals. To generate Tdrd5lox allele, heterozygous Tdrd5tm1a/loxP animals were bred with FLP-expressing [Jackson Laboratory] to remove FRT flanked sequences (Fig. 1a). Tdrd5lox/lox were bred with Tdrd5tm1a/+ females to generate homozygous Tdrd5tm1a/loxP mice. To generate Stra8-Cre Tdrd5 conditional knockout mice, Stra8-Cre transgenic mice (Jackson Laboratory) were bred with Tdrd5tm1a/fl mice using scheme described in Supplementary Fig. 2a. Primers for Tdrd5lox-352531103388-lox and Tdrd5lox-112715321220-lox genotyping PCR are: F1: 5′-AGGCTCCTAATCTCCTGAGATC-3′ and R1: 5′-CAGTTCCACACATCAACATTGAGCC-3′. Wild-type allele produced a 594 bp product; Tdrd5lox allele generated a 557 bp product. Primers for Stra8-Cre PCR (236 bp) were: 5′-GTCGAACGCTAGACAAAGACAG-3′ and 5′-AGGAACACAGATTGGAGTCT-3′. A set of primers was used as the internal control (324 bp) 5′-GCAGTTCCACACATCAACATTGAGCC-3′ and 5′-CTAGGGCCACAGAATTGAAAGATCT-3′.

TDRDS antibody generation. Complementary DNA corresponding to TDRD5 283–371 as a cloned in pET-28a (His-tag) and pGEX-4T-1 (GST-tag) vectors. His-tagged recombinant protein was used as an antigen to generate rabbit anti-TDRD5 polyclonal antiserum (Pacific Immunology). Antiserum were affinity purified with GST-tagged antigen immobilized on beaded agarose using AminoLink Plus immobilization kit (Thermo Scientific).

Histology. Testes and epididymides from adult wild-type and mutant mice were fixed in Bouin’s fixative and embedded in paraffin. For the histological analysis, sections of 5 μm were cut and stained with hematoxylin and eosin after dehydrating and rehydration.

Immunofluorescence. Testes were fixed in 4% PFA in PBS overnight at 4 °C and embedded in paraffin. For immunostaining, tissue sections of 5 μm were cut, dehydrated and rehydrated. Antigen retrieval was performed by microwaving the sections in 0.1 M sodium citrate buffer (pH 6.0) for 4 min. Tissue sections were blocked in 5% normal goat serum (NGS) for 30 min after rinsing with PBS. Testis sections were then incubated with primary antibodies diluted in 5% NGS at 37 °C for 2 h. Antibodies used were: rabbit anti-MIWI (1:100; 2079, Cell Signaling) or mouse anti-MILI (PM044, MBL) or anti-MIWI (ab12337, Abcam) or mouse anti-Miwi (1:500; Sigma, rabbit anti-MIWI (1:1000; ab9290, Abcam). Western blotting. RIPA buffer (50 mM Tris–HCl, pH 7.4, 1% Nonidet P-40, 0.5% Na deoxycholate, 0.01% SDS, 1 mM EDTA, and 150 mM NaCl) was used to homogenize and lyse mouse testes. Testis protein lysates were separated by 4–20% SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). After blocking in 5% non-fat milk, the membranes were incubated with primary antibodies in blocking solution at 4 °C overnight. Membranes were washed with TBST for three times and incubated with HRP-conjugated goat anti-rabbit IgG (1:5000; 1706515, Bio-Rad) or goat anti-mouse IgG (1:5000; 1706516, Bio-Rad) for 1 h. After rinsing with TBST for three times, chemiluminescent detection was performed. The primary antibodies used were: rabbit anti-TDRD5 (1:2000), mouse anti-β-actin (1:5000; A3854, Sigma), rabbit anti-MILI (1:2000; PM044, MBL), rabbit anti-MIWI (1:1000; 2079, Cell Signaling Technology). Uncropped versions of all blots are included as Supplementary Fig. 14.

Immunoprecipitation of piRNAs. Mouse testes were homogenized in lysis buffer (20 mM HEPES, pH 7.3, 150 mM NaCl, 2.5 mM MgCl2, 0.2 % NP-40, and 1 mM DTT). After proteinase inhibitors and RNase inhibitors (Promega). The lysates were centrifuged at 13,000 g for 10 min after sonication. The supernatants were collected and pre-cleared with protein-A agarose beads (Roche) at 4 °C for 2 h. Anti-MIWI (PM044, MBL) or anti-mIwi (ab13373, Abcam) antibodies were used for immunoprecipitation and protein-A agarose beads were added to the supernatants and incubated for 4 h to capture miRNAs. The beads were then collected and washed in lysis buffer for five times. Immuno-precipitated piRNAs were isolated using Trizol reagent (Thermo Scientific) for downstream piRNA labeling and small RNA library construction experiments.

Detection of piRNAs. Total RNA was isolated from mouse testes using Trizol reagent (Thermo Scientific). Total RNA (1 μg) or immunoprecipitated RNA was dephosphorylated with Shrimp Alkaline Phosphatase (NEB). RNA end-labeling was performed using T4 polynucleotide kinase (NEB) and [γ-32P] ATP. 32P-labeled RNA was separated on a 15% Urea-PAGE, and signals were detected by exposing the gel on phosphorimager screen. Images were obtained by scanning on the Typhoon scanner (GE Healthcare).

Cell sorting. Pachytene spermatocytes were isolated using flow cytometry according to a published protocol with modifications38. Briefly, testes were collected from adult mice, and the tunica was removed. Testes were digested for 10 min at 32 °C in HBSS with 50 μl/ml collagenase IV (Thermo Scientific, W26104) and RNase inhibitor (Promega). The lysates were centrifuged at 13,000 g for 10 min after sonication. The supernatants were collected and pre-cleared with protein-A agarose beads (Roche) at 4 °C for 2 h. Anti-MIWI (PM044, MBL) or anti-mIwi (ab13373, Abcam) antibodies were used for immunoprecipitation and protein-A agarose beads were added to the supernatants and incubated for 4 h to capture miRNAs. The beads were then collected and washed in lysis buffer for five times. Immuno-precipitated piRNAs were isolated using Trizol reagent (Thermo Scientific) for downstream piRNA labeling and small RNA library construction experiments.

Small RNA libraries and bioinformatics. Immunoprecipitated RNAs or total RNA were used to construct small RNA libraries. Small RNA libraries were prepared using NEBNext Multiplex Small RNA Library Prep Kit (E7300, NEB). Multiple libraries with different barcodes were pooled together and sequenced using Illumina HiSeq 2500 (Illumina, USA). Read length was 32 nt, unless otherwise indicated. The reads were then aligned to 5 sets of sequences: (1) 214 piRNA cluster5, (2) coding RNAs (Reseq coding gene mRNAs), (3) non-coding RNAs (Reseq non-coding gene mRNAs), (4) Repeats (LINE, SINE, LTR, DNA, Low_complexity, Satellite, Simple_repeat), and (5) Intron (Genic regions for Reseq genes). For alignment to each set, only sequence reads that were not aligned to any of the previous sets were included. Sequence reads not mapping to the above 5 sets of sequences were classified as ‘other’. Here we define “non-cluster” as all reads not mapping to the 214 piRNA clusters. Table 4 lists all subsets coding RNA, repeats, intron, and other. Alignments were performed using Bowtie (one base mismatch allowed). The Repeats sequence set used here is defined by the (2018) 9:127 | www.nature.com/naturecommunications

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For cDNA synthesis, quantitative RT-PCR was performed in triplicate wells using CFX96 Real-Time PCR detection system. Extracted RNA was transferred onto nitrocellulose membrane and exposed overnight. Nitrocellulose membrane was stained with SYBR Safe. DNA was extracted with QIAquick gel extraction kit and sequenced on an Illumina MiSeq at 300 cycles.

Table 2.

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Author contributions

D.D. and C.C. conceived the project; D.D. performed small RNA library constructions and CLIP-seq. J.L. and Y.W. performed imaging; U.M., K.D., and D.D. performed bioinformatics analysis. D.D. performed protein and antibody purification with assistance from Y.W. and A.M.; D.D., K.E.L., and C.C. wrote the manuscript; C.C. supervised the project.

Additional information

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