Mouse Piwi interactome identifies binding mechanism of Tdrkh Tudor domain to arginine methylated Miwi

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Tudor domains are protein modules that mediate protein–protein interactions, potentially by binding to methylated ligands. A group of germline specific single and multiTudor domain containing proteins (TDRDs) represented by *drosophila* Tudor and its mammalian orthologs Tdrd1, Tdrd4/RNF17, and Tdrd6 play evolutionarily conserved roles in germinal granule/nuage formation and germ cell specification and differentiation. However, their physiological ligands, and the biochemical and structural basis for ligand recognition, are largely unclear. Here, by immunoprecipitation of endogenous murine Piwi proteins (Miwi and Mili) and proteomic analysis of complexes related to the piRNA pathway, we show that the TDRD group of Tudor proteins are physiological binding partners of Piwi family proteins. In addition, mass spectrometry indicates that arginine residues in RG repeats at the N-termini of Miwi and Mili are methylated in vivo. Notably, we found that Tdrkh/Tdrd2, a novel single Tudor domain containing protein identified in the Miwi complex, is expressed in the cytoplasm of male germ cells and directly associates with Miwi. Mutagenesis studies mapped the Miwi–Tdrkh interaction to the very N-terminal RG/RA repeats of Miwi and showed that the Tdrkh Tudor domain is critical for binding. Furthermore, we have solved the crystal structure of the Tdrkh Tudor domain, which revealed an aromatic binding pocket and negatively charged binding surface appropriate for accommodating methylated arginine. Our findings identify a methylation-directed protein interaction mechanism in germ cells mediated by germline Tudor domains and methylated Piwi family proteins, and suggest a complex mode of regulating the organization and function of Piwi proteins in piRNA silencing pathways.

Tudor domains, together with Chromo, MBT, PWWP, and Agenet-like domains, comprise the "Tudor Royal Family" of domains (1). The core structure of this protein domain superfamily is characterized by an antiparallel β-barrel-like topology and mediates protein–protein interactions, in some cases by recognizing methylated lysine/arginine-containing ligands with a binding site composed of aromatic residues (2, 3). Their methylated target proteins are implicated in diverse biological processes such as chromatin remodeling and RNA splicing. For example, the Tudor domain of Smn binds to methylated arginine-glycine (RG) motifs on Sm proteins essential for spliceosome assembly (4), while the Tudor domains of Jmjd2a bind to methylated lysines in histone H4K20 (5).

Drosophila Tudor, the founding member of the Tudor domain family, is a germ cell-specific protein with multiple Tudor domains and is involved in germ plasm formation and germ cell specification (6). By analyzing the expression pattern of mammalian genes encoding Tudor domain proteins, we identified a group whose expression is highly enriched in germ cells, which we therefore term germline Tudor proteins (Tdrd1, Tdrkh/Tdrd2, RNF17/Tdrd4, Tdrd5, Tdrd6, Tdrd7, Stk31/Tdrd8, Tdrd9, Tdrd10, Akap1) (supporting information (SI) Fig. S1). While the physiological functions of germline proteins with a single Tudor domain (Tdrkh, Tdrd5, Stk31, and Tdrd9) are largely unknown, mouse knockout studies of Tdrd1, Tdrd4, and Tdrd6 have revealed crucial roles for these multiTudor domain proteins in nuage/chromatoid body formation, spermatogenesis, and small RNA pathways (7–9). However, the binding properties of these germline Tudor proteins are poorly understood.

Piwi proteins are conserved germline-specific Argonaute family members that are associated with Piwi-interacting RNAs (piRNAs), and thereby function in piRNA-mediated posttranscriptional silencing (10). Three murine Piwi paralogs, Miwi, Mili, and Miwi2, play pivotal roles in germ cell development, transposon silencing and spermatogenesis (11–13). The presence of multiple arginine-glycine and arginine-alanine (RG/RA)-rich clusters at the N-termini of these proteins prompted us to question whether these RG/RA motifs can be methylated in vivo and thereby serve as docking sites for the binding of various germline Tudor proteins.

To test this hypothesis, we performed a comprehensive proteomic analysis of Miwi and Mili complexes in adult male germ cells and determined the methylation status of these Piwi proteins. We show that several germline Tudor proteins are physiological binding partners of the Piwi family. In particular, we identify Tdrkh as a novel Miwi-interacting protein that binds Miwi through its single Tudor domain, likely via arginine methylation, as suggested by a combination of mass spectrometry, mutagenesis, and structural analysis.

Results

Tudor Domain-Containing Proteins Are Major Physiological Binding Partners of Piwi Family Proteins. To test whether Tudor domain family proteins comprise the in vivo binding partners of the Piwi proteins, we immunoprecipitated endogenous Miwi and Mili from lysates of adult testes and purified the complexes by acid elution. To obtain a comprehensive survey of the components of the Piwi complexes, we used a gel-free liquid chromatography coupled tandem mass spectrometry (LC-MS/MS) approach employing solid phase tryptic digestion. This technique allowed us to unambiguously identify an extensive list of candidate proteins that specifically associated with Piwi proteins (Fig. 1). Hierarchical clustering of 2 independent repeats of Miwi and Mili immunoprecipitations (IP) with their respective IgG control IPs reproducibly revealed distinct protein complex profiles for Miwi and Mili (Fig. 1). We found proteins that were specifically associated with either Miwi (Fig. 1*A*, blue box and Fig. S2) or Mili (Fig. 1*A*, red box), and proteins shared by both Miwi and Mili complexes (Fig. 1*A*, cyan box). All of these proteins were absent from the IgG control IPs, which contain nonspecific binding proteins (Fig. S2). In this analysis, we observed several previously known Piwi-interacting proteins or piRNA path-

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The authors declare no conflict of interest.

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 3fdr).

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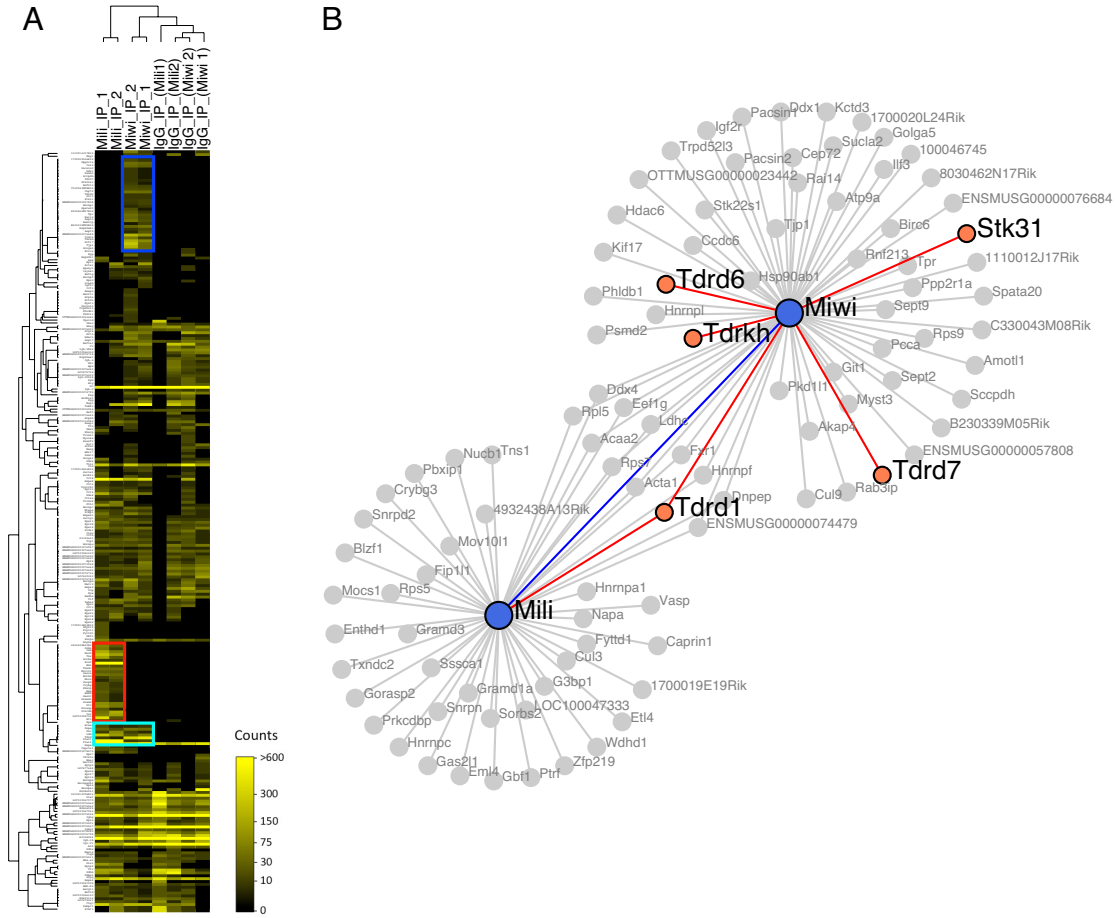

Fig. 1. Proteomic analysis of endogenous Piwi complexes identifies Tudor domain family proteins as physiological binding partners. (*A*) Hierarchical clustering of proteins identified by 2 independent immunoprecipitations (IP) of Miwi, Mili, and corresponding IgG controls through tandem mass spectrometry. Proteins for which 3 or more peptides were identified are shown. Proteins that interact specifically with Miwi or Mili are illustrated in blue and red boxes, respectively. The cyan box indicates common proteins associated with both Miwi and Mili. (*B*) Identification of multiple germline Tudor domain proteins in Miwi and Mili complexes. Miwi and Mili protein interaction networks are shown with baits in blue and Tudor domain proteins in orange. Proteins represented by 3 or more peptides and not present in the IgG control IP, and proteins represented by peptides with a 5-fold increase over the IgG control IP are shown.

way components such as Ddx4, Kif17, Fxr1, and Mov10l1 (12, 14–16).

Notably, several germline Tudor domain proteins (Tdrd1, Tdrkh, Tdrd6, Tdrd7, and Stk31) were present in Miwi immunoprecipitates (Fig. 1*B*). Among them, Tdrd1, Tdrkh, and Tdrd6 had high sequence coverage, suggesting a relatively higher abundance in the complex as compared with Tdrd7 and Stk31, which showed lower peptide numbers. Although Tdrd1 and Tdrd6 have very recently been implicated in binding Miwi (9), Tdrkh, Tdrd7, and Stk31 have not been characterized in association with Miwi. Only one Tudor protein, Tdrd1, was identified as one of the top hits in the Mili complex (Fig. 1*B*), consistent with recent findings characterizing a Mili-Tdrd1 interaction (17, 18). Due to the sensitivity and detection limit of the method, we cannot exclude the possibility that other germline Tudor proteins might also associate with Miwi or Mili complexes. In this report, we focus primarily on the cellular, biochemical, and structural characterization of the Tdrkh protein and its interaction with Miwi.

Identification of in Vivo Arginine Methylation Sites on Piwi Proteins by Mass Spectrometry. The interaction of Miwi and Mili with Tudor domain proteins raised the possibility that they are methylated. To determine whether the RG/RA-rich regions of Miwi and Mili are methylated in vivo, we immunoprecipitated Miwi and Mili and used acid elution and gel-free solid phase tryptic digestion followed by mass spectrometry to search for methylation sites. Mascot searches for monomethyl arginine (MMA) or dimethyl arginine (DMA) led to the identification of multiple in vivo methylation sites on Miwi and Mili with high confidence (Fig. 2 and Fig. S3). Overall, we observed more methylation sites on Mili than on Miwi. Specifically, R53 on Miwi was found in both monomethylated and dimethylated states. In Mili, R74, R83, R95, and R100 appeared both monomethylated and dimethylated and only dimethylation was found on R45, R146, R156, and R163. Interestingly, we found that 2 different arginines could be dimethylated simultaneously on a single peptide

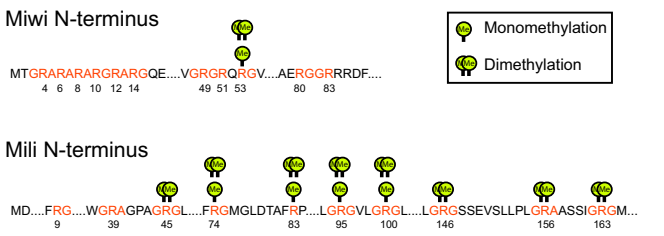

Fig. 2. Arginine methylation sites detected on endogenous Miwi and Mili by mass spectrometry. N-terminal RG/RA-rich sequences are show in red. Identified methylation sites (Me) are shown above the relevant arginine, with the residue numbers underneath.

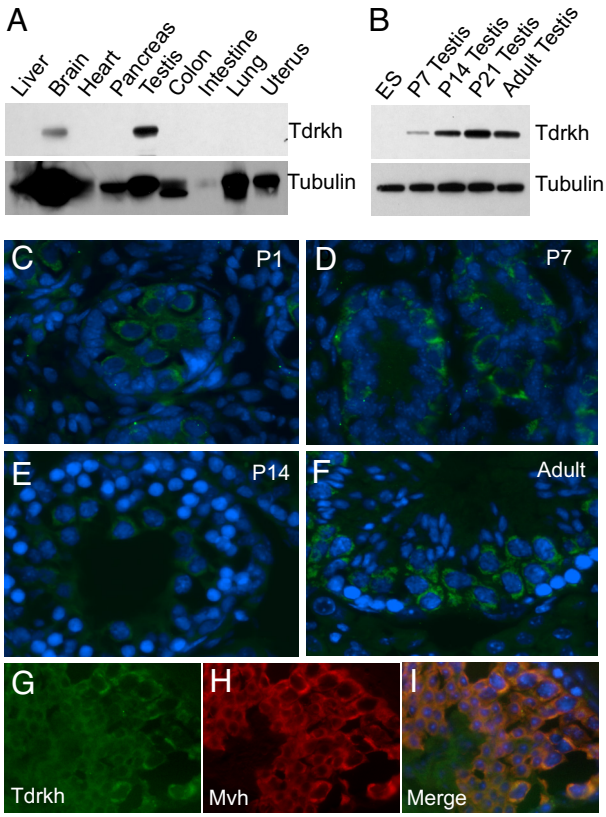

Fig. 3. Expression kinetics and cellular localization of Tdrkh in the mouse testis. (*A*) Tissue distribution of Tdrkh protein by Western blotting. Tissue lysates were probed with anti-Tdrkh antibody; anti-Tubulin antibody was used as a control. (*B*) Western blot analysis of temporal Tdrkh protein expression in mouse testes. ES: mouse embryonic stem cell, P: postnatal day. (*C–F*) Immunofluorescence staining of Tdrkh (green) in testes of different developmental ages. P: postnatal day. (*G–I*) Colocalization of Tdrkh and Mvh in the adult testis.

from Mili (Fig. S3), indicating a potential for one Piwi family protein to engage multiple docking partners, such as Tudor domains, at the same time. To distinguish whether the DMA sites we identified on the Piwi proteins were symmetric DMA (sDMA) or asymmetric DMA (aDMA), we used a method developed by Rappsilber et al. (19). By scanning the lower mass range of several DMA peptide MS/MS fragments, we were able to detect a dimethylcarbodiimidium ion (m/z = 71.06) but not an aDMA-specific dimethylammonium ion (m/z = 46.06), suggesting that these modifications are most likely symmetrically dimethylated (Fig. S4). Collectively, the identification of multiple methylation sites on Piwi proteins raises the possibility that they provide a platform for recruiting proteins with methyl arginine recognition modules, such as Tudor domains, to the piRNA silencing complex.

Temporal and Spatial Expression of Tdrkh in the Testis. We set out to further analyze the newly identified Tdrkh–Miwi interaction. The Tdrkh protein contains one Tudor domain and 2 tandem KH domains and has only been described in terms of gene cloning and mRNA expression (20). Since the tissue distribution and subcellular localization of the Tdrkh protein has not been studied, we used affinity purified anti-Tdrkh antibody to examine these points (Fig. 3). Western blot analysis of a number of mouse tissue lysates showed that Tdrkh (approximately 70 kDa) is highly expressed in the testis and to a lesser extent in the brain but was not detected, or is weakly expressed, in other tissues (Fig. 3*A*). Analysis of the temporal expression of Tdrkh in male gonads indicated a low expression level on postnatal day 7 (P7), but an elevated expression in P14, P21, and adult testes, correlating with the onset of meiosis (Fig. 3*B*). Tdrkh is not expressed in murine embryonic stem cells (Fig. 3*B*). To analyze the cell-type specific expression and subcellular localization of Tdrkh, we immunostained frozen testicular sections from P1, P7, P14, and adult testes. At P1 and P7, Tdrkh staining was enriched in the cytoplasm of spermatogonia in a granular pattern (Fig. 3 *C* and *D*). In contrast, no specific signal was detected in Sertoli cells or interstitial Leydig cells, supporting a germ cell-specific expression of Tdrkh. At P14, Tdrkh became strongly expressed in meiotic primary spermatocytes (Fig. 3*E*), and its high cytoplasmic expression was maintained in spermatocytes and round spermatids throughout adulthood (Fig. 3*F*). The expression dynamics of Tdrkh are similar to those of Tdrd1 (21, 22) but distinct from Tdrd6 (9, 22), since Tdrd6 is not expressed until P14, implicating a role for Tdrkh in both embryonic/neonatal prospermatogonia differentiation and adult meiosis. We observed coimmunostaining of Tdrkh and Mvh/Ddx4, an evolutionarily conserved RNA helicase and nuage component that shares a similar subcellular localization with Miwi (23), indicating that they are colocalized in the cytoplasm of spermatocytes and round spermatids in adult testis (Fig. 3 *G–I*) and suggesting the potential for physical and functional interactions between Tdrkh, Mvh, and Miwi.

Tdrkh Interacts with Miwi in Vivo and in Vitro. To validate the in vivo association of Tdrkh with Miwi, we immunoprecipitated Tdrkh from adult testis and analyzed its associated proteins by mass spectrometry (Figs. 4*A* and S5–S6). Consistent with our previous data, Miwi was one of the top hits in the Tdrkh pull-down. To test whether this interaction is RNA-dependent, we treated cell lysates with RNaseA before immunoprecipitation. Western blot analysis showed that RNaseA treatment did not affect the interaction between Miwi and Tdrkh, which is thereby RNA-independent (Fig. 4*B*). To further characterize and validate the interaction of Miwi and Tdrkh, we used HEK293T cells to coexpress GFP-tagged Tdrkh with either Flag-tagged wild-type Miwi or Miwi mutants with various arginine to lysine mutations, followed by immunoprecipitation with anti-Flag antibody and Western blotting with anti-GFP. There are 3 clusters of RG/RA repeats on the N terminus of Miwi. Our strategy for generating different Miwi mutants involved mutating all of the arginines, in all 3 clusters or any 2 clusters in combination, to lysine, as illustrated in Fig. 4*C Bottom*. While Tdrkh coprecipitated effectively with wild-type Miwi, this interaction was strongly attenuated by arginine to lysine mutations, especially the Miwi mutant in which all 3 clusters of arginines were mutated (Fig. 4*C*). We subsequently mapped the principal Tdrkh binding sites to the first cluster of RG/RA repeats, as mutations in clusters 1 + 2 or clusters 1 + 3 diminished binding. In contrast, a Miwi protein with cluster 2 + 3 mutations, but which possesses an intact cluster 1, still maintained Tdrkh association at a level equivalent to that of wild-type Miwi (Fig. 4*C*). These results suggested that the interaction between Miwi and Tdrkh is arginine-dependent, raising the possibility that Tdrkh binds Miwi through its Tudor domain, primarily through recognition of methylated RG/RA repeats in Miwi cluster 1. To address whether the Miwi–Tdrkh interaction is indeed arginine methylation dependent, we treated cells with MTA, a competitive inhibitor of methyltransferases, upon Flag-Miwi and GFP-Tdrkh cotransfection and analyzed the association of Tdrkh in Flag-Miwi immunoprecipitates (Fig. S7). MTA treatment reduced the overall cellular arginine methylation levels by approximately 50%, as evaluated by the antibody Sym10 (Anti-dimethyl-arginine, symmetric) immunoblotting, in accordance with a previous report (4), and there was a corresponding attenuation of Tdrkh binding to Miwi. To confirm that the Miwi–Tdrkh interaction is mediated through direct binding of the Tdrkh Tudor domain, we mutated 2 Tudor domain residues predicted to be important for ligand-binding. In this double mutant, D390, which is conserved across the TDRD family of Tudor domains, and F391, a component of the aromatic cage (see below), were substituted for alanine. These

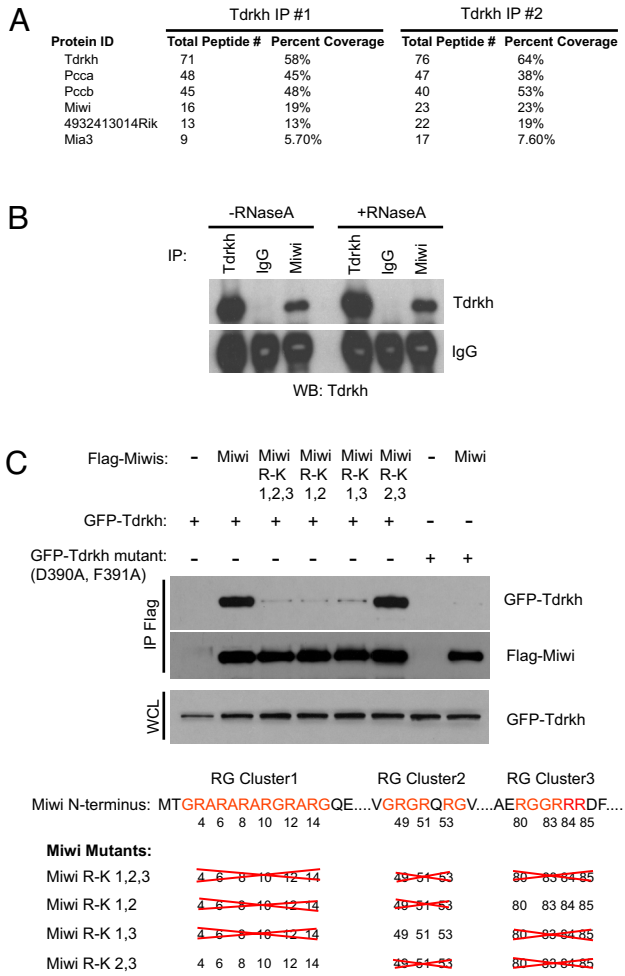

Fig. 4. Tdrkh directly interacts with Miwi in vivo and in vitro through its Tudor domain. (*A*) Miwi is among the top specific interaction partners complexed with Tdrkh. Immunoprecipitation of Tdrkh from adult testis lysate and gel-free mass spectrometry were performed. Specific binding proteins are ranked based on the total peptide number identified. The top 5 Tdrkh interacting proteins with total peptide numbers and percentage of sequence coverage are shown for 2 independent immunoprecipitation experiments. (*B*) The interaction between Tdrkh and Miwi is RNA independent. Endogenous Tdrkh and Miwi were immunoprecipitated from adult testis lysates treated with or without RNaseA using anti-Tdrkh and anti-Miwi antibodies, respectively and immunoblotted with anti-Tdrkh antibody. (*C*) Tdrkh binds to the first cluster of RG/RA repeats on Miwi via its Tudor domain. HEK293T cells were cotransfected with Flag-Miwi or Flag-Miwi (R-K) mutants and GFP-Tdrkh or GFP-Tdrkh Tudor domain mutant (D390A, F391A). Flag-tagged protein complexes immunoprecipitated from cell extracts and whole cell lysates (WCL) were probed with anti-GFP and anti-Flag antibodies. The scheme of Miwi arginine mutations is shown in the bottom panel, with a red cross indicating an R-K mutant.

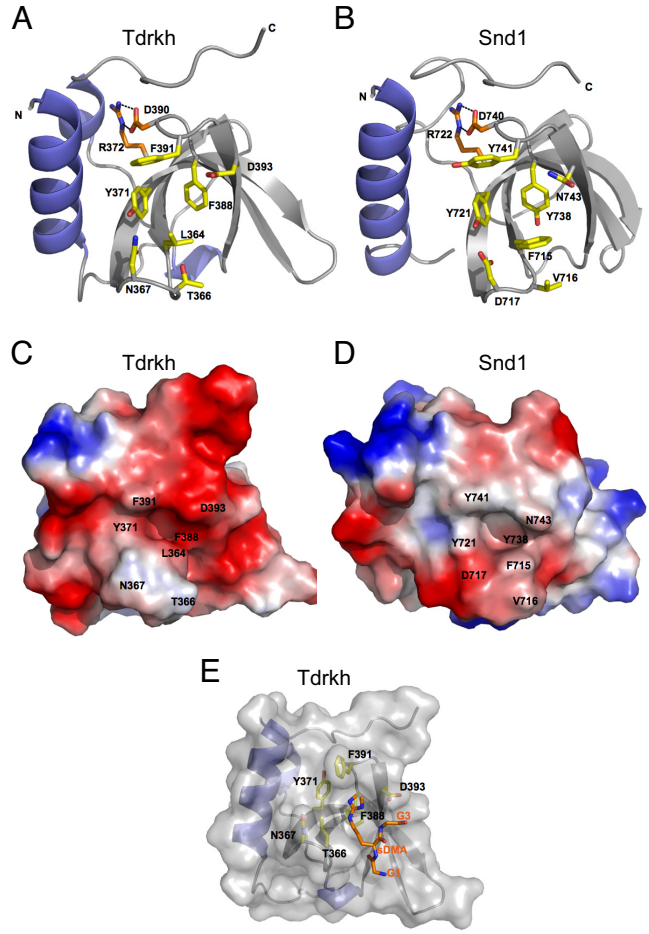

Fig. 5. Crystal structure of the Tudor domain of Tdrkh. (*A*) Ribbon representation of the Tdrkh Tudor domain crystal structure. The residues comprising the aromatic binding pocket are shown in yellow. (*B*) Ribbon representation of the Snd1 Tudor domain crystal structure. (*C*) Surface representation of the Tdrkh Tudor domain crystal structure. (*D*) Surface representation of the Snd1 Tudor domain crystal structure. (*E*) Molecular docking of a GRG peptide with sDMA into the aromatic cage of the Tdrkh Tudor domain.

mutations of the Tudor domain completely abolished the binding of Tdrkh to Miwi, consistent with the model that the Tudor domain is the protein-binding module involved in recognition of arginine methylated Miwi (Fig. 4*C*). Together, the endogenous coimmunoprecipitation results and in vitro cotransfection and immunoprecipitation data clearly indicate that a single Tudor domain can direct the binding of Tdrkh to Miwi and therefore may influence and regulate Miwi and piRNA function.

Crystal Structure of the Tudor Domain of Human Tdrkh. To pursue the structural basis for Tudor domain binding, the recombinant Tudor domain of Tdrkh was produced in *Escherichia coli* and its structure solved by x-ray crystallography (Table S1). Sparse matrix screening yielded crystals that diffracted to 1.75 Å and contained a single molecule in the asymmetric unit corresponding to residues 331 to 418 of the full-length human Tdrkh protein (PDB 3fdr). The crystallized protein consists of a single Tudor barrel flanked by α-helices at both termini. Overall, the Tdrkh Tudor domain exhibits excellent structural similarity to the Tudor domain of Snd1 (PDB 2hqx), with a root mean standard deviation of only 1.6 Å for the main-chain atoms despite the 30% sequence identity between the aligned regions (Fig. 5 *A* and *B*).

As with other Tudor domains, Tdrkh also appears to possess an intact aromatic cage that may be used for ligand recognition. The cage comprises residues L364, T366, N367, Y371, F388, F391, and D393 (Fig. 5*A*). In contrast, the aromatic cage of the Snd1 Tudor domain comprises residues F715, V716, D717, Y721, Y738, Y741, and N743 (Fig. 5*B*). Calculation of the vacuum electrostatic potential of the protein surface surrounding the putative ligand-binding cavity also suggests that the nature of the protein targets for these Tudor domains differs substantially. The Snd1 pocket is rather hydrophobic and is surrounded by regions of both positive and negative charge (Fig. 5*C*). That of Tdrkh, however, carries significant negative charges both within the pocket and over the entire surface of corresponding protein interface (Fig. 5*D*). This is in agreement with the possible interaction of the Tdrkh Tudor domain with the highly positively charged Miwi and Mili termini. As

the Snd1 Tudor domain may target different cellular substrates from Tdrkh, such as snRNP-U in the RNA splicing pathway (24), variations in the chemical nature of their protein-binding surfaces are anticipated to confer distinct ligand specificity between the Tudor domains of Tdrkh and Snd1.

Complementary to the biochemical and cell biology studies for the Tdrkh protein, molecular docking simulations also suggest that the aromatic cage of the Tudor is capable of accepting peptides carrying methylarginine modifications, although optimal docking requires simulation of flexibility of the residues lining this pocket. Docking simulations with a Gly-Arg-Gly (GRG) peptide carrying an sDMA suggest that the gamma carbons of L364, N367, Y371, and F391 would be displaced between 1.5–2.8 Å relative to the crystal structure conformation (Fig. 5*E*). Similar experiments carried out with MMA or aDMA variants of the GRG peptide resulted in less preferred energies, as compared to the sDMA peptide, with the lowest energy conformations excluding the arginine side chain from the binding pocket. This is consistent with the identified sites of sDMA on the N-termini of Miwi and Mili.

Discussion

A principal function of posttranslational modifications (PTM) is to create binding sites for specific protein interaction domains and thus to regulate the dynamic assembly of multiprotein complexes. Although originally established in the context of phosphorylation-dependent protein–protein interactions, it is apparent that a range of PTMs, including acetylation, methylation, hydroxylation, and ubiquitination, amongst others, can serve a similar purpose (25). Here, we have investigated the selective interactions of germline Tudor domain proteins with arginine methylated Piwi family proteins in the mouse testis. These interactions appear relatively specialized for germline Piwi proteins, as the related Argonaute proteins lack N-terminal RG/RA repeats, and are therefore unlikely to be regulated by Tudor domain interactions.

By undertaking a comprehensive proteomic survey of Miwi and Mili complexes in adult germ cells, we identified a group of germline Tudor proteins among the major physiological binding partners of the mouse Piwi proteins. We also found multiple in vivo sites of arginine methylation on the N-termini of Miwi and Mili, particularly symmetrically dimethylated arginines. These could therefore provide multiple modified motifs for binding the Tudor domains of the various proteins associated with the murine Piwi family. Consistent with this view, mutation of the aromatic cage observed in the structure of Tdrkh Tudor domain, which likely serves as the binding site for methylated arginines in Miwi, abolished the Tdrkh-Miwi interaction. In this regard, gel-free mass spectrometry of immunoprecipitated germline proteins has proven to be an efficient tool for analyzing the in vivo protein interaction network assembled around Piwi and Tudor domain proteins, which contains multiple components that are not naturally expressed in typical cultured cells.

The RG/RA clusters at the N-termini of Miwi and Mili are potentially methylated by specific protein arginine methyltransferases (PRMTs), especially PRMT5, which has a *Drosophila* counterpart (dPRMT5) with crucial roles in germ cell specification and maintenance, and gives a similar mutant phenotype to *Drosophila tudor* (26, 27). Kirino et al. (28) have demonstrated that dPRMT5 is required for arginine methylation of *Drosophila* Piwi proteins and their stability, reinforcing the functional significance of arginine methylation in germ cell biology. Our finding that multiple arginine methylation sites can be found on a single Mili peptide suggests that individual Mili and Miwi protein chains contain multiple methylation sites, reminiscent of multisite phosphorylation. Indeed, Miwi has 11 N-terminal RG/RA repeats, suggesting that it could accumulate a high density of methylated arginine sites, and undergo different modes of interaction with Tudor domain proteins. In one scenario, proteins with multiple Tudor domains could undergo multivalent interactions with methylated Piwi proteins. For example, a single multiTudor protein, such as Tdrd1 or Tdrd6, could simultaneously engage multiple sites on the same Piwi protein through its individual Tudor domains, or could cross-link distinct Piwi proteins; conversely, each Piwi protein with multiple methylated arginines could recruit Tudor domains from different Tudor proteins. Proteins with tandem Tudor domains may therefore serve as scaffolds that form multipoint contacts with Piwi proteins, and thereby coordinate the formation and operation of the nuage/chromatoid body important for germ cell differentiation and development (7, 9, 29).

Although multiTudor proteins potentially have scaffolding function in mammalian germinal granule formation, as discussed above, the means by which single Tudor domain proteins participate in germ cell development is still unexplored. In this study, we have identified and characterized a single Tudor domain containing protein, Tdrkh, and have shown that it interacts with Miwi in vivo and in vitro. Mutagenesis data indicate that this interaction involves the Tdrkh Tudor domain and the first cluster of Miwi N-terminal RG/RA repeats. Although our mass spectrometry analysis failed to detect arginine methylation sites on this first cluster of RG/RA repeats due to poor sequence coverage of the very N-terminal region of Miwi, the last residue of the first cluster (R14) is reportedly methylated (30), consistent with the possibility that the Tdrkh Tudor domain directly interacts with methylated Miwi through R14 or other unidentified arginine methylation sites. In addition, we have obtained a crystal structure of the Tdrkh Tudor domain, which provides the first insight into the structural basis for ligand recognition by the germline Tudor domain family. Given that the single Tdrkh Tudor domain interacts with a region of Miwi containing numerous RG/RA motifs, representing actual or potential sites for methylation, it is possible that the Tdrkh domain binds in a dynamic equilibrium to multiple methylated sites in Miwi, as we have previously shown for the Cdc4 F-box protein binding to multiply phosphorylated motifs in the Sic1 protein during the yeast cell cycle (31, 32). It will be of considerable interest to investigate whether this dynamic binding of a polyvalent ligand to a single site receptor applies to Tdrkh-Miwi interactions.

The observation of a direct association between Tdrkh and Miwi complexes could add another layer of regulation to Piwi/piRNA complex formation and assembly by the recruitment of associated functional protein domains. For example, Tdrkh has 2 KH domains, which are frequently associated with RNA recognition (33). Although it is not known whether the Tdrkh KH domains bind RNA, it is possible that there is an interplay between KH-mediated RNA interactions and Piwi protein-associated piRNAs, which therefore could influence piRNA biogenesis and functionality. It will be interesting to examine whether the loss of Tdrkh impacts the piRNA pathway through genetic and functional analysis.

Very recently, several groups have characterized the association of specific germline multiTudor proteins with the Piwi family proteins and have confirmed Mili–Tdrd1 and Miwi–Tdrd6 interactions, which coincides with our proteomic data (9, 17, 18). During the preparation of our manuscript, an elegant study has shown a comprehensive analysis of mouse Piwi complexes and demonstrated that germline Tudor proteins direct critical protein–protein interactions with Piwi proteins that are important for small RNA production and proper operation of the piRNA pathway (30). The profiles of Tudor domain proteins that associate with individual mouse Piwi proteins in this study are largely in accordance with our results, illustrating the biochemical and functional link between the Piwi family and the germline Tudor proteins. Here, we have focused on the single Tudor domain protein Tdrkh as a member of the repertoire of Piwi-binding proteins and have defined the potential biochemical and structural basis for this interaction as a prototype for germline Tudor-Piwi complexes.

In summary, our findings that various germline Tudor proteins are in complex with Piwi proteins, and our detection of in vivo Piwi methylation sites and biochemical and structural analysis of Tdrkh

Tudor domain binding indicates that arginine methylation underlies a critical protein interaction network in germ cell development. Future studies on germline Tudor domain binding properties and specificity, as well as the interaction dynamics of Tudor protein–Piwi complexes, will provide new insight into the construction and organization of mammalian piRNA silencing pathways.

Materials and Methods

Full details of material and methods are discussed in the *SI Text*.

Antibodies. Miwi (Abcam), Mili/Piwil2 (MBL), Tdrkh (Protein Tech), Ddx4 (Abcam), αTubulin (clone DM 1A, Sigma), Flag M2 agarose (Sigma), GFP (A290, Abcam), Sym10 (Millipore).

Tissue Immunoprecipitation and Western Blot Analysis. Adult testes were homogenized in 1% Triton lysis buffer (10 mM Tris-HCl pH7.5, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 10 μg/ml Leupeptin, 10 μg/ml Aprotinin, 10 μg/ml Pepstatin) using Dounce homogenizer. After centrifugation, the supernatant was filtered through 0.45 μm filter, precleared with Protein A Sepharose for 2 h, and incubated with 3 μg of antibodies overnight at 4 °C. The immunoprecipitates were recovered by incubation with 80 μl 10% Protein A slurry for 3 h. After extensive washing with the lysis buffer, the samples were eluted using 50 mM H_3PO_4 for mass spectrometry sample preparation or eluted using 1% SDS sample buffer for Western blotting (34). For tissue extract used for Western blotting, mouse tissues and testes from different aged animals were lysed in RIPA buffer using Polytron tissue homogenizer.

Immunofluorescence. Testes from different aged animals were fixed in natural buffered 10% formalin for 24 h and embedded in CYRO-OCT compound (Sakura Finetek). Frozen sections (6 μm) were cut, air dried, fixed, rehydrated with PBS, and blocked with 5% goat serum in PBS for 1 h. Primary antibody incubation (anti-Tdrkh, 1:200) was performed at room temperature for 2 h followed by 3 × 10 min wash with PBS and 1 h secondary antibody incubation. After extensive washing with PBS, sections were stained with DAPI and mounted. For double staining of Tdrkh and Ddx4, Zenon Rabbit IgG Labeling Kit (Invitrogen) was used according to manufacturer's instruction. Images were acquired with Leica DM IRE2 Microscope and processed with OpenLab software.

Plasmid Constructs. Miwi cDNA (BC129857) was cloned into pcDNA3 vector in frame with a N-terminal Flag-tag. Mouse Tdrkh cDNA (BC049363) was cloned into AscI and PacI sites of a modified pDNR-Dual donor vector and shuttled to an acceptor vector with N-terminal GFP-tag. Tudor domain mutation (D390A, F391A) was generated by site directed mutagenesis. For Miwi R-K mutagenesis, various N-terminal Miwi mutant cDNA fragments encoding the first 101 aa of Miwi were generated by DNA synthesis (Genscript) and cloned into XhoI and BlpI sites of parental pcDNA3-Flag-Miwi plasmid.

Cell Transfection and Immunoprecipitation. HEK293T cells were cultured in DMEM supplemented with 10% FBS and transfected with Flag-Miwi, Flag-Miwi mutants, and GFP-Tdrkh or GFP-Tdrkh mutant using polyethyleneimine; 24–36 h after transfection, cells were harvested and lysed using 1% Triton buffer as mentioned above. Flag-Miwi complex was immunoprecipitated by incubating with anti-Flag M2 agarose (Sigma) for 4 h at 4 °C and extensive washing. Proteins eluted by SDS/PAGE sample buffer were subject to Western blotting. For methylation inhibition experiment, cells cotransfected with Flag-Miwi and GFP-Tdrkh were treated with 5′-deoxy-5′-(methyl-thio) adenosine (MTA) (Sigma) at final concentrations of 250 μM, 750 μM, and 1 mM for 24 h before lysis.

Mass Spectrometry. Immunoprecipitated endogenous Miwi and Mili complexes were analyzed by gel-free LC-MS/MS. All experiments were performed on a QSTAR Elite QqTOF mass spectrometer equipped with a nanospray III ion source (Applied Biosystems/MDS Sciex). The details on sample preparation, experimental setup and data analysis are described in the *SI Text*.

Crystallization and Structure Determination. Amino acids 327 to 420 of human Tdrkh was expressed and purified for crystallization. For details on protein purification, crystallization, structure determination and docking simulation, see *SI Text*.

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