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Novel role of specific Tudor domains in Tudor - Aubergine protein complex assembly and distribution during *Drosophila* oogenesis

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Abstract

Germ cells give rise to the next generation and contain ribonucleoprotein particles, germ granules. In these granules, Piwi protein Aubergine has been shown to interact with Tudor protein in *Drosophila*. Tudor protein has eleven Tudor domains and it has been unclear to what extent all these domains are involved in the interaction with Aubergine. Here we present direct biochemical evidence that Tudor - Aubergine interaction surface is composed of different Tudor domains including those that have not been previously implicated in Aubergine recognition. Furthermore, we show that specific single Tudor domains determine localization of Tudor complex to different sites in ovarian germ cells. Our data suggest that multiple Tudor domains of germline proteins from various species are redundantly used for interaction with the same protein partner during germline development.

Keywords

Germline development; Germ granules; Piwi proteins; RNP particles; Tudor domain

Introduction

Germline (germ) cells constitute an intriguing class of stem cells that are both highly specialized and capable of giving rise to entirely new organisms after fertilization [1,2]. These cells contain ribonucleoprotein (RNP) particles, germ granules [2-4]. Germ granules are dynamic structures and their composition, assembly and function in germline development are not well understood.

Proteins with Tudor (Tud) domains are found in germ granules of many organisms [5-8]. Tud domains belong to the Tudor domain 'Royal Family' which includes chromatin-binding (Chromo), PWWP, MBT, and plant Agenet domains [9]. Frequently, germline Tud domain-containing proteins have multiple Tud domains and although different Tud domains exhibit similar β -barrel structures, these domains show significant sequence variations when

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compared to each other [10,11]. Tud domains are protein-protein interaction modules known to interact with methylated arginines or lysines of target proteins [3]. Recently, Tud domains from several organisms have been implicated in the recognition of methylated arginines of Piwi proteins which protect the germline DNA from transposons [10,12-21].

In *Drosophila*, Tud protein is a component of two types of germ granules: nuage, which is assembled around the nurse cells' nuclei during ovary development, and polar granules, which form at the posterior cytoplasm of the oocyte (germ plasm) and are maintained in early embryos [22]. Nuage has been implicated in the degradation of transposon mRNAs through the direct cleavage of these mRNAs by the Piwi protein Aubergine (Aub) which uses small antisense Piwi-interacting RNAs (piRNAs) to guide the protein to the transposon mRNAs [23]. Germ plasm is necessary and sufficient for germ cell formation at posterior during early embryogenesis and Tud is absolutely required for this process [5,6,24].

Tud protein contains eleven Tud domains and binding of Aub to Tud is dependent on symmetrically dimethylated arginines (sDMAs) of Aub [13,16]. In addition to full-length Tud, a fragment missing Tud domains 2-6 (mini-Tud $\Delta 3$) [5] was shown to bind to Aub *in vitro* [13] and recently the structure between the last Tud domain (domain 11) and Aub peptide (amino acids 6-18) containing sDMAs was solved which provided the structural visualization of the interaction network between a Tud domain and a methylated Aub peptide [10].

At this time there is no direct biochemical evidence available as to whether full-length Aub interacts with multiple Tud domains of Tud protein and whether other Tud domains (domains 2-6) are involved in Aub binding. Although the role of individual Tud domains of full-length Tud in localization of germline factors to germ plasm during early embryogenesis has been analyzed [5], the importance of specific domains on distribution of full-length Tud protein during ovary development has not been evaluated. Here we analyze binding of Aub to several full-length Tud proteins, which have mutations in different single Tud domains or lack several Tud domains altogether. Our study provides the first direct evidence that interaction surface of Tud for Aub is composed of multiple Tud domains and our data suggest that in addition to the segment containing domains 7-11, the other Tud domains are used for Aub interaction. Also, we show for the first time that specific single Tud domains control the localization of Tud to nuage during ovary development and demonstrate that normal localization of Aub to the oocyte's germ plasm can take place in the presence of very small amounts of localized Tud protein.

Materials and methods

Fly stocks

Lines producing HA-tagged full-length and mini-Tud $\Delta 3$ have been described [5]. For binding and immunostaining experiments, ovaries were dissected from females transheterozygous for a particular *tud* allele and a *tud* deletion [*Df(2R)Pur^{P133}*]. *uas-gfp-aub* line and *aub^{N11}* and *aub^{HN2}* mutants have been described [25].

Tud – Aub complex isolation

Tud – Aub complex was isolated during a biochemical screen to identify Tud-interacting proteins in *Drosophila* ovary after chemical crosslinking, purification and mass spectrometry identification of crosslinked HA-tagged full-length and mini-Tud $\Delta 3$ protein complexes essentially as described for isolation and identification of Tud embryonic complexes [4]. Details of the ovarian screen will be published elsewhere (T.M.C, S.N.L. and A.L.A, unpublished results).

Binding of Aub to mutant Tud proteins

Ovaries from *tud* mutants were dissected in protein lysis buffer (PBS, 10% glycerol) and ovarian extracts were prepared in the presence of the protease inhibitors (Roche). Rabbit anti-Aub antibody (1:800) [4] was used for coimmunoprecipitations of mutant Tud proteins. Protein extracts were incubated with anti-Aub antibody at 4°C for 4 hr with subsequent incubation with Protein A beads (Sigma) at 4°C for 1 hr. Beads were washed three times with PBS and bound Tud protein was detected using Western blotting with anti-Tud antibody (1:700) [24]. The immunoblotting membrane was re-probed with anti-Aub antibody (1:7000). As a control for immunoprecipitation, rabbit IgG (Millipore) was used (1:150).

Immunopurification of GFP-Aub and HA-Tud proteins

Ovaries from 1600 flies expressing GFP-Aub were homogenized in protein lysis buffer and diluted to a protein final concentration of 2 mg/ml. GFP-Aub was immunoprecipitated with rabbit anti-GFP antibody (Abcam) (1:900) at 4°C for 4 hr and then with Protein A beads at 4°C for 1 hr in the presence of 2% Triton X-100. Proteins nonspecifically bound to the beads were removed by subsequent washes with 1M NaCl+PBS, 0.5M NaCl+PBS, and twice with PBS.

HA-Tud proteins were isolated from ovaries of 1200 flies using anti-HA affinity matrix beads under the same conditions as for purification of GFP-Aub. The HA-Tud proteins were eluted from the beads with 40 µl of HA-peptide (1mg/ml).

Aub-Tud direct binding assay

Equimolar amounts (5 µl) of immunopurified full-length HA-Tud or HA-mini-Tud $\Delta 3$ were added to 20 µl of purified Protein A-bound GFP-Aub beads and 20 µl of Protein A beads as a control. These samples were incubated in PBS and 0.05% NP-40 at 4°C for 1 hr and washed three times with the same solution. HA-Tud proteins bound to the GFP-Aub were detected with Western blotting. Protein transfer onto the immunoblotting membrane was complete as verified by monitoring the transfer of stained standard proteins with molecular weights similar to Tud and GFP-Aub proteins. For each of the three experiments, intensities of Tud bands were measured using ImageJ software (<http://rsbweb.nih.gov/ij/>) and slight background intensities, given by these proteins bound to protein A beads alone, were subtracted. Intensities corresponding to specific binding were normalized to the Aub-GFP amounts present in each reaction and to the total amounts of Tud proteins added to Aub-GFP beads (input intensities). The normalized values of mini-Tud bound to Aub-GFP were divided by the corresponding normalized values of full-length Tud and quantified as percentage values that show amount of mini-Tud bound to Aub-GFP as a percentage of Aub-GFP-associated full-length Tud.

Immunohistochemistry

These methods have been described [26]. Whole-mount immunostaining of ovaries was performed with rat anti-Aub antibody (1:1000) [4] and rabbit anti-Tud antibody (1:500) [24].

Results and discussion

Aub binds to different Tud domains of Tud protein

Piwi protein Aub has been shown to interact with a Tud domain of Tud protein [13,16]. However, Tud protein contains eleven Tud domains (Fig. 1) and it is not yet clear whether each of these domains or a specific subset of Tud domains are involved in binding with full-

length Aub. A recent study has shown that a Tud fragment containing five C-terminal domains (domains 7–11) is important for Aub localization to the germ plasm *in vivo* and binds to methylated peptides composed of Aub amino acids 6–18 [10]. Also, a mini-Tud $\Delta 3$ fragment lacking Tud domains 2–6 can interact with Aub *in vitro* [13]. Taken together, these data indicated that at least one of the Tud domains from domain 7–11 segment of Tud binds directly to methylated full-length Aub. However, the importance of other Tud domains in Aub binding has not been evaluated and direct biochemical evidence for the involvement of multiple Tud domains in Aub binding is missing.

We carried out biochemical screens for Tud-interacting proteins in embryos [4] and more recently in ovaries (see Materials and methods) to isolate proteins associated with both full-length Tud and mini-Tud $\Delta 3$ (Fig. 1) and identified Aub in both Tud protein complexes in ovary and in full-length Tud complex in embryos. mini-Tud $\Delta 3$ can rescue germ cell formation in embryos from a protein-null *tud* mutant females albeit with lower efficiency than the full-length Tud protein [5]. In this study we used several different full-length Tud proteins which have mutations in single Tud domains or Tud protein fragments that lack several Tud domains (Fig. 1) to analyze the direct involvement of different Tud domains in binding with full-length Aub protein.

First, we asked whether mutations in a specific Tud domain would interfere with direct binding to Aub. Fig. 2A shows that mutations in Tud domains 1, 7, and 10, each of which has been isolated based on their strong effect on germ cell formation [5,6], did not affect Tud binding to Aub. Recent analysis of Tud domain 11 – Aub peptide complex highlighted the important structural role of conserved arginine residue changed in Tud domain 1 and 10 mutants analyzed in our study and lead to conclusion that this residue belongs to a signature motif for Piwi-binding Tud domains since it is absent in non-Piwi-interacting Tud domains (see below) [10]. Tud domain 7 mutation is a deletion of three consecutive amino acids (Fig. 1) which is likely to cause a strong structural effect on this domain [5,10]. Thus, our binding results show that germline defects caused by mutations in these domains are not caused by the inability of Aub to bind to mutated full-length Tud proteins and suggest that Aub does not exclusively bind to either of these domains.

Therefore, we designed an *in vitro* binding assay to test the possibility that Aub may bind to multiple Tud domains as follows. HA-tagged full-length, mini-Tud $\Delta 3$ and functional GFP-tagged Aub were immunopurified from ovarian extracts. Correct folding of isolated GFP-Aub bound to the anti-GFP antibody/protein A beads was verified by measuring GFP fluorescence in a plate reader of a real-time PCR instrument using an appropriate filter. HA-Tud proteins were eluted with HA peptide and added to GFP-Aub bound to the beads. Fig. 2B shows that full-length Tud binds much stronger to Aub *in vitro* than mini-Tud $\Delta 3$ which lacks Tud domains 2–6. The amount of mini-Tud $\Delta 3$ bound to Aub was just 2.6% of the full-length Tud associated with Aub when equimolar amounts of full-length and mini-Tud were added to Aub (Fig. 2B). Recently, Kirino *et al.* reported that *in vitro* translated mini-Tud $\Delta 3$ binds to Aub and suggested that Tud domains 7–11 present in this mini-Tud associate with Aub [13]. Subsequently, Liu *et al.* showed that a fragment containing Tud domains 7–11 was able to bind methylated Aub peptides containing Aub amino acids 6–18 [10]. Consistent with these data, we detected mini-Tud $\Delta 3$ - full-length Aub binding in our assay using ovarian proteins. However, we could detect substantial mini-Tud $\Delta 3$ - Aub interaction when the molar amount of mini-Tud $\Delta 3$ added to the binding reaction was ~40 times higher than that of full-length Tud (Fig. 2C). Our binding experiments using full-length wild-type Tud, mutant Tud proteins and mini-Tud $\Delta 3$ suggest that Aub binds to different Tud domains and indicate that the region that includes Tud domains 2–6, which are absent in mini-Tud $\Delta 3$, is an important binding site for Aub.

Tud domains from domain 2–6 segment of Tud protein contain Aub/Piwi binding motifs

Next we asked whether any of the Tud domains 2-6 contain residues shown to form the binding site of Tud domain 11 for methylated Aub peptide [10]. In Tud domain 11 five amino acids form the binding pocket, namely four aromatic residues (tyrosine or phenylalanine) and one asparagine. We observed that Tud domain 2 contains all four aromatic residues in the corresponding positions and domain 6 – three aromatic residues, and both domains have the asparagine (Supplementary Fig. S1), indicating that similarly to Tud domain 11, domains 2 and 6 may efficiently bind to methylated Aub. Interestingly, a Tud domain from a human protein, Tdrkh, which, similarly to *Drosophila* Tud was shown to bind arginine-methylated Piwi protein Miwi, contains three aromatic residues in the binding cage [12] indicating that there is some variability in the number of aromatic amino acids in Tud domains which recognize sDMAs of Piwi proteins (Supplementary Fig. S1).

In addition, two other directly interacting residues of Tud domain 11 were recently proposed to be included in a signature motif specific for Piwi-interacting Tud domains, namely Arg2411 and Asp2429, since they are absent in Tud domains that recognize non-Piwi protein targets [10]. Importantly, we observe that all Tud domains 2-6 have these two residues (Supplementary Fig. S1).

Specific Tud domains control Tud complex localization during oogenesis

Our data showed that mutations in specific Tud domains allow binding of mutant full-length Tud proteins to Aub (Fig. 2A) and therefore indicated that these domains may be dispensable for the formation of Aub - Tud complex within germ granules during oogenesis. These mutations in Tud domains caused embryonic phenotypes [5], however, the effect of changes in individual domains in the context of the full-length Tud on ovary development has not been investigated. In addition to localizing to germ plasm, which is present in both oocytes and early embryos, full-length Tud protein is component of nuage in germline nurse cells in the ovary. Therefore, we analyzed Tud - Aub complex assembly and distribution during ovary development in detail.

Our analysis showed that the point mutation affecting the same amino acid in different Tud domains cause remarkably different effects on accumulation of mutant Tud proteins in nuage and germ plasm of the oocyte. Particularly, the mutation in Tud domain 1 (*tud*^{A36}) severely reduced nuage localization but did not affect germ plasm localization of the mutant protein (Fig. 3). However, in Tud domain 10 mutant (*tud*^{B42}), change of the same Arg residue resulted in Tud protein nuage localization that was stronger than that of the wild-type protein (Fig. 3; 16 egg chambers for each mutant were analyzed). At the same time, localization of Tud domain 10 mutant protein was significantly reduced in the germ plasm. These data indicate that single Tud domains control accumulation of Tud protein in nuage. Furthermore, the increased accumulation of Tud mutant protein in nuage was an unexpected result and suggests that Tud domain 10 determines the stoichiometry of Tud complex in nuage structure. Tud domain 7 mutant (*tud*^A) localized to nuage similarly to the wild-type protein (Fig. 3), however, germ plasm localization of the mutant protein was reduced. Nuage and germ plasm localization of other mutant proteins that lack Tud domains 10 and 11 (*tud*^{B45}) or small C-terminal segment (*tud*^{A7}) was either diminished or absent (Fig. 3). However, the decrease in the accumulation of these proteins in nuage and germ plasm may be due to significantly reduced levels of these polypeptides in the ovary [5].

Mutant analysis of Aub and Tud distribution

The direct requirement of Aub for Tud localization to nuage and germ plasm has not been tested. Therefore, we decided to examine whether the removal of Aub from the egg chamber would affect Tud localization (Fig. 3). In ovaries from protein-null *aub* mutant females, Tud

failed to localize to nuage and germ plasm. Absence of Tud localization to *aub* mutant germ plasm can be explained by the requirement of Aub for translation of *osk* mRNA [27] since Osk protein acts upstream of Tud during germ plasm assembly [28]. However, the lack of Tud localization in *aub* mutant nuage suggests that Aub may directly recruit Tud protein to nuage since Osk is not present there.

In *tud* mutants with point mutations in Tud domains, Aub accumulation in nuage and germ plasm was not reduced, consistent with our data that showed association of these mutant proteins with Aub (Fig. 2A). It is interesting to note that the increase in Tud localization to nuage, detected in Tud domain 10 mutant (*tud*^{B42}), correlated with the enhanced amount of Aub in nuage (Fig. 3). This indicates that higher amounts of Tud mutant protein can associate and assemble with extra Aub in nuage.

tud mutants with low levels of Tud proteins (*tud*^{A7} and *tud*^{B45}) showed a normal accumulation of Aub in nuage (Fig. 3). 60.2% of *tud*^{A7} and 79.2% of *tud*^{B45} mutant stage 10 oocytes showed wild-type localization of Aub in the germ plasm. The rest of the mutant egg chambers exhibited reduced Aub localization in the germ plasm (70-100 egg chambers were counted).

Conclusion

Tud proteins interact with Piwi proteins in flies, frogs and mice [4,10,12-21] demonstrating that Piwi - Tud interaction has been remarkably evolutionarily conserved. In this work we present direct biochemical evidence that Piwi protein Aub binds to different Tud domains including those which have not been implicated in Aub recognition. Our data suggest that repeated Tud domain architecture, exhibited by many germline Tud proteins, may be required to bind to several molecules of the same germ granule components. At the same time, we find for the first time that a single Tud domain of Tud protein specifically controls its localization to nuage. In addition, our study shows that Aub localization to germ plasm can proceed normally in the presence of very low amounts of localized Tud protein as detected in *tud*^{B42}, *tud*^{A4}, and *tud*^{B45} mutants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Tud mutant proteins analyzed in this study. Wild-type (wt) Tud protein and proteins with mutant Tud domains (empty squares) are shown. *tud*^{A36} and *tud*^{B42} had mutations changing equivalent arginines in Tud domains 1 and 10 respectively (Arg91Trp in *tud*^{A36}, Arg2228Cys in *tud*^{B42}); *tud*⁴ had a deletion of three amino acids in Tud domain 7 (Asp1708, Ile1709, Lys1710); *tud*^{B45} lacked Tud domains 10 and 11; *tud*^{A7} had a deletion of a small C-terminal segment caused by premature UAG stop codon; mini-Tud Δ3 lacks Tud domains 2 - 6 [5].

Fig. 2.

Involvement of different Tud domains in Aub binding. (A) Mutations in single Tud domains 1, 7, and 10 in full-length (FL) Tud protein do not affect Aub binding in coimmunoprecipitation experiments with anti-Aub antibody. Western blot detections of bound Tud or Aub are shown. Top panel demonstrates levels of Tud and Aub present in extracts from wt and mutant ovaries. *tud*¹ is a protein-null. Bottom panel shows coimmunoprecipitation of Tud and Aub from wt and Tud domain mutants. As a control, rabbit IgG instead of anti-Aub antibody was added to wt extracts. (B) *in vitro* direct binding experiments using immunopurified FL Tud protein, mini-Tud Δ3 and functional GFP-Aub. Tud proteins were added to GFP-Aub-associated beads and, as a control, to Protein A beads. Bound Tud and Aub were detected with anti-HA and anti-Aub antibodies respectively. The total (input) amounts of Tud proteins are indicated. From three experiments using equimolar amounts of FL Tud and mini-Tud, binding of mini-Tud to Aub was 2.61% of that of FL Tud to Aub (standard error was 1.69). (C) This mini-Tud Δ3/Aub binding experiment was performed as described for (B) except the input amount of mini-Tud was ~40-fold higher.

Fig. 3.

Mutations in single Tud domains of full-length Tud protein differently affect Tud accumulation in ovarian nuage or germ plasm but do not interfere with Aub localization. *tud*, *aub* mutants and wild-type (wt) stage 7 egg chambers (Nuage columns) and stage 10 oocyte posterior (Germ plasm columns) were co-stained with rabbit anti-Tud (green) and rat anti-Aub (red) antibody. Overlay images are shown. Anterior is to the left. *tud*^{A36}, *tud*^{A4} and *tud*^{B42} mutants had mutations in specific Tud domains (Fig. 1); *tud*^{B45} lacked Tud domains 10 and 11 and *tud*^{A7} had a deletion of a small C-terminal segment (Fig. 1). In the bottom wt row, closer views of wt nuage and germ plasm are shown. Tud is not localized to nuage and oocyte germ plasm in protein-null *aub*^{N11}/*aub*^{HN2} ovary (*aub*^{mut}).