Tudor-related proteins TDRD1/MTR-1, TDRD6 and TDRD7/TRAP: Domain composition, intracellular localization, and function in male germ cells in mice

Mihoko Hosokawa a, Masanobu Shoji a, Kouichi Kitamura a, Takashi Tanaka a, Toshiaki Noce b, Shinichiro Chuma a,⁎, Norio Nakatsuji a

⁎ Corresponding author. Fax: +81 75 751 3890.
E-mail address: schuma@frontier.kyoto-u.ac.jp (S. Chuma).

a Department of Development and Differentiation, Institute for Frontier Medical Sciences, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan
b Mitsubishi Kagaku Institute of Life Sciences, 11 Minamiooya Machida-shi, Tokyo 194-8511, Japan

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Abstract

The germ-line cells of many animals possess a characteristic cytoplasmic structure termed nuage or germinal granules. In mice, nuage that is prominent in postnatal male germ cells is also called intermitochondrial cement or chromatoid bodies. TDRD1/MTR-1, which contains Tudor domain repeats, is a specific component of the mouse nuage, analogously to Drosophila Tudor, a constituent of polar granules/nuage in oocytes and embryos. We show that TDRD6 and TDRD7/TRAP, which also contain multiple Tudor domains, specifically localize to nuage and form a ribonucleoprotein complex together with TDRD1/MTR-1. The characteristic co-localization of TDRD1, 6 and 7 was disrupted in a mutant of mouse vasa homologue / DEAD box polypeptide 4 (Mvh/Ddx4), which encodes another evolutionarily conserved component of nuage. In vivo over-expression experiments of the TDRD proteins and truncated forms during male germ cell differentiation showed that a single Tudor domain is a structural unit that localizes or accumulates to nuage, but the expression of the truncated, putative dominant negative forms is detrimental to meiotic spermatocytes. These results indicate that the Tudor-related proteins, which contain multiple repeats of the Tudor domain, constitute an evolutionarily conserved class of nuage components in the germ-line, and their localization or accumulation to nuage is likely conferred by a Tudor domain structure and downstream of Mvh, while the characteristic repeated architecture of the domain is functionally essential for the differentiation of germ cells.

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Introduction

In a wide variety of animals, germ cells exhibit particular cytoplasmic structures called nuage or germinal granules (Eddy, 1975). The structures are characterized by an amorphous shape, the absence of surrounding membranes, the abundance of RNAs and proteins and a close association with mitochondria or nuclei. In Drosophila, several products of posterior-group genes such as oskar, vasa and tudor, which function in pole cell and abdominal formation, localize to polar granules, a form of nuage in oocytes and early embryos, and these granules are asymmetrically partitioned to germ cell precursors (Mahowald, 1962; Lehmann and Ephrussi, 1994; Saffman and Lasko, 1999). Similarly, P granules in C. elegans (Strome and Wood, 1982) and germinal granules in Xenopus (Czolowska, 1969) are segregated to prospective germ cells during early development, and these structures are thought to participate in the partitioning and/or accumulation of germ cell determinants.

In mice, prospective germ cells are induced among pluripotent epiblast cells at around gastrulation stages (Lawson and Hage, 1994; Lawson et al., 1999; Tam and Zhou, 1996; McLaren, 2003), and the presence of nuage during this determination process remains unclear. On the other hand,
mammalian nuage becomes clearly discernible at the later stages of differentiation of the germ-line, such as in spermatogonia and developing oocytes, and in mice, nuage becomes most prominent in postnatal meiotic spermatocytes and haploid spermatids (Fawcett et al., 1970; Eddy, 1974; Russell and Frank, 1978; Parvinen, 2005). Nuage in spermatogonia, spermatocytes and oocytes is seen among clusters of mitochondria and is called “inter mitochondrial cement/material/bar,” whereas in spermatocytes and spermatids, larger solitary aggregates of nuage, termed “chromatoid bodies,” are prominent in the cytoplasm. Mammalian nuage does not appear to be asymmetrically partitioned in these cells, thus its developmental function may differ from nuage in early embryos of other species. Meanwhile, close morphological similarities among nuages of divergent species, including mice, at different developmental stages suggest that they share common properties that are essential and conserved in the germ-line.

Vasa protein, a DEAD-box RNA helicase, is a component of Drosophila polar granules, and its homologies are widely conserved components of nuage (Raz, 2000). In mice, the mouse vasa homologue/DEAD box polypeptide 4 (Mvh/Ddx4) is expressed in differentiating germ cells rather than during germ cell specification, and the MVH protein localizes to chromatoid bodies (Fujiwara et al., 1994; Toyooka et al., 2000). Interestingly, the targeted disruption of Mvh leads to male-specific sterility due to postnatal defects in early spermatocytes (Tanaka et al., 2000), although the gene is expressed in both male and female germ cells.

Tudor is another component of polar granules in Drosophila and is genetically downstream of vasa in respect to its intracellular localization. The tudor gene maternally functions in pole cell and abdominal formation, as well as participating in the localization of mitochondrial RNAs to polar granules. The protein contains 11 Tudor domains, but the biochemical and physiological importance of the domain repeats remains unknown (Boswell and Mahmoud, 1985; Golombek et al., 1991; Bardsley et al., 1993; Ponting, 1997; Amikura et al., 2001; Thomson and Lasko, 2004). We previously reported that the Tudor domain containing 1/mouse tudor repeat 1 (Tdrd1/ Mtr-1; hereafter referred to as Tdrd1 according to Mouse Genome Informatics) is expressed in differentiating germ cells, and encodes four Tudor domains and a zinc-finger MYND domain (Wang et al., 2001; Chuma et al., 2003). The TDRD1 protein localizes to both intermitochondrial cement in male and female germ cells and to chromatoid bodies in the male (Chuma et al., 2003; Chuma et al., 2006). The germ-line expression, domain composition and intracellular localization to nuage are analogous features shared by Drosophila Tudor and mouse TDRD1.

In this study, we report the characterization of TDRD6, a putative orthologue of Drosophila Tudor, and TDRD7/TRAP (Hirose et al., 2000) which also contains Tudor domain repeats. TDRD6 and 7 specifically localize to nuage and form a complex together with TDRD1. The co-localization of the TDRD proteins was disrupted in a mutant of Mvh, and this is analogous to the relationship between Drosophila vasa and Tudor, suggesting that the Tudor related proteins retain an evolutionarily conserved mechanism that regulates their intracellular localization. To investigate the possible correlation between the repeated architecture of the Tudor domain and their localization and function, we carried out in vivo over-expression experiments of the TDRD proteins and truncated forms in male germ cells. The results showed that a single Tudor domain can localize or accumulate to nuage, while the Tudor domain repeats are essential for meiotic spermatocyte differentiation. Our results demonstrate that Tudor-related proteins constitute a novel class of nuage components, with their characteristic Tudor domains being important for their localization and function in the germ-line.

Materials and methods

Mice

Jcl: ICR mice were obtained from CLEA Japan and maintained in a controlled environment with 12:12 light: dark cycles. Mvh gene-targeted mice were genotyped as previously described (Tanaka et al., 2000). All experiments on mice were carried out in accordance with the institutional guidelines and regulations.

Cloning of Tdrd6 cDNA

The mouse genomic sequence Genbank/EMBL/DDBJ AZ647796 was found to contain an open reading frame (ORF) for two Tudor domains. An approximately 260 bp fragment of this partial ORF was PCR-amplified from mouse testis cDNA with the primers 5′-TTTATCAGATTGTGGCAAACATGTCT-3′ and 5′-ACCTGCTAATCATCCTCGCTA-3′. A lambda gt11 library of mouse testis cDNA (a kind gift from Dr. M. Nozaki) was probed with this cDNA fragment, and four overlapping clones were obtained. 5′-cap structure dependent rapid amplification of cDNA ends (5′-RACE) was carried out (Gene Racer, Invitrogen, USA) using adult testis mRNA (Dynabeads mRNA direct kit, Dynal, Norway). Amplified products of four different lengths were cloned into pBlueScript SK (Stratagene, USA), and at least 12 clones were sequenced for each transcript variant. The consensus sequence of transcript variant 1 assembled from the lambda library clones and 5′-RACE products was submitted to Genbank/EMBL/DDBJ under the accession number AB097085.

Northern blot and RT-PCR analyses

20 μg of total RNA isolated from tissues of Jcl: ICR mice using a modified AGPC method (Trizol, Invitrogen, USA) was electrophoresed in a 0.9% formaldehyde gel, transferred to a nylon membrane (Hybond-N+, Amersham, USA) and probed with a 1.3 kb 3′-fragment of Tdrd6 cDNA labeled with [32P]dCTP. Signals were detected with X-ray film (Kodak, USA). For RT-PCR, 1 μg of total RNA was treated with DNase I (Promega, USA) and reverse-transcribed using random 9 mer and SuperScript II (Invitrogen). PCR primers were 5′-TTTATCAGATTGTGGCAACATGTCT-3′ and 5′-ACCTGCTAATCATCCTCGCTA-3′ for Tdrd6 and 5′-GTCGTCCTACCGGCATTGTGATGG-3′ and 5′-GCAATGCCTGGGTACATGGTGG-3′ for β-actin. Amplified products were gel-electrophoresed and stained with ethidium bromide.

Production of anti-TDRD6 and 7 antibodies, and Western blot analysis

A Tdrd6 cDNA fragment encoding amino acids 1911–2134 with a 6xHis tag was cloned into pGEX-6P-1 (Amersham). The fusion protein produced in E. coli BL21 was purified using Glutathione Sepharose 4B and PreScission protease (Amersham). Rabbits were immunized with the TDRD6 C-terminal fragment, and specific antibodies were affinity-purified from the antiserum using the same antigen coupled to cellulose beads (Chisso, Japan). A Tdrd7 cDNA
fragment encoding amino acids 1–246 was cloned into pQE-32 (Qiagen, Germany). The 6×His-tagged TDRD7 N-terminal fragment produced in E. coli M15[pREP4] was purified using Ni-NTA agarose under denaturing conditions. Rabbits were immunized with the denatured protein, and specific antibodies were affinity-purified from the antisera using the same antigen. For Western blotting of TDRD6, lysates of adult testes and NIH/3T3 cells transfected with pCAG-TDRD6 or control PCXN2 (Niwa et al., 1991) were subjected to a 5–20% gradient SDS-PAGE and transferred to a nitrocellulose membrane (Protran BA, Schleicher and Schuell, Germany). The blots were probed with anti-TDRD6 antibodies followed by alkaline phosphatase-conjugated secondary antibodies. Signals were detected with CDP-Star with alkaline phosphatase and X-ray film.

**Immunofluorescence and immunoelectron microscopy**

10 μm cryosections of testes fixed in 2% paraformaldehyde (PFA) in 1× phosphate buffered saline (PBS) were immunostained with anti-TDRD1 (Chuma et al., 2003), TDRD6, TDRD7, SYCP3/SCP3 (Chuma and Nakatsuji, 2001), Mvh (Toyooka et al., 2000), Sm (Y12) (Lab vision, USA), FLAG (Sigma) and 6×His (Bethyl laboratories, USA). The secondary antibodies used were FITC-conjugated anti-rat immunoglobulin (Ig), Rhodamine B-anti-rabbit Ig (BioSource, USA), Alexa Fluor 488 anti-rabbit IgG, and Alexa Fluor 568 and 555 anti-mouse IgG (Molecular Probes, USA). For double-immunostaining using two rabbit antibodies, Zenon Rabbit IgG Labeling Kits for Alexa Fluor 555 and 568 (Molecular Probes) were used. Nuclei were stained with 1 μg/ml Hoechst 33258 dye (Sigma). For immunoelectron microscopy of TDRD6, 8 μm cryosections of testes fixed in 4% PFA, 0.05% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) were incubated with anti-TDRD6 antibodies or preimmune serum as a control, followed by incubation with 1.4 nm gold-conjugated secondary antibodies (Nanoprobes, USA). The signals were intensified using a silver enhancement kit, HQ silver and sections were post-fixed in 1% OsO4 in 0.1 M phosphate buffer, dehydrated, embedded in epoxy resin and cut into 70–90 nm sections for electron microscopy. For TDRD7, testes were fixed in 4% PFA, 0.02% glutaraldehyde in 0.1 M phosphate buffer, and embedded in epoxy resin. 70–90 nm sections were incubated with anti-TDRD7 antibodies, followed by 15 nm gold-labeled secondary antibodies. After postfixation with 2% glutaraldehyde in PBS, sections were stained with uranyl acetate followed by lead citrate. Samples were examined using an electron microscope H7000 (Hitachi, Japan).

**Immunoprecipitation**

Single cell suspension of adult testes was prepared by collagenase and trypsin treatment (Shoji et al., 2005). For Western blotting, the cells were suspended in hypotonic buffer (50 mM KCl, 10 mM Tris–Cl (pH 7.4), 5 mM MgCl2), homogenized using a Dounce homogenizer, added with one volume of 280 mM KCl, 60 mM NaCl, 10 mM Tris–Cl, 5 mM MgCl2, then centrifuged at 160×g. The supernatant was supplemented with 0.1% NP-40, a protease inhibitor cocktail (Sigma) and SUPERaseIn (Ambion, USA). Immunoprecipitation was carried out using anti-TDRD1, 6 and 7 antibodies and a normal rabbit immunoglobulin G bound to Protein G coupled magnetic beads (Dyna). The precipitates were eluted in standard SDS-PAGE buffer with 6 M urea, and Western blots were probed with anti-TDRD1, 6 and 7 and anti-MVH (Abcam, UK) antibodies, followed by alkaline phosphatase-conjugated secondary antibodies. Signals were detected with CDP-Star with NitroBlock II and X-ray film. For protein and RNA detection, single cell suspensions of testes fixed in 2% paraformaldehyde in PBS, sections were stained with uranyl acetate followed by 15 nm gold-labeled secondary antibodies and embedded in epoxy resin. 70–90 nm sections were incubated with anti-TDRD7 antibodies, followed by 15 nm gold-labeled secondary antibodies. After postfixation with 2% glutaraldehyde in PBS, sections were stained with uranyl acetate followed by lead citrate. Samples were examined using an electron microscope H7000 (Hitachi, Japan).

**Results**

**Tdrd6 encodes seven Tudor domains and is abundant in the testis**

Tblastn search was carried out for cDNAs and ESTs encoding multiple Tudor domains, and the human cDNA fragment Genbank/EMBL/DDBJ AF039442 was found to contain an incomplete ORF for two Tudor domains. By blastn search, the mouse genomic sequence AZ647796 was found to contain the mouse homologue of this human cDNA. A corresponding mouse cDNA fragment, amplified by RT-PCR using primers designed based on the genomic sequence, was then used to probe a mouse testis cDNA library, and four overlapping clones were obtained. The 5′-ends of the cDNAs were determined by 5′-RACE, and the assembled sequences revealed four transcript variants of 4.8–7.1 kb (Fig. 1A). The longest transcript variant 1 was most abundant in the RACE analysis. Correspondingly, Northern blotting showed a predominant band of about 7 kb in the testis, indicating that transcript variant 1 is the major transcript (Fig. 1B). The transcripts were not detected by RT-PCR in fetal male and female gonads or in adult ovaries (Fig. 1C).

Transcript variant 1 contained an ORF encoding 2134 amino acids. Dot matrix plotting and motif searches using the PROSITE and Pfam databases identified seven copies of the Tudor domain (Figs. 1D, E), and the gene was designated tudor domain containing 6 (Tdrd6) according to the Mouse Genome Informatics. The Tdrd6 gene consists of a long first exon followed by four short exons (Fig. 1A) spanning about 50 kb on chromosome 17. The promoter region of the Tdrd6 gene lacked TATA box sequences, similarly to several other genes expressed in spermatogenic cells (Kleene, 2005). Tdrd6 is a putative orthologue of Drosophila tudor as was predicted by a unique best reciprocal hit by BLAST, Ensemble v.32.

**TDRD6 is a component of intermitochondrial cement and chromatoid bodies in male germ cells**

We raised polyclonal antibodies against a C-terminal fragment of TDRD6. Western blotting of adult testes using plasmids and in vivo electroporation of the testis

pCAG-TDRD1-TDx4, -ΔTDRD1-TDx2 and -ΔTDRD1-TDx1, which contain four, two, and one Tudor domain(s), respectively, and tagged with a 6×His, were described previously (Chuma et al., 2003). pCAG-TDRD6-TDx7, -ΔTDRD6-TDx2 and -ΔTDRD6-TDx1 contained the full-length or truncated Tdrd6 cDNAs, encoding amino acids 1–2134, 1–415 and 1–160 tagged with FLAG, respectively, in an XhoI site downstream of the CAG enhancer/promoter of pcXN2. Point mutations Tyr1019 to Asn (TAT to AAT) and Glu1023 to Lys (GAA to AAA) were introduced into the Tudor domain of pCAG-ΔTDRD1-TDx1 by a PCR-based over-lap extension method (Sambrook and Russell, 2001) using Pfu DNA polymerase (Stratagene). These plasmids were mixed with the transfection marker pCAG-enhanced cyan fluorescent protein (ECFP) and injected into seminiferous tubules of testes of juvenile mice undergoing the first wave of spermatogenesis. The testes were subjected to in vivo electroporation as described (Shoji et al., 2005). Spermatogenic cells were identified by their morphology and position in seminiferous tubules and by immunostaining for germ cell marker proteins.
the antibody showed a band of approximately 250 kDa, the size of the putative molecular mass of TDRD6 (Fig. 2A). By immunofluorescence staining of adult testis sections, TDRD6 signals were observed as fine cytoplasmic granules or larger solitary aggregates in germ cells (Figs. 2B, C). Double-staining for TDRD6 and SYCP3, a component of meiotic synaptonemal complex (Lammers et al., 1994), showed that the cells with fine TDRD6 granules were pachytene spermatocytes (Fig. 2C, arrows). Larger aggregates of TDRD6 were observed in haploid round spermatids (Fig. 2C, arrowheads). No significant signal was detected in adult ovaries and in male and female fetal gonads (data not shown).

To further localize TDRD6, immunoelectron microscopy was carried out. TDRD6 signals were predominantly observed at intermitochondrial cement in spermatocytes (Fig. 2D, arrows) and chromatoid bodies in both spermatocytes and round spermatids (Figs. 2D, E, arrowheads). Thus, TDRD6 is a novel specific component of nuage in male germ cells, and this localization agrees with the granular distribution of TDRD6 signals observed by immunofluorescence staining (Figs. 2B, C).

TDRD1, 6 and 7 co-localize to nuage and form an ribonucleoprotein complex in male germ cells

TDRD7/TRAP is another protein that contains Tudor domain repeats and is abundant in the testis (Hirose et al., 2000). To examine the intracellular localization of TDRD7, we produced anti-TDRD7 antibodies and carried out immunofluorescence

Fig. 1. Tdrd6 encodes seven Tudor domains and is abundant in the testis. (A) Exon structure and transcript variants of Tdrd6. The first ATGs and stop codons are indicated by vertical arrows and arrowheads. Numbered boxes represent regions encoding Tudor domains. The horizontal arrow indicates the position of Tdrd6-specific primer used in 5′-RACE. The right panel shows the 5′-RACE products. TAP, tobacco acid pyrophosphatase. (B) Upper panel, Northern blot of total RNA isolated from adult tissues probed with Tdrd6 cDNA. Lower panel, 18S rRNA stained with ethidium bromide. (C) RT-PCR of Tdrd6 and beta-actin from total RNA of adult and fetal 13.5 days post coitum (dpc) testes and ovaries. (D) Domain composition of mouse TDRD1, 6 and 7 and Drosophila Tudor. Black and shaded boxes represent Tudor domains and a MYND domain. (E) Alignment of selected Tudor domain sequences by ClustalW. Identical and similar residues are in reverse and shaded fonts, respectively. The residue numbers are on the right. Accession numbers: mouse TDRD6, AB097085; Drosophila Tudor, X62420; human SMN, Q16637.
staining of testis sections. TDRD7 was diffusely observed in spermatogonia (Figs. 3A, C, open arrowheads), but the signals diminished in leptotene-zygotene spermatocytes. TDRD7 became detectable again in early-pachytene spermatocytes, then in mid-late pachytene spermatocytes, the signals exhibited fine granular appearance (Figs. 3A, B, arrows). Then in spermatids, TDRD7 formed larger solitary aggregates similarly to TDRD1 and 6 (Figs. 3A, B, arrowheads). The intracellular localization of TDRD7 was further examined by immunoelectron microscopy. TDRD7 signals were enriched in chromatoid bodies in spermatocytes and spermatids (Figs. 3D–F) and to a lesser extent, to intermitochondrial cement in spermatocytes. The signals were also observed in particulate cytoplasmic structures whose characteristics were unclear (data not shown).

These results showed that TDRD7, together with TDRD1 (Chuma et al., 2003) and 6, constitute a novel class of nuage components in male germ cells in mice. The immunostaining results of TDRD1, 6 and 7 appeared mostly similar (Figs. 4A–C). However, the precise localization patterns of the TDRD proteins differed from each other. In spermatogonia, TDRD1 exhibited a fine granular appearance that corresponds to intermitochondrial cement (Chuma et al., 2006), while TDRD7 was diffused (Fig. 4E, asterisks) and TDRD6 was undetectable. The three TDRD proteins were not detected in leptotene-zygotene spermatocytes (Figs. 2B, and 3A, and data not shown). In pachytene spermatocytes, TDRD1 again showed granular distribution, which corresponds to both intermitochondrial cement and chromatoid bodies, and this
Localization of TDRD1 was observed at earlier stages than TDRD6 and 7 (for TDRD1 and 6, Fig. 4D, arrows). TDRD6 and 7 subsequently co-localized with TDRD1 (for TDRD6 and 7, Fig. 4E, arrows), but considering the immunoelectron microscopy observations (Figs. 2D, and 3D), TDRD6 becomes localized to nuage earlier than TDRD7. In haploid round spermatids, TDRD1, 6 and 7 strongly co-localized to chromatoid bodies. However, TDRD1 became undetectable earlier than TDRD6 and 7 (Fig. 4D, arrowheads), and TDRD6 disappeared prior to TDRD7 (Fig. 4E, arrowheads). Taken together, the TDRD proteins are nuage components in common, but they also show differential localization or the accessibility of their epitopes change, as summarized in Fig. 4F. This suggests that nuage undergoes compositional changes along with germ cell differentiation and the TDRD proteins may take part in this molecular rearrangement of nuage.

Fig. 4. Nuage localization and a complex formation of TDRD1, 6 and 7 in male germ cells. (A–C) Sections of an adult testis immunostained for TDRD1 (A), 6 (B) and 7 (C) (green) and counterstained with Hoechst dye (blue). Spermatocytes and spermatids are indicated by arrows and arrowheads, respectively. (D, E) Double immunostaining of adult testis sections for TDRD1, 6 (D) and for TDRD6, 7 (E) counterstained with Hoechst dye (blue). (D) In spermatocytes, TDRD1 shows granular distribution at earlier stages than TDRD6 (arrow), while in spermatids, TDRD6 remains aggregated longer than TDRD1 (arrowheads). (E) TDRD6 and 7 exhibit granular appearances at about the same stage in spermatocytes (arrows), while in spermatids, TDRD7 remains detectable later than TDRD6 (arrowheads). Spermatogonia that show diffused signals of TDRD7 are also indicated by asterisks. In panels A–E, dotted lines demarcate seminiferous tubules. Scale bars: 10 μm. (F) Sequential localization of TDRD1, 6 and 7 to nuage. The arrows depict the differentiation stages of spermatocytes and spermatids when nuage localization is discernible. (G) Immunoprecipitation of TDRD1, 6 and 7 from adult testis. (Left panel) SDS-PAGE of immunoprecipitates with anti-TDRD1, 6 and 7 antibodies and normal rabbit immunoglobulin G as a control from adult testis lysate cross-linked with formaldehyde. Immunoglobulin heavy and light chains are indicated with arrowheads. Proteins were detected with SyproRuby dye. (Middle) Denaturing PAGE of RNAs extracted from the immunoprecipitates and end-labeled with 32P. (Right) Western blots of the immunoprecipitates obtained under a non-cross linked condition. The blots were probed with anti-TDRD1, 6 and 7 (upper panel) or with anti-MVH (lower panel) antibodies. The input lanes represent 1 (left), 0.1 (middle) and 3 (right) % of the input, respectively.
We then examined whether the TDRD proteins are physically associated in vivo. Adult testis lysate was subjected to immunoprecipitation with anti-TDRD1, 6 and 7 antibodies and normal rabbit immunoglobulin G as a control. The immunoprecipitates contained several proteins and also, RNAs, as shown in Fig. 4G. Western blotting demonstrated that the TDRD proteins reciprocally co-precipitated each other together with MVH/DDX4, another characteristic component of nuage. Thus, the TDRD proteins are in a large ribonucleoprotein complex, consistently with their characteristic localization to nuage. Future identification of the protein and RNA species in the TDRD complex would help address the molecular architecture of nuage structure.

**Co-localization of TDRD proteins is disrupted in Mvh mutant**

*Mvh/Ddx4* is a mouse homologue of *vasa* and encodes a well conserved component of nuage (Toyooka et al., 2000). In mice, Mvh1098/1098 gene-targeted mutants are male-sterile and arrest spermatogenesis during meiotic prophase of spermatocytes (Tanaka et al., 2000). In Mvh1098/1098 spermatocytes, intermitochondrial cement is not discernible and the localization of TDRD1 is disrupted (Chuma et al., 2006). Here, we compared the intracellular localization of TDRD1, 6 and 7 in Mvh1098/1098 spermatocytes. While all the TDRD proteins were detected, they failed to co-localize in Mvh1098/1098 spermatocytes (Fig. 5). TDRD1 was diffusely distributed or exhibited peri-nuclear accumulation, while TDRD6 and 7 formed discrete cytoplasmic granules. However, the distribution of TDRD6 and 7 granules was distinct and independent. The TDRD proteins in Mvh1098/1098 spermatocytes did not co-localize with Sm proteins of small nuclear ribonucleoproteins (snRNPs) (data not shown), which also are conserved components of nuage (Biggiogera et al., 1990; Moussa et al., 1994; Chuma et al., 2003). This indicates that each TDRD complex was compositionally distinct from or incomplete compared to wild-type nuage. The results showed that TDRD1, 6 and 7 require *Mvh* for their proper localization and assembly, and that each TDRD protein likely functions in distinct complexes at different intracellular loci before their co-localization to nuage.

*A single Tudor domain can localize or accumulate to nuage, but Tudor domain repeats are essential for germ cell differentiation*

It was speculated that Tudor domain repeats are closely associated with intracellular localization and possible function. To address this issue, we carried out in vivo over-expression experiments of the TDRD proteins and their truncated forms in male germ cells (Fig. 6A). Testes of premature mice on different postnatal days were used for in vivo electroporation of the constructs to achieve preferential transfection into mitotic spermatogonia, meiotic spermatocytes and haploid round spermatids (Fig. 6B) (Shoji et al., 2005).

![Fig. 5. Co-localization of TDRD1, 6 and 7 is disrupted in Mvh mutant. Sections of Mvh1098/1098 (A, B) and Mvh+/1098 (C, D) testes were immunostained for TDRD1, 6 and 7 as indicated. (A, B) In control Mvh+/1098 spermatocytes, the granules of TDRD1, 6 and 7 show precise co-localization that corresponds to nuage (arrowheads). (C, D) In Mvh1098/1098 spermatocytes, TDRD1, 6 and 7 exhibit separate, distinct distributions. TDRD1 is diffusely distributed or shows peri-nuclear accumulation, while TDRD6 and 7 form cytoplasmic granules, but they do not merge each other. Nuclei were counterstained with Hoechst dye (blue). Scale bar: 10 μm.](image-url)
In spermatogonia, full-length TDRD1-TDx4 (TDx4 denotes four Tudor domains) was primarily diffused in the cytoplasm. The fine granular pattern, as seen for endogenous TDRD1 (Chuma et al., 2006), was not discernible, possibly due to the large increase in the diffused signal (Fig. 6C). Full-length TDRD6-TDx7 was also diffusely observed in the cytoplasm (Fig. 6D). The expression of these two full-length constructs did not trigger aggregate formation of MVH (Figs. 6C, D) and Sm proteins of snRNPs (data not shown), both of which show granular localization to nuage in spermatocytes and spermatids.

ΔTDRD1-TDx1 and ΔTDRD6-TDx1, that contained a single Tudor domain, were detected both in the cytoplasm and nuclei (Figs. 6E, F). The nuclear localization is presumably due to passive diffusion through the nuclear pore because of their reduced molecular masses. ΔTDRD1-TDx1 was also localized to speckle-like structures in the nucleus similarly to full-length (Fig. 6C, E) and endogenous TDRD1. The nuclear bodies of TDRD1 co-localize with those of Sm proteins of snRNPs that
are called Cajal bodies (Chuma et al., 2003). ΔTDRD6-TDx1, on the other hand, occasionally showed mitochondrial localization (data not shown). This difference between ΔTDRD1-TDx1 and ΔTDRD6-TDx1 suggests that these Tudor domains associate with different binding partners located at different intracellular loci, or that the fragments contain distinct localization signals to each intracellular destination, although we did not find canonical signal peptide sequences in these TDRD fragments.

In spermatocytes, full-length TDRD1-TDx4 and TDRD6-TDx7 showed discrete cytoplasmic granules that co-localized with endogenous nuage components, TDRD1, 6, MVH (Figs. 7A–D, arrowheads) and Sm proteins of snRNPs (data not shown). This indicated that the two full-length constructs were properly targeted to nuage. In contrast, ΔTDRD1-TDx1 and ΔTDRD6-TDx1 did not show particular localization, and the distribution of endogenous nuage components was disrupted (Figs. 7E–H). The spermatocytes expressing these truncated constructs were morphologically impaired, and mostly solitary among surrounding non-transfected spermatocytes. Deleterious effects of ΔTDRD1-TDx1 and ΔTDRD6-TDx1 on spermatocytes were also observed as disorganization of meiotic synaptonemal complexes (Figs. 8A–F). Similar results were also seen for ΔTDRD1-TDx2 and ΔTDRD6-TDx2 (data not shown). Quantification of the disorganization of synaptonemal complexes in spermatocytes by the expression of each construct is shown in Fig. 8G. The data clearly demonstrated that the truncated TDRD1 and 6, which presumably functioned as dominant negative forms, were detrimental to meiotic spermatocytes.

In round spermatids, TDRD1-TDx4 and TDRD6-TDx7 were localized to chromatoid bodies, as demonstrated by co-localization with MVH and Sm proteins of snRNPs (Figs. 9A–D, arrowheads). ΔTDRD1-TDx2 and ΔTDRD1-TDx1 also localized to chromatoid bodies (Figs. 9E–H, arrowheads), while these truncated forms exhibited diffused signals both in the cytoplasm and nuclei as was also seen in spermatogonia. The over-expression of both full-length and truncated proteins showed no discernible differences in deleterious effects on spermatids.

The single Tudor domain of the Survival of Motor Neuron (SMN) protein interacts with its binding partners via aromatic and charged residues at the binding surface (Selenko et al., 2001; Cote and Richard, 2005). These amino acids are conserved among Tudor domain sequences, and we introduced point mutations at residues, Y1019N and E1023K, in ΔTDRD1-TDx1 (Fig. 6A). The expression levels of the two mutated ΔTDRD1-TDx1 were confirmed to be comparable to that of wild-type ΔTDRD1-TDx1 when examined in NIH/3T3 cells (data not shown). In round spermatids, the mutated ΔTDRD1-TDx1-Y1019N and -E1023K were both diffusely distributed and did not exhibit specific localization, despite that endogenous chromatoid bodies were clearly observed (Figs. 10A–F, and quantification in Fig. 10G). This result suggests that the single Tudor domain functions as a structural unit that localizes or accumulates to chromatoid bodies/nuage.

Discussion

We showed that TDRD1, 6 and 7 are abundant in the testis and constitute a novel class of nuage components in male germ cells. The molecular property and function of mammalian nuage remain largely unclarified. The chromatoid body in spermatids has been relatively well described among the mammalian nuage, because of its prominence by light and electron microscopy. This structure is implicated in RNA regulation based on the abundance of RNA and RNA binding proteins such as transition protein 2 mRNA (Saunders et al., 1992) and p48/p52 protein (Oko et al., 1996). Recently, Kotaja et al. (2006) reported that the chromatoid body in spermatids contains Dicer and microRNAs, proposing that the structure is involved in a microRNA pathway. Intracellular and intercellular movements of the structure also suggest that it functions in RNA/protein trafficking and gene dosage compensation (Parvinen, 2005). In contrast, nuage in germ cells at other stages of development, including spermatocytes, is less studied, and only a few components have so far been reported. TDRD1, 6 and 7 are common components of nuage in both spermatocytes and spermatids, and these proteins sharing Tudor domain repeats may represent characteristics of nuage in these cells. Meanwhile, TDRD1 also localizes to nuage in oocytes, while TDRD6 and 7 were only detectable in the male. The TDRD proteins may confer more diversity of components and functions onto the male nuage than female.

In this report, we addressed the possible correlation of Tudor domain repeats with their intracellular location and function. The result obtained by in vivo over-expression experiments showed that the truncated, but not wild-type TDRD1 and 6 proteins, were deleterious to meiotic spermatocytes. The truncated proteins that contained only one or two Tudor domains presumably functioned as dominant negative forms that interfered with full-length proteins, providing evidence that Tudor domain repeats are actually essential for germ cell differentiation. It is probable that the phenotype observed in spermatocytes resulted from functional inhibition of the TDRD proteins localized to nuage, although it remains possible that smaller diffused fractions of the TDRD proteins are responsible for the phenotype. In spermatids, on the other hand, both the full-length and truncated TDRD proteins showed clear localization to chromatoid bodies. Thus, Tudor domain repeats are not a prerequisite for nuage localization or accumulation. It is reported that multiple domains or motifs of several proteins make up one contiguous unit of a substrate binding site, such as the Armadillo repeats of importin alpha that associate with nuclear localization signals (Conti et al., 1998). The data on ΔTDRD1-TDx1 in spermatids suggest that this is not the case for Tudor domains in TDRD1 and that a single Tudor domain can be a unit for nuage localization or accumulation.

The single Tudor domain in the SMN protein binds to Sm proteins of snRNPs (Buhler et al., 1999; Selenko et al., 2001) and two neighboring Tudor folds of 53BP1, which comprise a single globular domain, associate with histone H3 (Charier et al., 2004; Huyen et al., 2004). These Tudor domains have been
Fig. 7. In vivo over-expressions of TDRD1, 6 and truncated forms in spermatocytes. Sections of testes transfected with the constructs indicated for postnatal days 15–17 or 16–18 were immunostained for 6xHis or FLAG and for TDRD1, 6 or MVH. Transfected and adjoining non-transfected spermatocytes are demarcated by dotted and solid lines, respectively. (A–D) TDRD1-TDx4 (A, B) and TDRD6-TDx7 (C, D) formed granules that co-localized with endogenous nuage components as indicted (arrowheads). (E–H) Spermatocytes expressing ΔTDRD1-TDx1 (E, F) and ΔTDRD6-TDx1 (G, H) showed aberrant morphologies, and endogenous nuage components were irregularly distributed or the signals were undetectable. Nuclei were counterstained with Hoechst dye (blue). Scale bar: 10 μm.
Fig. 8. Truncated TDRD1 and 6 are detrimental to meiotic spermatocytes. (A–F) Sections of testes, transfected as described in Fig. 7, were immunostained for 6xHis or FLAG and for meiotic synaptonemal complex protein SYCP3. Spermatocytes expressing TDRD1-TDx4 (A) and TDRD6-TDx7 (D) were clustered and showed normal fibrous structures of synaptonemal complexes. In contrast, spermatocytes expressing ΔTDRD1-TDx1 (B, C) and ΔTDRD6-TDx1 (E, F) were mostly solitary, and synaptonemal complexes were disorganized, or SYCP3 signals were undetectable. (G) The percentages of spermatocytes showing different patterns of synaptonemal complexes stained by SYCP3. Spermatocytes transfected with each construct were immunostained for 6xHis or FLAG and for SYCP3, and classified as follows: normal, fibrous structures of SYCP3 were observed as in non-transfected spermatocytes; disorganized, fibrous patterns of SYCP3 were fragmented or disrupted; and signal (−), SYCP3 signals were invisible despite adjoining non-transfected spermatocytes showed normal SYCP3 staining. Data were collected from at least 50 spermatocytes from two independent experiments. Scale bar: 10 μm.
Fig. 9. In vivo over-expressions of TDRD1, 6 and truncated forms in spermatids. Sections of testes, transfected with the constructs indicated for postnatal days 18–23, were immunostained for 6xHis or FLAG and for endogenous nuage components MVH or Sm proteins of snRNPs. Full-length TDRD1-TDx4 (A, B) and TDRD6-TDx7 (C, D), and also truncated ΔTDRD1-TDx2 (E, F) and ΔTDRD1-TDx1 (G, H) all clearly localized to chromatoid bodies (arrowheads). Dotted lines demarcate transfected spermatids. Scale bar: 10 μm.
Fig. 10. A Tudor domain is a structural unit that localizes or accumulates to chromatoid bodies. (A–F) Spermatids transfected with wild-type ΔTDRD1-TDx1 (A, B), mutated ΔTDRD1-TDx1-Y1019N (C, D) and -E1023K (E, F) were immunostained for 6xHis and for endogenous nuage components MVH (A, C, E) or TDRD6 (B, D, F). The point mutations in ΔTDRD1-TDx1 (C–F) disrupt the localization to chromatoid bodies (arrowheads). Dotted and solid lines demarcate transfected and adjoining non-transfected spermatids. (G) The percentages of spermatids in which transfected constructs localize to chromatoid bodies. Spermatids expressing 6xHis signals were classified as follows: TDRD6 cb (+)/6xHis cb (+), aggregates of endogenous TDRD6, that corresponds to chromatoid bodies (cb), are visible and 6xHis signals of transfected constructs merge with the TDRD6 aggregates; TDRD6 cb (+)/6xHis cb (-), TDRD6 aggregates are seen, but 6xHis signals are diffused and do not co-localize with the TDRD6 aggregates; TDRD6 cb (-)/6xHis cb (-), both TDRD6 and 6xHis signals are diffused and no specific localization is observed. Data were collected from at least 100 spermatids from two independent experiments. Scale bar: 10 μm.
shown to interact with the dimethylated arginines of their binding partners, and that aromatic clusters and negative charges at the binding pocket surfaces are essential for these interactions (Selenko et al., 2001; Charier et al., 2004; Huyen et al., 2004; Cote and Richard, 2005). Point mutations at the corresponding residues, Y1019N and E1023K, in ΔTDRD1-TDx1 abolished its localization to chromatoid bodies. This strongly suggests that the Tudor domain structure and its interaction with dimethylated arginines of putative binding partners are essential for nuage localization or accumulation of the TDRD proteins.

A possible function of Tudor domain repeats is to assemble molecules that associate with each Tudor domain into macromolecular complexes. This notion agrees with the result observed in spermatocytes that the localization of endogenous nuage components was disrupted following over-expression of truncated TDRD proteins carrying one or two Tudor domains. The scaffold function of Tudor domain repeats is also in agreement with the reduced number and size of polar granules in hypomorphic and null mutants of Drosophila tudor (Boswell and Mahowald, 1985; Thomson and Lasko, 2004). However, over-expression of full-length TDRD1 and 6 did not trigger the formation of nuage-like aggregates in spermatogonia. Nuage is enriched in cytoplasmic granules similar to nuage, but the TDRD proteins to nuage, as shown in Fig. 4, may then bring about the different distribution of RNF17 from that of the TDRD proteins.

Recently, RNF17, originally identified as a RING finger protein, was shown to contain four Tudor domains. RNF17 is abundant in the testis, and a targeted disruption of Rnf17 results in spermiogenic defect (Pan et al., 2005). Interestingly, RNF17 is enriched in cytoplasmic granules similar to nuage, but the RNF17-granules are distinct from intermitochondrial cement and chromatoid bodies where TDRD1, 6 and 7 accumulate. Provided that a Tudor domain has the activity to localize or accumulate to nuage, a RING finger domain may bring about the different distribution of RNF17 from that of the TDRD proteins.

The characteristic co-localization of TDRD1, 6 and 7 was abrogated in Mvh1098 spermatocytes. Since Tudor localization is also regulated by vasa in Drosophila oocytes and embryos, the molecular pathway, vasa/Mvh-Tudor/TDRD, is likely evolutionarily conserved, suggesting its importance in the germ-line of diverse species and at different stages of development. The independent, separate distribution of each TDRD protein in Mvh1098 spermatocytes indicates that the TDRD proteins possess distinct molecular properties that function at different intracellular loci before their trafficking or accumulation to nuage. The sequential localization of the TDRD proteins to nuage, as shown in Fig. 4, may then bring about compositional changes of the structure during germ cell differentiation. Future investigation to identify molecules that associate similarly and differently with each TDRD protein would help study nuage and its assembly at the molecular level.

Technically, this study involves an attempt to apply in vivo transfection of the testis to characterize gene functions in male germ cells. To achieve preferential transfection into spermatogenic cells at the intended stages of differentiation, testes of juvenile mice during the first wave of spermatogenesis were subjected to in vivo electroporation, as we recently reported (Shoji et al., 2005). When many constructs are to be examined, this approach can be an alternative to producing transgenic mice. However, a disadvantage of this technique is its limited transfection efficiencies. Thus, the method is more suitable for analyzing genes that function cell-autonomously, such as those encoding organelle components, rather than genes that function via cell–cell interactions, like those encoding growth factors etc.

In summary, this study showed that the Tudor-related proteins constitute a novel class of nuage components in the germ-line and that the characteristic domain architecture is closely associated with their intracellular localization and function. The analogy between vasa-Tudor in Drosophila and Mvh-the TDRDs in mice suggests that the molecular pathway has been retained and plays an important role in germ-line cells. Nuage is a site of assembly of these evolutionarily conserved molecules, and the Tudor-related proteins would serve as valuable probes to study this intriguing structure, whose molecular and developmental characteristics remain largely unknown.

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