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

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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.

Keywords: Germ cell; Spermatogenesis; *Tdrd*; Tudor; *Mvh*; Vasa; Intermitochondrial cement; Chromatoid body; Nuage; Electroporation

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,

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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 "intermitochondrial 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 homologues 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 Mahowald, 1985; Golumbeski 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′-TTTATCGATTATGGCAACATGTCT-3′ and 5′-ACCTGCTAATCATATCTTCAGCTA-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 [^{32}P] 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′-TTTATCGATTATGGCAACATGTCT-3′ and 5′-ACCTGCTAATCATATCTT-CAGCTA-3′ for *Tdrd6* and 5′-GTCGTACCACGGGCATTGTGATGG-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 antisera using the same antigen coupled to cellufine beads (Chisso, Japan). A *Tdrd7* cDNA