Germ-line specific variants of components of the mitochondrial outer membrane import machinery in *Drosophila*

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Abstract A search of the *Drosophila* genome for genes encoding components of the mitochondrial translocase of outer membrane (TOM) complex revealed duplication of genes encoding homologues of Tom20 and Tom40. Tom20 and Tom40 were represented by two differentially expressed homologues in the *Drosophila* genome. While *dtom20* and *dtom40* appeared to be expressed ubiquitously, the second variants, called *tomboy20* and *tomboy40*, were expressed only in the male germ-line. Transcripts for *tomboy20* and *tomboy40* were detected in primary spermatocytes as well as post-meiotic stages. Transcription of *tomboy20* and *tomboy40* in spermatocytes was not dependent on the transcription factor Cannonball, which is responsible for controlling expression of gene products exclusively required for post-meiotic germ cell differentiation. Epitope-tagging and transient expression of dTom20 and Tomboy40 in mammalian cell culture showed proper targeting to mitochondria.

Keywords: Mitochondrial protein; Spermatogenesis; Gene duplication; Transcription; *Cannonball*; Mitochondrial import; TOM complex; Testis-specific; *Drosophila*

1. Introduction

Spermatogenesis in *Drosophila* provides an excellent model system to study how general cellular processes are modified by the developmental program to achieve differentiation of specialized cell types. During spermatogenesis, a multi-step differentiation program converts a diploid stem cell to a highly specialized haploid sperm cell. An extraordinary, cell type specific transcriptional and post-transcriptional gene expression program ensures the synthesis and deposition of all gene products needed for the post-meiotic spermatid differentiation process (spermiogenesis) before transcription is shut down after meiosis [1]. During spermiogenesis, spermatid cells undergo dramatic changes at both the cellular and the subcellular levels, including nuclear shaping, building of the flagellar axoneme, formation of the mitochondrial Nebenkern formation and the subsequent differentiation of the two mitochondrial derivatives.

Genetic and molecular studies revealed that a specialized set of germ-line specific proteins replaces many generally expressed housekeeping proteins during development of male germ cells. For example, the germ-line specific β2-tubulin isoform replaces the more broadly expressed β1-tubulin in primary spermatocytes and is essential for meiosis and generation of the axoneme [2–4]. In addition, the *cdc25* homologue *twine* is expressed in primary spermatocytes and controls the meiotic cell cycle in males [5,6] replacing its more widely expressed homologue *string*, which mediates cell-cycle progression in embryos and mitotically dividing spermatogonia. Likewise, the male germ-line specific mitochondrial GTPase Fzo exclusively regulates controlled mitochondrial fusion for Nebenkern formation in early spermatids [7]. Finally, expression of many male-specific proteins involved in spermatid differentiation coordinated by tissue-specific transcription factors like the TAF5 homologue Cannonball (*can*) [8]. Duplication of nuclear genes encoding mitochondrial proteins has only been reported so far for the *fzo* gene and its generally expressed counterpart *dmfn* [9].

We screened the *Drosophila* genome for other conserved nuclear genes encoding mitochondrial proteins and began investigating genes for subunits of the mitochondrial outer membrane translocator (TOM complex). In yeast, the TOM complex consists of seven subunits (Table 1) [10,11], which are divided into two classes: the so-called receptors and the core of the general import pore, respectively. Only one homologue of the TOM complex, the Tom22 homologue Maggie, has been characterized in *Drosophila* so far [12]. Here, we report the identification and expression of duplicated genes for both the Tom20 and Tom40 subunits. In each, one homologue is generally expressed, while the second homologue is expressed specifically in primary spermatocytes.

2. Materials and methods

2.1. Identification of dtom20, dtom40, tomboy20 and tomboy40

The *dtom20*/CG7654 gene was identified by searching for EST (expressed sequence tag) sequences homologous to yeast Tom20. EST clone SD03699 was sequenced and found to contain the *dtom20* encoding open reading frame. *Tomboy20*/CG14690 was identified in a

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Table 1
TOM complex subunit genes of *D. melanogaster*

Function	*S. cerevisiae*	*Drosophila*	CG-Number	Map position
Receptors	Tom70	*dtom70*	CG6756	2L-33B9
	Tom20	*dtom20*	CG7654	3L-76E1
		dtomboy20	CG14690	3R-86C5
General import pore	Tom40	*dtom40*	CG12157	X-7B7
		dtomboy40	CG8330	2R-42A12
	Tom22	*maggie(mge)*	CG14981	3L-63F6
	Tom7	*dtom7*	CG8226	2R-44F11
	Tom6	?		
	Tom5	?		

tBlastn search for *Drosophila* ESTs homologous to the previously identified *dtom20*. The *tomboy20* open reading frame was obtained by PCR amplification from genomic DNA. *dtom40*/CG12157 and *tomboy40*/CG8330 were identified in a tBlastn search by sequence homology to the rat Tom40 protein [13]. The *tomboy40* cDNA sequence was obtained by sequencing EST clone AT23456). The *dtom40*-cDNA was amplified by RT-PCR using primers designed to amplify the coding region. Amplification of the *dtom40* open reading frame by RT-PCR was carried out using Qiagen OneStep RT-PCR kit with 700 ng of total RNA from adult female flies using appropriate primer pairs. RNA was isolated from flies using the Trizol reagent following the manufacturer's protocol. All PCR products were subcloned into the pCRII-TOPO vector (Invitrogen) and sequenced. All EST-clones were obtained from Research Genetics.

2.2. In situ hybridization on testes and Northern blot analysis

In situ hybridization to whole adult wild-type *Drosophila* testes was carried out using DIG-labeled riboprobes [14]. Sense and antisense riboprobes were generated by in vitro transcription using a linearized plasmid of pCRII containing the coding sequence of *tomboy20* or *tomboy40*. *can*-mutant males transheterozygote for can^2/can^{12} were used to study the effect of *can* mutants on expression of *tomboy20* and *tomboy40*. Northern blot for Fig. 2A has been described in detail in [9]. The same filter has been used earlier to show differential expression of *dmfn* and *fzo* [9]. For the developmental Northern blot, one gram of embryos from each stage was homogenized with RNAzol and total RNA was extracted, followed by poly(A)$^+$ mRNA purification using the polyATract mRNA isolation kit (Promega).

2.3. Expression constructs, transfection and immunofluorescence

The *dtom20* open reading frame was cloned in frame into the appropriate EGFP Living colors vector (Clontech). His-tagged Tomboy40 was constructed by first amplifying the *tomboy40* coding sequence using primers designed to remove the stop codon, then subcloned directly into the pcDNA3.1/V5-His-TOPO vector (Invitrogen). All expression constructs were sequenced (Stanford PAN facility) to confirm correct fusion of the open reading frames. Constructs were transfected into HeLa or NIH3T3 cells using the Superfect reagent (Qiagen) and processed for microscopy as described [15].

3. Results

3.1. Identification of genes encoding subunits of the mitochondrial translocase of outer membrane in Drosophila

Homologues of the TOM complex from *Saccharomyces cerevisiae* have been found in higher organisms such as human and rat, suggesting that they are evolutionarily conserved [13,16]. While surveying the *Drosophila* genome for homologues of mitochondrial proteins, we identified homologous genes encoding components of the mitochondrial TOM complex. One gene each was identified for Tom70, Tom22, and Tom7. No homologous sequences for either Tom6 or Tom5 were found. However, two gene copies were identified for both Tom40 and Tom20 (summarized in Table 1). We characterized the two homologues of the receptor subunit Tom20 and the import pore Tom40 in more detail to investigate whether the two homologues showed different expression patterns.

The *dtom20*/homologue (CG7654) was located on chromosome 3L at position 76E1. Sequencing of EST clone SD03966 derived from CG7654 revealed a 0.6-kb *dtom20* cDNA consisting of three exons (Fig. 1C). The EST was verified by amplifying *dtom20* cDNA directly from adult total RNA by RT-PCR. The resulting PCR product was sequenced, confirming the initially determined cDNA sequence. The *dtom20* cDNA encoded a 168 amino acid predicted polypeptide, which is 23% identical and 36% similar to yeast Tom20. The predicted protein shared all conserved protein motifs with the characterized human hTom20 (NP_055580; 51% identity, 66% similarity), including the transmembrane domain and TPR-domain (tetratrico peptide repeat), but showed sequence differences in the C-terminus (Fig 1A). The *dtom40* homologue (CG12157/*ms(1)15*; Xu, E. and Wu, C.I.; A. Dros. Res. Conf., 37:237, 1996) was located on the X-chromosome (7B7). The two predicted mRNAs, *tom40-RA* and *tom40-RB*, consisted of four and five exons, respectively (Fig. 1C), encoding a predicted 344 amino acid polypeptide sharing 28% identity and 48% similarity with the yeast homologue, but 54% identity and 70% similarity to hTom40 (NP_006105; Fig. 1B).

Duplication of genes encoding TOM components is not restricted to *Drosophila melanogaster* (Fig. 1D). Two predicted *tom40* genes, *tom40-1* and *tom40-2*, were also found in the genome of *Drosophila pseudoobscura* and *Apis mellifera* as deduced from the preliminary genomic sequence (Human Genome sequencing center at Baylor College of Medicine; http://hgsc.bcm.tmc.edu/projects/). The deduced Tom40 proteins from these predicted genes were highly homologous to dTom40 and Tomboy40. Interestingly, in both species the predicted *tom40-1* gene appeared to be encoded by a single predicted exon, whereas the coding sequence for *tom40-2* appeared to be encoded by several exons (not shown). Strikingly, the recent release of the complete *Anopheles gambiae* genome revealed that mosquitoes apparently express only one form of Tom20 (*A.g.* XP_320211) and Tom40 (*A.g.* XP_317621), which are more homologous to the generally expressed variant in *Drosophila* (*A.g.*Tom20, 55% identical to dTom20 and 46% identical to Tomboy20; *A.g.*Tom40, 69% identical to dTom40 and 59% identical to Tomboy40). The existence of two putative *tom40* genes in *A. mellifera* suggests that gene duplication might have happened early during evolution.

3.2. Two isoforms of tom20 and tom40 are present in Drosophila

Analysis of the *Drosophila* genome by tBlastn revealed second homologues for both *dtom20* and *dtom40*. The second *dtom20* homologue, called *tomboy20*, corresponded to the predicted gene CG14690 on chromosome 3R at position 86C5. The predicted Tomboy20 protein was 47% identical and 72% similar to dTom20 sharing all the conserved motifs (including TM and TPR), and also showed comparable similarities to human Tom20 (47% identity and 66% similarity) (Fig. 1). The second *dtom40* homologue, named *tomboy40*, corresponded to the annotated gene CG8330, located on chromosome 2R at position 42A12. The Tomboy40 protein predicted from EST sequence AT23456 was 64% identical and 75% similar to dTom40, with less homology at the N-terminus (Fig. 1B). Unlike dTom20 and dTom40, both Tomboy20 and Tomboy40

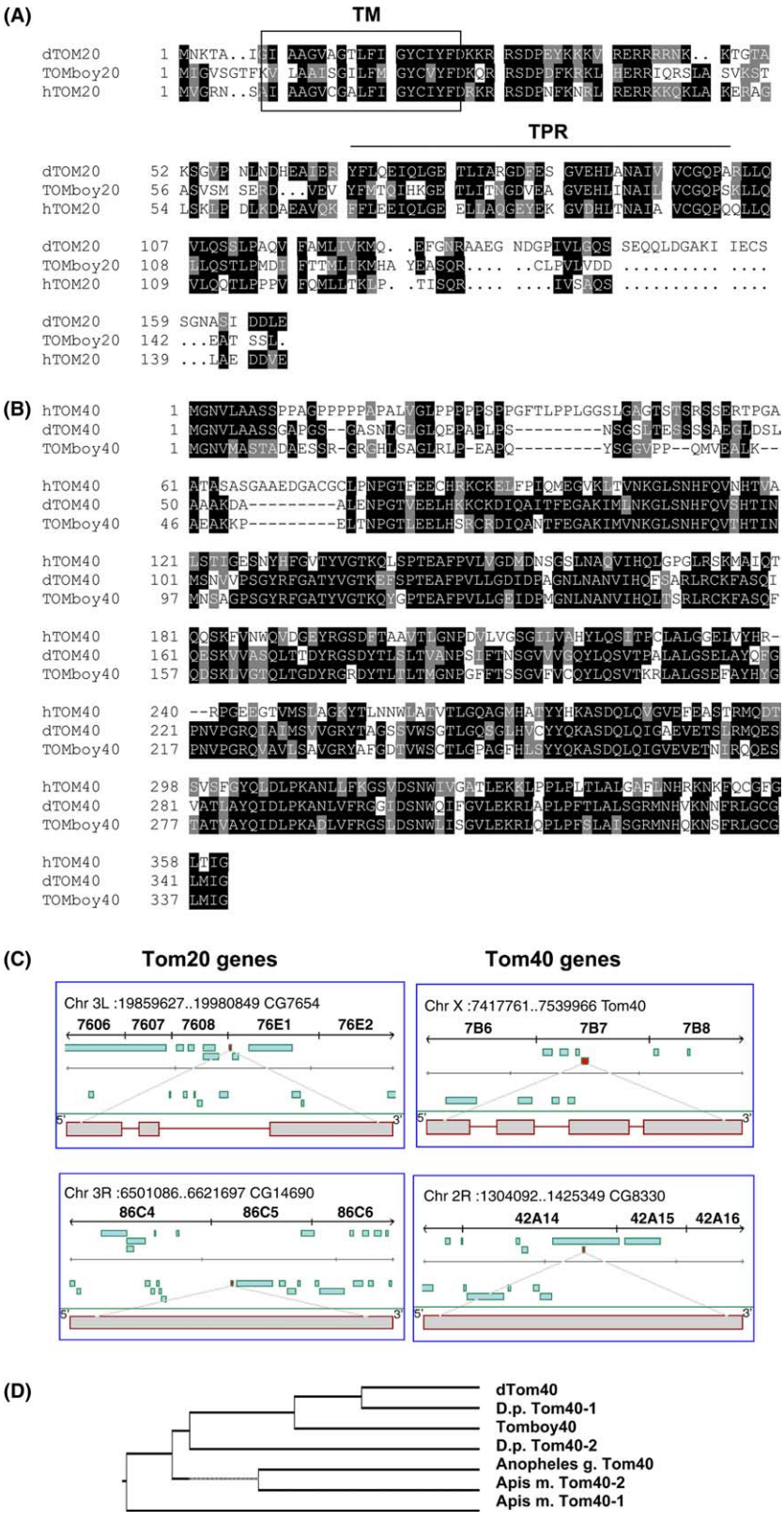

Fig. 1. Two homologues of *Drosophila* Tom20 and Tom40 were identified. Alignment of the predicted dTom20/Tomboy20 (A) and dTom40/Tomboy40 proteins (B) with human homologues. (C) Gene organization of corresponding *Drosophila* Tom20/Tom40 homologues as predicted according to Flybase [30]. For *tom40*, the predicted *tom40-RA* transcript is shown. (D) Phylogenetic tree of predicted Tom40 homologues based on ClustalW alignment (default parameter; MegAlign program DNAstar).

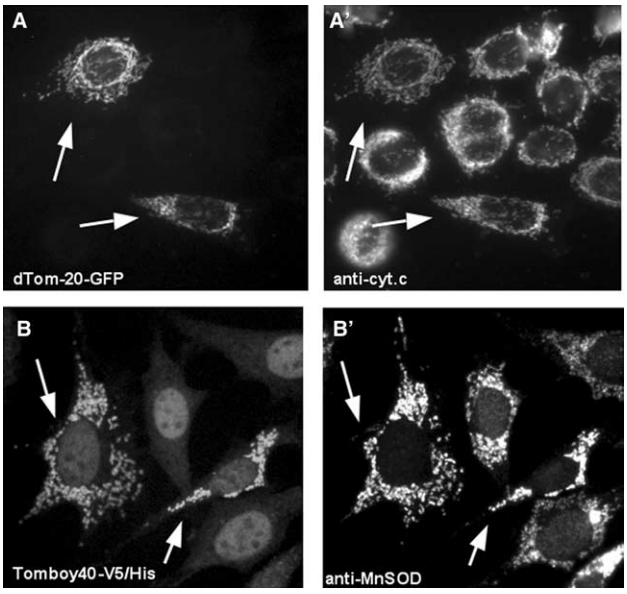

Fig. 2. dTom20 and Tomboy40 localized to mitochondria. (A) Localization of dTom20 to mitochondria in mammalian tissue culture cells. Transiently transfected HeLa cells expressing dTom20-GFP counterstained with anti-cytochrome *c* (A′) to demonstrate mitochondrial localization (arrows). (B) Localization of Tomboy40 to mitochondria. NIH3T3 cells transfected with Tomboy40-V5/His detected with anti-His (arrows) (anti-His also stains nuclei unspecifically) and countertstained with anti-MnSOD to label mitochondria (B′).

were encoded by a single exon with no intervening intron sequences (Fig. 1C). For both homologues, corresponding ESTs were found only in a testis cDNA library [6,17], suggesting specificity to the male germ-line.

3.3. *Drosophila Tom20 and Tom40 isoforms localized to mitochondria after transient expression in mammalian cells*

Drosophila Tom20 and Tom40 homologues encoded mitochondrial proteins that localized to mitochondria in mammalian cells. Transient expression of dTom20, fused to GFP at the C-terminus (dTom20-GFP) in mammalian cells, showed that dTom20-GFP co-localized with the mitochondrial marker anti-cytochrome *c*, a protein specific to the mitochondrial inner membrane space (Fig. 2A and A′). Some cells (approximately 10%) exhibited clustered mitochondria after transfection instead of the normally more dispersed structures (not shown). To determine whether Tomboy40 can associate with mitochondria, the Tomboy40 open reading frame was fused to a V5/His-tag (Tomboy40–V5/His) and the corresponding expression construct was transfected into NIH3T3 cells. Immunofluorescence staining demonstrated that Tomboy40–V5/His was targeted to mitochondria, as assessed by counterstaining with anti-manganese superoxide dismutase (anti-MnSOD) antibodies to label mitochondria (Fig. 2B and B′). Tomboy40, fused to GFP at either the N-terminal or C-terminal end, failed to localize to mitochondria (data not shown).

3.4. *Differential expression of the two genes for Tom20/Tom40*

Expression of *tomboy20* and *tomboy40* was detected only in the male germ-line. Northern blot analysis detected the ~0.6 kb *tomboy20* and the ~1.3-kb *tomboy40* transcripts only in poly(A)$^+$-mRNA from adult males, but not adult females, adult males without germ-line cells (agametic testis) or embryos. (Fig. 3A). In contrast, *dtom20* and *dtom40* appeared to be ubiquitously expressed (Fig. 3B and C). For example, both *dtom20* and *dtom40* were expressed during all stages of embryogenesis including very early embryonic stages, suggesting maternal deposition of mRNA. The single *dtom20* mRNA had a size of ~1.3 kb. Interestingly, two transcripts for *dtom40* (~2.3 and ~1.4 kb) were observed during embryonic development (Fig. 3B) and in adult flies (Fig. 3C), which is consistent with the predicted annotation of two transcripts (*tom40-RA*, *tom40-RB*) by FlyBase. In addition, corresponding ESTs were found in cDNA libraries derived from different developmental stages (adult males, females, testis, ovary, head, larval and S2 cells) confirming the general expression.

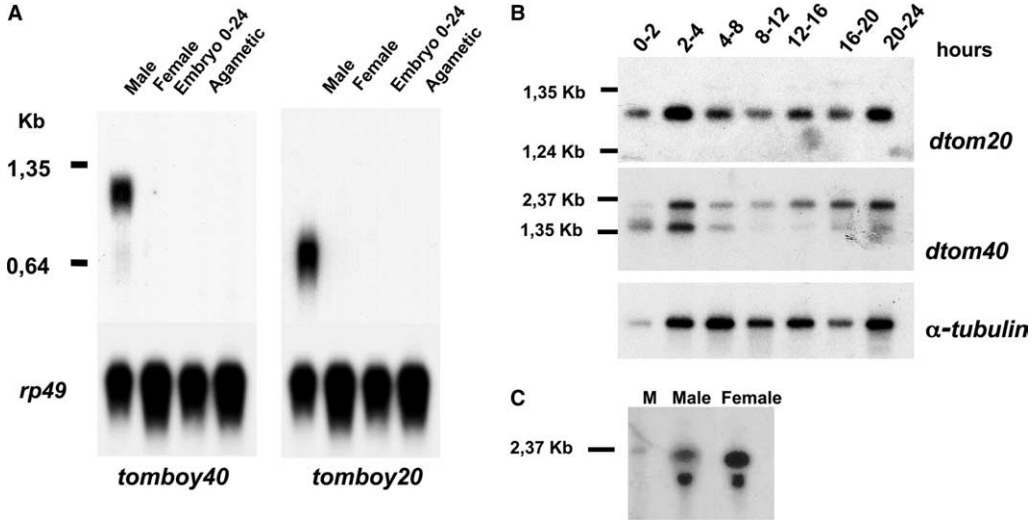

Fig. 3. Differential expression of *Drosophila* Tom20/40 homologous genes. (A) Northern blot of poly(A)$^+$ selected mRNA from males, females, embryos, and males lacking a germ-line (agametic) hybridized with specific probes for *tomboy20* and *tomboy40*; *rp49*, loading control. (B) Developmental Northern blot with polyA$^+$ selected mRNA (1μg per lane) derived from different embryonic stages (hours after egg laying) probed with specific probes for *dtom20* and *dtom40*. A probe for α-*tubulin* served as a loading control. The size standards are indicated. (C) Expression of *dtom40* in adult males and females revealed by Northern blot using total RNA.

3.5. Expression of tomboy20/tomboy40 in the male germ-line is independent of can

The temporal–spatial expression pattern of *tomboy20* and *tomboy40* during spermatogenesis was determined by in situ hybridization to adult testes (Figs. 4 and 5). *Tomboy20* (Figs. 4 and 5) and *tomboy40* (Fig. 5) were expressed in primary spermatocytes and spermatids. *Tomboy20* expression began during the growth phase of primary spermatocytes (Fig. 4A, double arrow). *Tomboy20* transcripts were clearly absent from stem cells and spermatogonia (Fig. 4A). Expression of *tomboy20* persisted after meiotic division and transcripts were detected in round haploid spermatids (Fig. 4B, arrow) and in elongating spermatid bundles (Fig. 4B, double arrow) but, disappeared later during late spermiogenesis in fully elongated and individualized spermatids (Fig. 4A, arrowheads). In contrast, for *dtom20* and *dtom40* mRNA expression was clearly detected earlier in premeiotic spermatogonia and persisted to the end of meiosis (Fig. 4C and D). No or very little staining was detectable in stem cells and early elongating spermatids. *Tomboy40* showed a similar expression pattern to *tomboy20*, with mRNA expressed in primary spermatocytes (Fig. 5C, arrow), but not detected in stem cells and spermatogonia near the tip of the testis (Fig. 5C), double arrow).

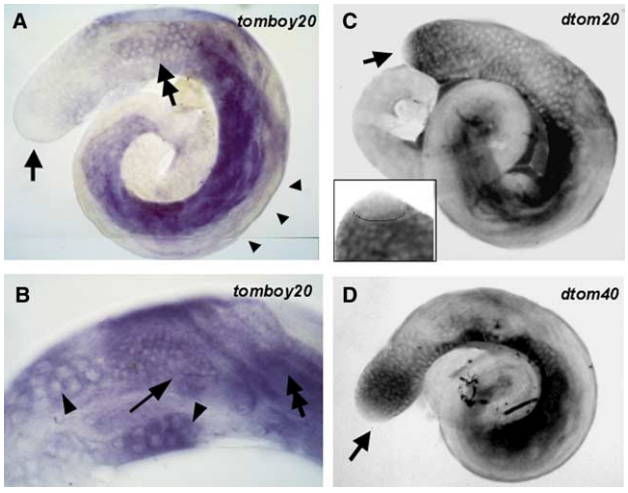

Fig. 4. Expression of tomboy20 during spermatogenesis. Whole mount in situ hybridization of adult *Drosophila* testis shows *tomboy20* (A,B), *dtom20* (C) and *dtom40* (D) expression in the male germ-line. (A) Adult testis tube: apical tip (arrow), spermatocytes (double arrow), region with elongated spermatids (arrow heads). (B) Magnified view of expression in different stages of germ cell differentiation premeiotic stages (arrowheads), cyst of 64 early spermatids (arrow), elongating spermatids (double arrow). (C,D) Arrow: apical tip with stem cell region (inset in C with dotted line).

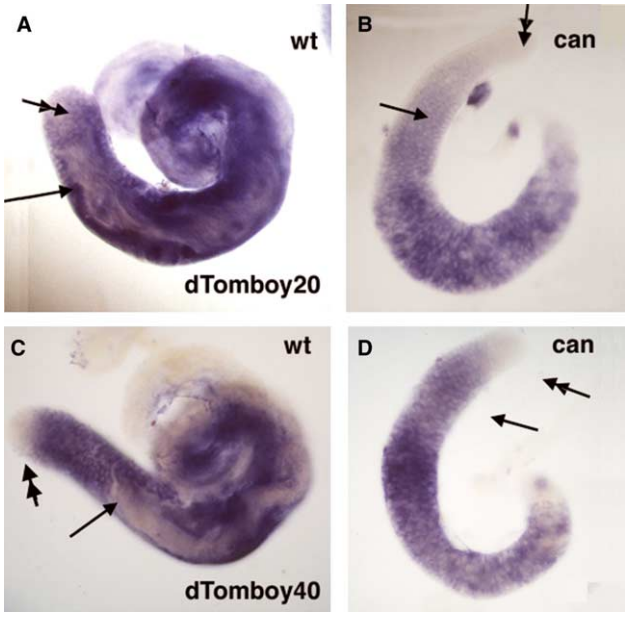

Fig. 5. Expression of *tomboy20* and *tomboy40* in the male germ-line is not under control of *can*. Expression of *tomboy20* (A,B) and *tomboy40* (C,D) in wild type (A,C) and male sterile *can* mutant (B,D) adult testis.

Tomboy20 and *tomboy40* mRNA expression was independent of the male-specific transcription factor *can*. For both genes, transcripts were detected by in situ hybridization in male sterile *can* mutants, which arrest as mature primary spermatocytes. Expression was as strong in the primary spermatocytes of *can* mutant testes as in wild-type spermatocytes treated in parallel, indicating that, unlike many other genes expressed specifically in the male germ-line involved in spermiogenesis, transcription of both *tomboy* genes was not under control of *can.*

4. Discussion

Two homologues of the Tom20 and Tom40 subunits of the TOM-complex were identified in *Drosophila*. Of the organisms where TOM subunits have been characterized, including *S. cerevisiae*, *Neurospora crassa*, rat and human, expression of duplicated genes encoding alternate forms of the protein has been documented only in *Drosophila*. A search of the entire yeast genome revealed only one gene encoding Tom20 and one gene encoding Tom40. In humans, five Tom20 genes have been identified [18]. However, four of these appear to be processed pseudogenes that no longer encode full-length proteins [19,20]. Interestingly, multiple variants of Tom20 and Tom22 exist in plants (*Arabidopsis*) [21,22].

Tomboy20 and *Tomboy40* homologues are expressed specifically in the male germ-line. Other examples of generally expressed genes with homologues encoding variants expressed only in the male germ-line have been found in *Drosophila.* The *can* gene encodes a male germ-line specific homologue of the TAF5 (TBP-associated factor) subunit of the TFIID transcription factor required for normal levels of transcription of certain genes expressed at the primary spermatocyte stage of spermatogenesis. *Can* mutant spermatocytes do not develop past the mature spermatocytes stage, presumably because they do not express normal levels of target genes required for meiotic cell cycle progression and spermatid differentiation [14,23]. Dependence of transcriptional expression on *can* is a common feature of a suite of gene products that are used exclusively after meiosis for spermiogenesis. Expression of spermatid differentiation genes such as *fzo*, *dj*, *mst35B* and *mst87F* [9,24–27] is dramatically lowered in *can* mutant males. Since expression of both *tomboy* genes was independent of *can*, it may be that Tomboy20 and Tomboy40 function is not restricted to post-meiotic spermatid differentiation processes, but might be used premeiotically like other *can*-independent genes such as β2-*tubulin* or *twine* [8,14]. Other examples of generally expressed genes that have second homologues expressed only

in the male germ-line in *D. melanogaster* include several of the subunits of the proteosome [28], other subunits of the TFIID (Hiller et al., in preparation) and the mitochondrial fusion transmembrane GTPase Fzo [7].

As in the case of the *dtom/tomboy* gene pair, it is not clear why one homologue is expressed in a testis-specific pattern while the other is more generally expressed. One explanation might be differential gene regulation; both forms of the gene may perform the same function, but the testis-specific variant may allow further spatial and temporal control of gene expression. For example, many transcripts that are used post-meiotically for spermiogenesis are transcribed premeiotically during the primary spermatocyte stage and stored for later translation [1,26,27]. As such, spermatid differentiation genes must be heavily transcribed during the spermatocyte stage, having a second copy that is specific to the testis, which may allow high expression in spermatocytes while maintaining lower levels of expression elsewhere. Alternatively, the testis-specific transcripts may incorporate features of the mRNA that allow translational repression to coordinate the proper time for expression of the protein.

It is also possible that there are functional differences between the generally expressed and testis-specific variants, and that the general and testis-specific isoforms perform related but distinct functions. For example, incorporation of a Tomboy20 and Tomboy40 subunit in place of Tom20 and Tom40 could modify how the TOM complexes operate in male germ-cells. In particular, since mitochondria dramatically change morphology in post-meiotic male germ-cells to the cell type specific Nebenkern and minor and major mitochondrial derivatives [29], it is conceivable that the Tomboy20 and Tomboy40 proteins are incorporated into a specialized TOM complex that distinguishes between different proteins to be imported into mitochondria at different stages of mitochondrial morphogenesis. It may even be imagined that two different forms of the TOM complex act in spermatids, one for import into the major mitochondrial derivative and one into the minor mitochondrial derivative to set up different characteristics of the two mitochondrial compartments.

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