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RESEARCH ARTICLE

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The *ThioredoxinT* and *deadhead* gene pair encode testis- and ovary-specific thioredoxins in *Drosophila melanogaster*

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Abstract So far, two thioredoxin proteins, DHD and Trx-2, have been biochemically characterized in *Drosophila melanogaster*. Here, with the cloning and characterization of TrxT we describe an additional thioredoxin with testis-specific expression. *TrxT* and *dhd* are arranged as a gene pair, transcribed in opposite directions and sharing a 471 bp regulatory region. We show that this regulatory region is sufficient for correct expression of the two genes. This gene pair makes a good model for unraveling how closely spaced promoters are differentially regulated by a short common control region. Both TrxT and DHD proteins are localized within the nuclei in testes and ovaries, respectively. Use of a transgenic construct expressing TrxT fused to Enhanced Yellow Fluorescent Protein reveals a clear association of TrxT with the *Y* chromosome lampbrush loops *ks-1* and *kl-5* in primary spermatocytes. The association is lost in the absence of the *Y* chromosome. Our results suggest that nuclear thioredoxins may have regulatory functions in the germline.

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Introduction

Thioredoxins constitute a family of small thiol proteins that are present in all organisms studied so far, and are characterized by the sequence of their conserved active site (WCGPC) (Holmgren 1989). Reduction of the two cysteines from the disulfide ($TrxS_2$) to the dithiol [$Trx(SH)_2$] protein form is catalyzed by the NADPH-dependent flavoenzyme thioredoxin reductase [TrxR; $NADPH+H^+ + TrxS_2 \rightarrow NADP^+ + Trx(SH)_2$] (Holmgren 1995). Thioredoxin reductase is structurally and mechanistically related to glutathione reductase (GR) (Arscott et al. 1997). Kanzok et al. (2001) have shown that in *Drosophila melanogaster* GR is absent and glutathione reduction is supported by the thioredoxin system. The Trx system therefore has a critical role in the regulation of intracellular redox homeostasis. Several cellular functions are based on thioredoxins (for reviews see Arner and Holmgren 2000; Powis 2001): they have important roles in DNA synthesis as electron donors for ribonucleotide reductase (Holmgren 1989) and are needed for the cellular response to oxidative stress (Nishinaka et al. 2001). Disulfide bonds participating in protein folding and/or in the redox control of transcription are effectively and selectively reduced by thioredoxins (for review see Arner and Holmgren 2000), e.g., deactivation of the OxyR transcription factor (Zheng et al. 1998) and the redox regulation of transcription factor NF-κB (Hirota et al. 1999). Thioredoxin also sometimes functions as a structural component of another enzyme by forming a complex, e.g., $Trx(SH)_2$ binds with high affinity to the T7 DNA polymerase in a 1:1 stoichiometric ratio, promoting high processivity (Huber et al. 1987). A similar function has also been shown for thioredoxin in mammalian cells where $Trx(SH)_2$ participates in an inhibitory complex with apoptosis-signaling kinase 1 (ASK1) (Saitoh et al. 1998). In a number of different ways redox reactions, like phosphorylation, can be used to regulate protein activity.

In mammals, five proteins with the active site CGPC have so far been described: Trx-1, which is mostly cytosolic but can under certain conditions be translocated

to the nucleus (Powis 2001); Trx2, a mitochondrial thioredoxin (Spyrou et al. 1997); Sptrx-1 and Sptrx-2, specific to the flagellum of the sperm (Miranda-Vizuete et al. 2001; Sadek et al. 2001); and Txl-1, a ubiquitous protein with unknown function (Miranda-Vizuete et al. 1998). In addition to this, seven thioredoxin pseudogenes have been identified in humans. Based on phylogenetic analysis the pseudogenes originated after the rodent-human radiation (Spyrou et al. 2001). In *D. melanogaster* two thioredoxins, DHD and Trx-2, have so far been biochemically characterized (Pellicena-Palle et al. 1997; Bauer et al. 2002). Trx-2 is expressed in larvae and in adults as well as in Schneider cell lines and represents 0.3%–1% of extracted protein (Bauer et al. 2002). DHD on the contrary is, as deduced from RNA blotting results, expressed in ovaries (Salz et al. 1994). Trx-2 but not DHD can function as a reducing substrate for thioredoxin peroxidase-1, suggesting functional differentiation of the two thioredoxins (Bauer et al. 2002). Mutations in *dhd* cause female sterility. Salz et al. (1994) found that the majority of eggs laid by *dhd* mothers showed a set of condensed chromosomes that suggested an arrest in meiosis; however, subsequent studies showed that eggs laid by *dhd* mothers arrested after completion of meiosis but before initiation of the S-M cycles (Page and Orr-Weaver 1996; Elfring et al. 1997). In addition, the maternal function of *dhd* is required also for early mitotic divisions (Salz et al. 1994). Mutations in the thioredoxin active site of DHD (CGPC) fail to rescue the *dhd* loss of function, suggesting that *dhd* function in vivo is dependent on its disulfide-reducing activity (Pellicena-Palle et al. 1997).

Here we present the characterization of a third *D. melanogaster* thioredoxin, TrxT. *dhd* and *TrxT* are organized as a gene pair sharing a 471 bp regulatory region, which is sufficient for the correct expression of the two genes. Using a transgenic construct expressing DHD fused to Enhanced Cyan Fluorescent Protein (ECFP) and TrxT fused to Enhanced Yellow Fluorescent Protein (EYFP), we show that DHD is expressed only in the ovary while TrxT is testis specific. Furthermore, both proteins are nuclear and TrxT is specifically associated with the *Y* chromosome loops. Our results suggest that the reversible formation of disulfide bonds may regulate gene expression from the *D. melanogaster Y* chromosome and that nuclear thioredoxins may have regulatory functions in the germline.

Materials and methods

Fly strains and crosses

The established mutants and fly lines used in this study have previously been described (Lindsley and Zimm 1992; Salz et al. 1994; Larochelle et al. 1998). To study the consequences of a lack of *TrxT* in sperm competition one *w* female was crossed with two males, either wild type or *w Df(1)J5*. After 2 days the males in each vial were removed and two new males of the other strain were added. The female progeny were counted and divided into classes according to eye color, which corresponds to the fertilizing sperm. Offspring from 100 crosses were counted. To generate males lacking the *Y* chromosome, we used the strain *X^Y, y v f*^{1}*/0/C(1)RM, y su(w*^{a}*) w*^{a}. We crossed *C(1)RM/0* females to males (wild-type Oregon R or *Df(1)J5; P[w*$^{+}$ *TrxT-EYFP]*) and isolated *X/0* male offspring. *XXY* females and *XYY* males were isolated from the *z*^{1} *In(1)w* ^{is}*/Y* ^{bb} stock where these karyotypes can be isolated based on eye color (Rasmuson-Lestander et al. 1993). Dr. Helen Salz, Dr. Maurizio Gatti and the Umeå *Drosophila* Stock Centre kindly provided stocks. Flies were cultivated in vials with potato mash-yeast-agar medium at 25°C.

Transgenic flies

The *P[w*$^{+}$ *TrxT-EYFP]* construct was made as follows: primer (TAT**GGTACC**GTGTGCTCCAGAACGGCATTCTC), which contains a KpnI site, was used together with primer (GT**GGATCC**GCCTTCACCAGCTTGGCCAT) to amplify the *TrxT-dhd* region using wild-type DNA as template for the *pfu*$^{\text{TURBO}}$ polymerase (Stratagene). The polymerase chain reaction (PCR) product was purified and cut with KpnI and BamHI. The *EYFP* -encoding fragment was excised from the pEYFP-N1 plasmid (Clontech) using KpnI/XbaI digestion. This fragment was ligated to the amplified *TrxT-dhd* fragment. The product was purified and ligated into the XbaI/BamHI sites in the P{CaSpeR-4} vector (Pirrotta 1988). The *P[w*$^{+}$ *dhd-ECFP]* construct was made by ligating the *ECFP* KpnI/NotI fragment to the amplified *TrxT-dhd* fragment and into the BamHI/NotI sites in the P{CaSpeR-4} vector. The *P[yellow*$^{+}$ *hsp70:FLAG-TrxT]* construct expressing the TrxT protein with a FLAG-corresponding peptide at the N-terminal region was made as follows: Primer (aa**ctcgag**catggattacaaggacgatgacgataagCCGCGGAACAAGGACGATCTT), which contains a XhoI site followed by a FLAG epitope tag (Met-DYKDDDK-) (Sigma), fused to the open reading frame of *TrxT* was used in a PCR together with the T7 primer and a *TrxT* cDNA cloned in pBluescript as template for the *pfu*$^{\text{TURBO}}$ polymerase (Stratagene). The product was cut with XhoI and cloned into the corresponding site of the C4Y-hs vector (Poux et al. 2001). This vector uses the *yellow* gene as a marker and places the *FLAG-TrxT* sequence under the control of the *hsp70* promoter. A strong overexpression of the FLAG-TrxT fusion is seen using immunoblot analysis following heatshock induction. The constructs were sequenced to confirm that they were correct. DNA for transformation was prepared using Qiagen Maxi Prep cartridges. Germline transformation of the construct was done according to the methods of Spradling (1986) using the *Df(1)w*^{67c23}*, yw* strain as host.

Molecular biology

For RNA blot analysis, poly(A)$^{+}$ RNA was isolated using Dynabeads Oligo $(dT)_{25}$ (Dynal). Adult flies, pupae, larvae and embryos were frozen at −70°C and homogenized in 0.1 M TRIS-HCl, pH 8.0, 0.5 M LiCl, 10 mM EDTA, 1% SDS, 5 mM DTT, and the instructions of the bead manufacturer were then followed. The samples were separated on a 1.0% formaldehyde-agarose gel and blotted onto a MagnaCharge filter (MSI) using the VacuGene Vacuum Blotting System. Prehybridization and hybridization were performed with Ultrahyb solution (Ambion) at 42°C. A *TrxT* cDNA clone (1.1 kb), *dhd* genomic DNA (2 kb XhoI/XbaI) fragment, *α-tubulin* cDNA (1.6 kb), *RpS3* cDNA (1.0 kb) and a mixture of ECFP/EYFP cDNA (0.8 kb), ^{32}P-labeled by random priming and purified with a Pharmacia S-200 HR column, were used as probes. The membrane was washed twice for 5 min each in 2×SSPE at room temperature, three times for 15 min each in 2×SSPE, 2% SDS at 65°C and once in 0.1×SSPE at room temperature for 15 min. In situ hybridization to polytene chromosomes was performed according to described methods (Heino 1994) using digoxigenin-labeled *TrxT* cDNA as probe. Predictions concerning the *TrxT* gene product were obtained using software at the following electronic

sites: http://jura.ebi.ac.uk:8765/gqsrv/submit (GeneQuiz), http://www.expasy.ch/.

Testis and ovary preparations

For live testes squashes testes from young adults were dissected in TB (183 mM KCl, 47 mM NaCl, 10 mM TRIS-HCl, 1 mM PMSF, 1 mM EDTA, pH 6.8) and prepared according to Bonaccorsi et al. (2000). To determine which *Y* chromosome loops were decorated by TrxT-EYFP, *Df(1)J5; P[w$^+$ TrxT-EYFP]* females were crossed to the following *Y* chromosome rearrangement males: *X.YL, y v/Df(Y)S7 Δkl-5 kl-3*; *X.YL, y v/Df(Y)G28 Δkl-5 kl-3*; *X.YL, y v/In(Y)S16 Δkl-3*; and *X.YS, y v/In(Y)S17 Δks-1*.

For whole mount live testis preparation (Fig. 5A, B, G, H), testes from adult males were dissected in TB and mounted in halocarbon oil 27 (Sigma). Live ovaries were dissected and mounted in halocarbon oil 27 as described by Hazelrigg (2000) and the stages were determined based on King (1970), Mahowald and Kambysellis (1980) and Spradling (1993). For indirect immunofluorescent staining of testes squashes, methanol-acetone fixation was done according to Bonaccorsi et al. (2000). The slides were then washed for 30 min in 1×PBT, transferred to blocking solution (0.1 M maleic acid, 0.15 M NaCl, 1% Boehringer blocking reagent) and incubated for 30 min at room temperature. The slides were incubated overnight at 4°C with 1:300 diluted anti-Trx-2 rabbit polyclonal primary antibody (kindly provided by H. Bauer). The slides were washed twice for 10 min each in 0.1 M maleic acid, 0.15 M NaCl, 0.3% Tween 20 and blocked for 30 min. As a secondary antibody, donkey anti-rabbit antibody conjugated with Cy3 was used (Jackson Laboratories), diluted 1:300 and incubated at room temperature for 2 h. The squashes were washed twice for 10 min each before mounting with Vectashield (Vector). In situ hybridization of whole testes from wild type and *Df(1)J5* (negative control) was done using a digoxigenin-labeled *TrxT* cDNA as probe, in principle according to Lehmann and Tautz (1994). Testis and ovary preparations were analyzed using phase contrast, Nomarski and fluorescence microscopy with a Zeiss Axiophot microscope equipped with a KAPPA DX30C CCD camera. Images were assembled, contrasted and merged electronically using Adobe Photoshop. The filter sets 31044v2 and 41028 (Chroma) were used to detect ECFP and EYFP, respectively.

Results

Cloning and characterization of *TrxT*

A partial *TrxT* cDNA was originally isolated in a two-hybrid screen for Zeste-interacting proteins (Chen 1992). The in vivo relevance of this interaction is at present not known. Screening of an embryonic cDNA library failed to isolate additional clones and we therefore used the expressed sequence tag (EST) clones GH21036 and GH14562, which are identical to the original *TrxT* cDNA, for further analysis. The sequence was used to search the Berkeley Genome Project database of ESTs and predicted genes. The *TrxT* gene is identical to gene CG3315 (predicted genes, release 3), is organized in three exons and encodes a 157 amino acid polypeptide (Fig. 1A, C). The genome sequence and in situ hybridization to polytene chromosomes showed that the *TrxT* gene was located on the *X* chromosome at 4F (results not shown). Analysis of the deduced amino acid sequence with conventional software tools predicts that the TrxT protein is likely a thioredoxin. Surprisingly, the *TrxT* gene is located 471 bp upstream of *deadhead*, also encoding a thioredoxin homolog (Salz et al. 1994). *TrxT* is most probably identical to the male-specific transcript next to *dhd* identified by Salz et al. (1994).

A ClustalW alignment of the three *D. melanogaster* thioredoxins, DHD, Trx-2 and TrxT, is shown in Fig. 1C. TrxT shows higher identity to Trx-2 (58% identity, amino acids 1–104) than to DHD (42%), suggesting a closer functional relation. However, it is also clear from the alignment that DHD is less related to Trx-2 than to TrxT (38% and 42% identity, respectively). At the nucleotide level the homology between the three genes is restricted to the coding region. In its dithiol form Trx-2 but not DHD is proposed to be the reducing substrate for thioredoxin peroxidase-1 (Bauer et al. 2002). Whether TrxT can act as a reducing substrate for thioredoxin peroxidase-1 has not yet been determined.

Loss of *dhd* function causes female sterility. Phenotypic analysis of two different mutations that eliminate function suggests that *dhd* is essential for female meiosis (Salz et al. 1994). To study the consequences of a lack of *TrxT* we used a deletion of *TrxT*, i.e., *Df(1)J5* , which completely uncovers the TrxT coding region (Fig. 1A). Since loss of *TrxT*, i.e., *Df(1)J5* , causes neither any male phenotype nor sterility, we tested *Df(1)J5* males for sperm competition. We failed to show any decreased competitive function of sperm from *Df(1)J5* males compared with sperm from wild type when a female is mated to two different males (results not shown). Neither does overexpression of TrxT by heatshock of a line containing the transgenic construct *P[yellow $^+$ hsp70:FLAG-TrxT]* cause any visible phenotype or affect fertility (results not shown).

Expression of *dhd* and *TrxT*

The two thioredoxin-encoding genes *TrxT* and *dhd* are divergently transcribed. Blots of poly(A)$^+$ RNA from different stages of development, hybridized with *TrxT* cDNA, revealed that the transcript has no maternal component, is not expressed in the embryo but becomes progressively more strongly expressed during larval and pupal development. In adults, the transcript is exclusively found in males (Fig. 2A). When RNA from sexed larvae and pupae was used, no transcript was detectable in females at these stages (Fig. 2B). In contrast, *dhd* is exclusively expressed in females (Figs. 2B, 3A). To test the control of the sex-specific expression of *TrxT* and *dhd*, we made a blot using RNA from males and females with different karyotypes regarding presence and number of *Y* chromosomes. As seen in Fig. 3A, the presence of a *Y* chromosome does not influence the expression of *TrxT* nor of *dhd*; the expression of both is strictly sex dependent and complementary. The arrangement of two homologous genes in opposite directions prompted us to look more into the regulation of *TrxT* and *dhd*. We therefore made blots using RNA from *Df(1)J5* individuals with or without a *P[w$^+$ snf$^+$ TrxT$^+$ dhd$^+$]* transgene (Larochelle et al.

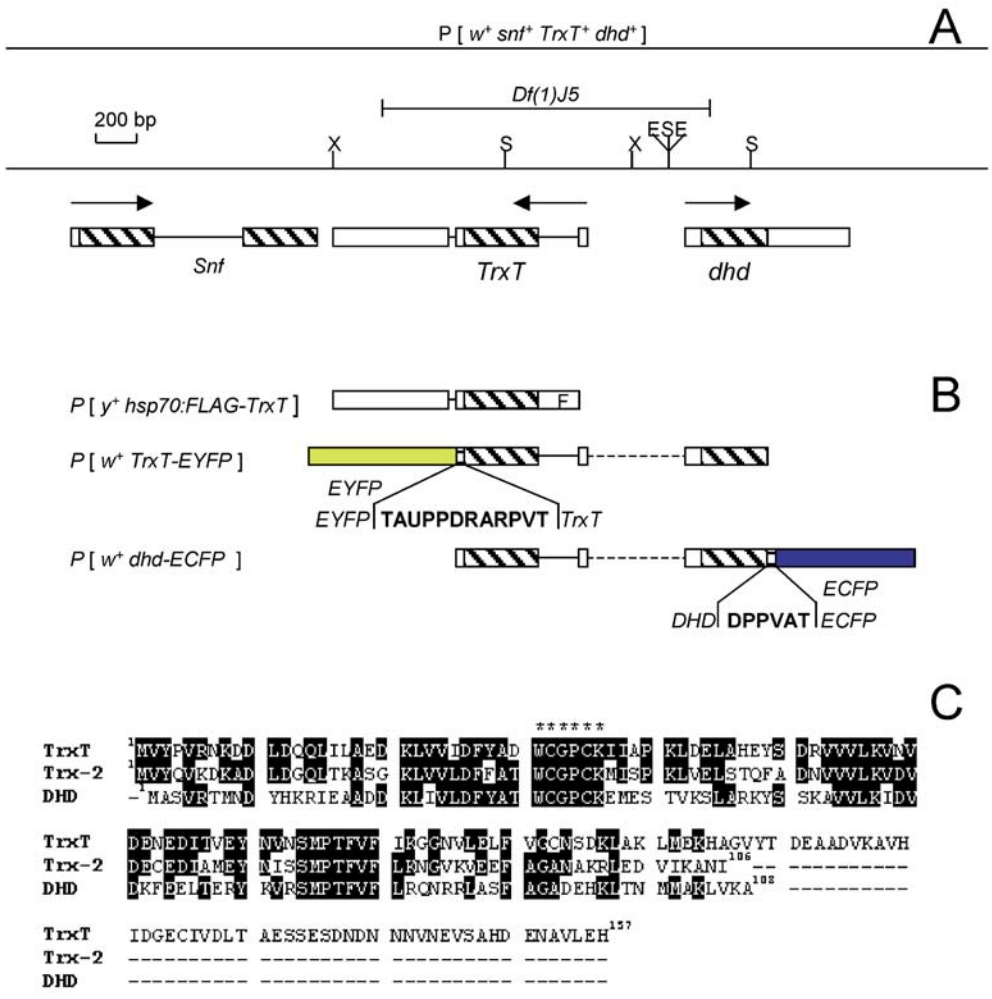

Fig. 1A–C Map of the *TrxT-dhd* genomic region. **A** The exon-intron structure of the *TrxT* and *dhd* genes is shown below the genomic DNA line. *Filled boxes* represent coding regions and *open boxes*, untranslated sequences. *Arrows* indicate direction of transcription. The lengths of the deletion *Df(1)J5* and of the genomic sequence in the transgenic construct *P[w+ snf+ TrxT+ dhd+]* are shown. (*E* EcoRI, *S* SphI, *X* XhoI) **B** Transgenic constructs used in this study. The amino acid sequences linking TrxT and DHD to EYFP and ECFP, respectively, derived from the cloning are shown. (*F* FLAG) **C** Multiple sequence alignment of DHD, Trx-2 and TrxT (Accession numbers P47938, AAF52794 and AJ507731, respectively). Identical amino acids are shown as *black boxes*. *Asterisk* indicates the active site motif WCGPC

1998). This transgenic construct consists of the genomic region including the *TrxT-dhd* region and an additional 2 kb and 1 kb downstream of *TrxT* and *dhd*, respectively. The results show that *Df(1)J5* is, as expected, a null mutation of both *dhd* and *TrxT* in females and males, respectively (Figs. 3B, 4A, lane 2). Furthermore the *P[w+ snf+ TrxT+ dhd+]* construct restores the correct expression pattern of *TrxT* and *dhd* both when located on the *X* chromosome (lane 4) and when inserted on to an autosome (lanes 5 and 6), suggesting that the regulatory region is within the gene complex and the endogenous *X* chromosome location of *TrxT-dhd* is not essential for transcription control.

Considering the male- and female-specific expression of *TrxT* and *dhd*, respectively, we were interested to test transcription control in relation to the sex-determination pathway (for review see Schütt and Nöthiger 2000). To test whether *TrxT* is under the control of *doublesex* we also tested for expression in tra^1/tra^1 pseudomales (*XX* individuals, phenotypically males), dsx^D/+ and dsx^1/dsx^{19} intersex individuals. The results show that neither *TrxT* nor *dhd* is expressed properly in tra^1/tra^1 and dsx^D /+ individuals. A weak *TrxT* transcript is detected in the tra^1/tra^1 pseudomales and a weak *dhd* transcript is detected in the dsx^D intersex individuals. This means either that *TrxT* control is not downstream of *tra* but through the MSL dosage compensating system or, more likely, that *TrxT* and *dhd* are expressed specifically in testes and ovaries, respectively. Testes are not properly developed in *tra* and *dsx* mutants and testes-specific expression of *TrxT* would therefore be lost in tra^1/tra^1 pseudomales and dsx^D/+ intersex individuals.

To study the regulation and the regulatory region of *TrxT* and *dhd* in more detail and also to determine the cellular localization of the protein products we made genomic constructs in which the *TrxT* genomic sequence was fused to the EYFP-encoding sequence and *dhd* to the ECFP-encoding sequence (Fig. 1B). This allows us to test whether the 471 bp between the genes is sufficient for correct regulation and also where in testes and ovaries the protein products are localized. RNA blot analysis confirms that *TrxT* and *dhd* fusion proteins are testis and ovary specific, respectively (Fig. 4A, B). Furthermore, the expression of the *TrxT-EYFP* and *dhd-ECFP* fusion transcripts phenocopies the expression of the endogenous transcripts. The truncated *TrxT* transcript seen in the *dhd-ECFP* transgenic line and the truncated *dhd* transcript seen in the *TrxT-EYFP* line are also expressed as the

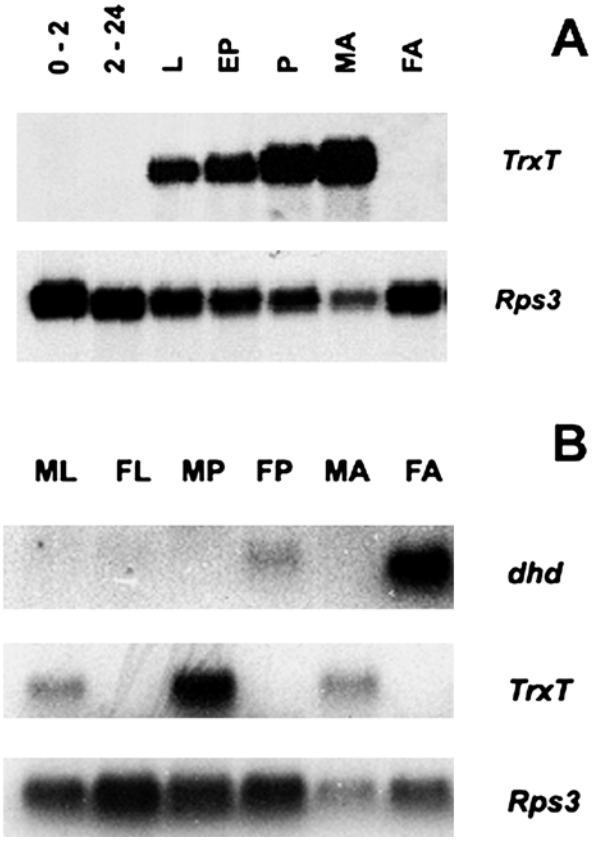

Fig. 2A, B Developmental RNA blot analysis. **A** The blot was hybridized with a *TrxT* cDNA probe and then reprobed with *RpS3* as a control for RNA loading. RNA samples are from 0–2 h and 2–20 h embryos (*0–2*, *2–24*); third instar larvae (*L*); early pupae (*EP*); late pupae (*P*); adult males (*MA*) and females (*FA*). **B** mRNA from third instar larvae, male (*ML*) and female (*FL*); pupae, male (*MP*) and female (*FP*); and adults were used and probed as in **A**. An additional reprobing to *dhd* is also shown

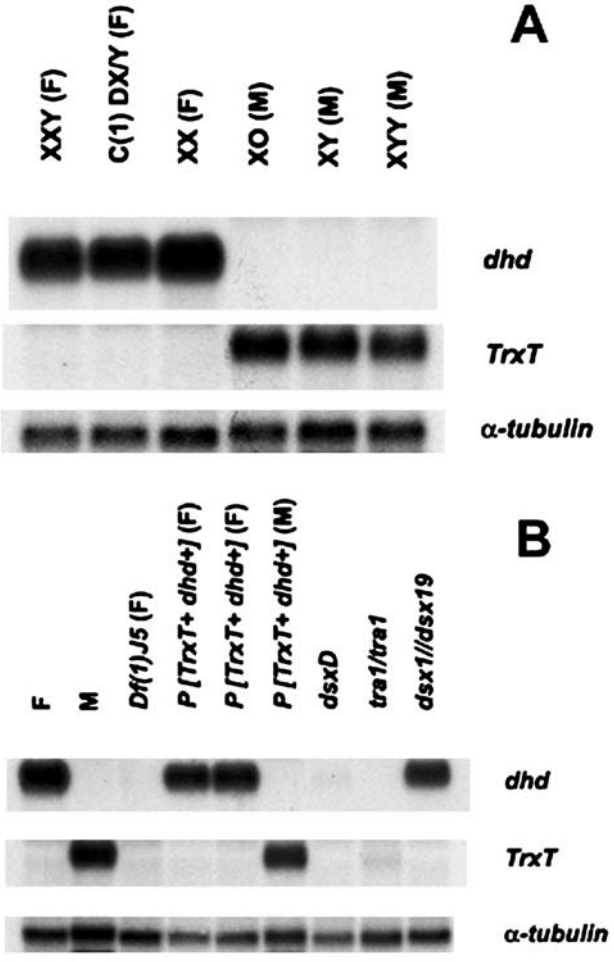

Fig. 3A, B RNA blot analysis. **A** Females with the karyotype *XXY*, *C(1)DX/Y* and *XX* were used together with *X/0*, *XY*, *XYY* males (*F* females, *M* males). **B** Expression of *dhd* is restored with a transgenic *dhd* copy on *X* (*lane 4*) and on an autosome (*lane 5*). *TrxT* expression is restored by an autosomal transgenic copy (*lane 6*). Small amounts of *dhd* and *TrxT* are seen in dsx^{D}/+ and tra^{1}/tra^{1} (*XX*) individuals, respectively. The weaker *dsx* transheterozygote dsx^{1}/dsx^{19} mutants express *dhd* at levels comparable to wild-type females. The filters were probed with *TrxT* followed by *dhd* and finally *α-tubulin* to control RNA loading

corresponding wild-type genes (Fig. 4A, lane 8; B, lane 1). This experiment confirms that the 471 bp separating *TrxT* and *dhd* together with the first intron of *TrxT* is sufficient for correct testis- and ovary-specific control and is not dependent on an *X* chromosome location. Whether the 1st intron of *TrxT* is needed for proper regulation has not been tested.

Localization of the TrxT and DHD proteins

To study the localization of TrxT and DHD proteins we established fly lines with the *TrxT-EYFP* and *dhd-ECFP* transgenes in a *TrxT*- and *dhd* -null background, i.e., *Df(1)J5; P[w+ TrxT-EYFP]* and *Df(1)J5; P[w+ dhd-ECFP]* (Fig. 1B). As shown in Fig. 5A, B the testis apical tip where the stem cell and the spermatogonial cells are located remains unstained. In young spermatocytes, a localized nuclear fluorescent signal is seen. In the postmeiotic spermatid stages, TrxT-EYFP is seen as distinct nuclear staining (Fig. 5E, F) until the nuclear elongation stage, when the staining becomes less intense (not shown). Strong staining is detected in the waste bag (not shown) in which material no longer needed for the mature sperm is eliminated (Tokuyasu et al. 1972; Fuller 1993). To confirm that TrxT-EYFP expression is really representative of endogenous expression we did an in situ hybridization using a digoxigenin-labeled *TrxT* probe against wild-type testes. The expression of *TrxT* is restricted to the spermatocytes; the stem cells and spermatogonial cells remain unstained, identical to what is seen with TrxT-EYFP (Fig. 6A).

During spermatogenesis the *Y* chromosome and other parts of the genome become activated in primary spermatocytes. This stage is also characterized by the

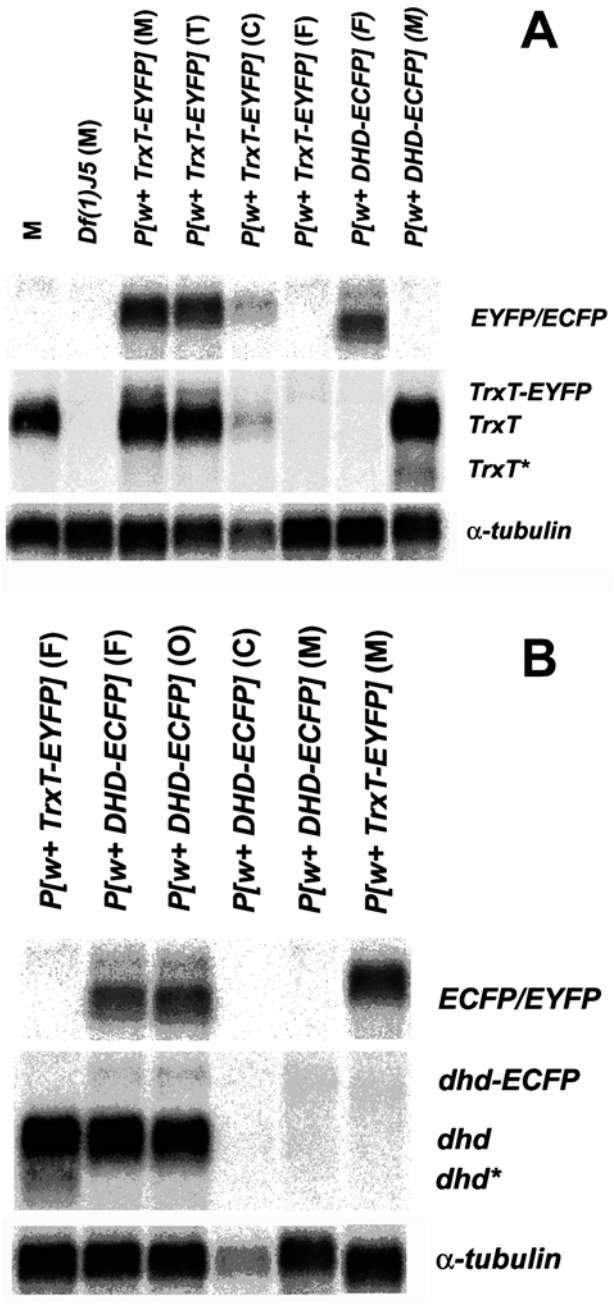

Fig. 4A, B RNA blot analysis of transgenic constructs probed with *EYFP/ECFP* (*top*) *TrxT* or *dhd* (*middle*) and *α-tubulin* (*bottom*). **A** The *TrxT* and *TrxT-EYFP* transcripts are both expressed specifically in testes and no expression is seen in females. The weak band seen in *lane 5* is probably due to testis tissue remaining in the carcasses. *Lane 2* confirms that the *Df(1)J5* deletion causes a null mutation in *TrxT*. (*M* males, *T* testes, *C* carcasses from dissection of testes, *F* females). Note the male-specific truncated *TrxT* transcript (*TrxT**) from the *P[w*$^{+}$ *dhd-ECFP]* construct, *lanes 7*, *8*. **B** The *dhd* and *dhd-ECFP* transcripts are expressed ovary specifically. (*O* ovaries, *C* carcasses from dissection of ovaries). Note the female-specific truncated *dhd* transcript (*dhd**) from the *P[w*$^{+}$ *TrxT-EYFP]* construct (*lanes 1, 6*)

first appearance of *Y* chromosome loops. In *D. melanogaster* there are three lampbrush-like loops, formed by the *kl-5, kl-3* and *ks-1* fertility factors, with *kl-5* and *ks-1* appearing earlier than the *kl-3* loop (Bonaccorsi et al. 1988). The TrxT-EYFP staining in primary spermatocytes strongly suggests association with the lampbrush loops (Fig. 5C, D). To test this potential association, males without *Y* chromosomes were constructed by crossing *Df(1)J5; P[w*$^{+}$ *TrxT-EYFP]* males to *C(1)RM/0* females and preparing testes from the *Df(1)J5/0; P[w*$^{+}$ *TrxT-EYFP]/+* male offspring. A whole mount of testes from *X/0* males is shown in Fig. 5G, H. Note the difference in the primary spermatocytes compared with *X/Y* males (Fig. 5A, B). The lampbrush-like association of TrxT-EYFP seen in *Df(1)J5/Y* males is not seen in *Df(1)J5/0* males lacking the *Y* chromosome (Fig. 5I, J), suggesting that TrxT-EYFP associates with the *Y* chromosome lampbrush loops. However, localized but weaker nuclear staining is seen also in *Df(1)J5/0* primary spermatocytes. The localization is spherical at first and, by comparing the phase contrast image with the fluorescent image, it seems to be localized to the nucleolus (Fig. 5I, J). The postmeiotic association of TrxT-EYFP with the spermatid nuclei (Fig. 5E, F) is seen also in males lacking the *Y* chromosome (not shown). An examination of Fig. 5C, D reveals that not all thread-like material within the primary spermatocyte nuclei is fluorescent, suggesting that one of the *Y* loops is not labeled. To determine which loops TrxT-EYFP decorates we crossed *Df(1)J5; P[w*$^{+}$ *TrxT-EYFP]* females to males with *Y* chromosome rearrangements (see Materials and methods). On *Y* chromosomes Δ *kl-5 kl-3* and Δ *ks-1*, labeled loop material is significantly reduced. Furthermore, in Δ *kl-5 kl-3* and Δ *kl-3*, no unlabeled loop material is seen in contrast to Δ *ks-1* (results not shown). These results suggest that the unlabeled material seen in Fig. 5C, D originates from the *kl-3* loop and that both the *ks-1* and *kl-5* loops are labeled. Examination of the Δ*kl-3 Y* chromosome confirms this (Fig. 5K, L). In these spermatocyte nuclei the amount of labeled material is similar to wild type (Fig. 5D); however, in contrast to the wild-type *Y* chromosome only small amounts of unlabeled thread-like material are detected.

Mutations in the fertility factor genes cause male sterility. The spermatids will undergo the elongation process but the sperms degenerate before maturation (Lindsley and Tokuyasu 1980). However, *Df(1)J5* males are fertile, suggesting that TrxT is redundant for male fertility. We were interested to test whether another thioredoxin, Trx-2 (Bauer et al. 2002), may substitute for TrxT in spermatocytes. Staining of testes squashes with anti-Trx-2 shows that Trx-2 is indeed expressed in primary spermatocytes and localized to the nuclei (Fig. 6B). Although it is not clear whether Trx-2 is associated with *Y* loops, this result makes it possible that Trx-2 performs the TrxT role in *TrxT* mutants.

The female-specific thioredoxin gene *dhd* is expressed exclusively in the germline in a fashion similar to *TrxT*. We used the *Df(1)J5; P[w*$^{+}$ *dhd-ECFP]* line to determine

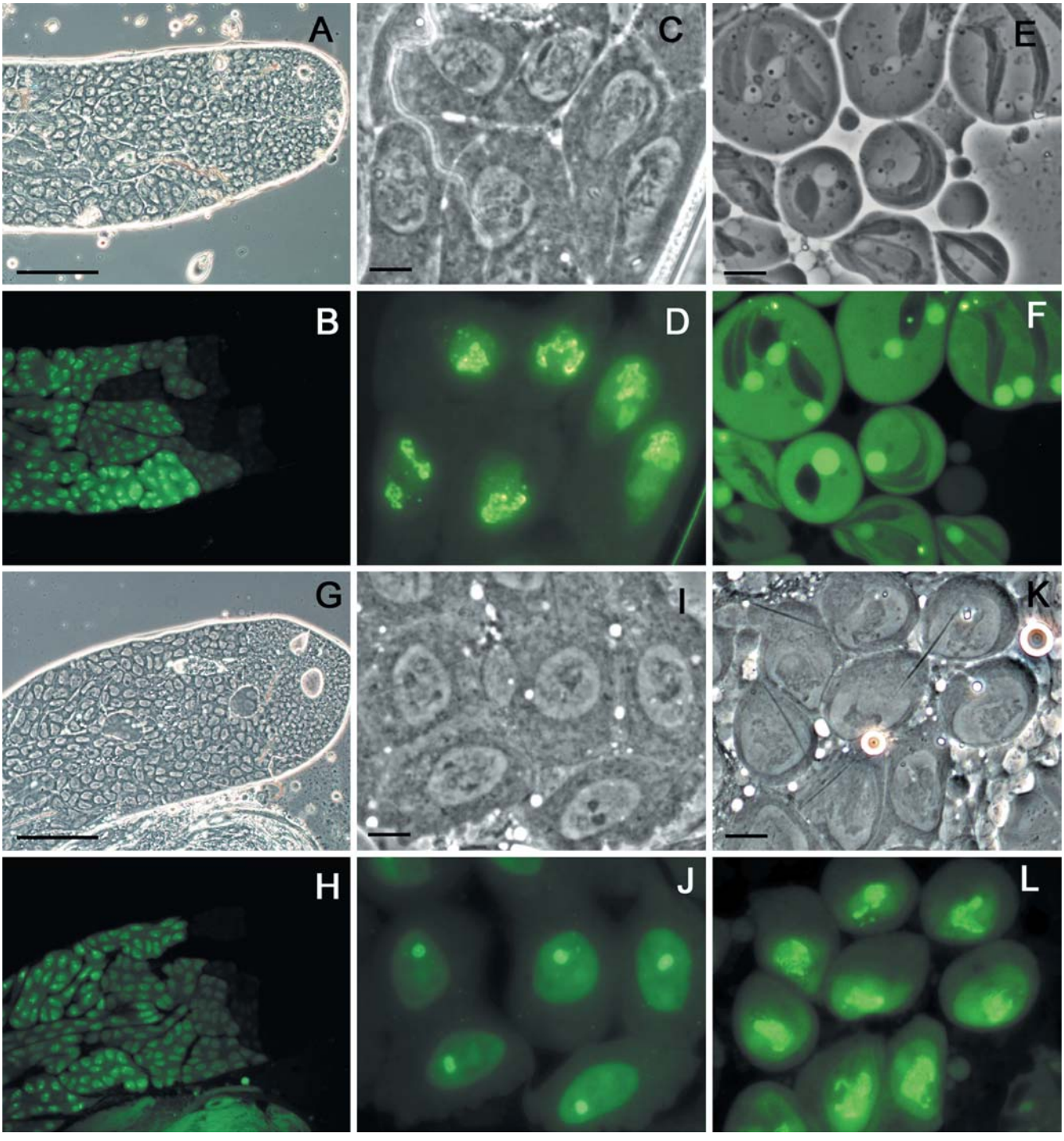

Fig. 5A–L Expression of TrxT-EYFP in unfixed adult testis preparations. Phase contrast (first and third row) and fluorescent (2nd and 4th) images from *Df(1)J5/Y; P[w+ TrxT-EYFP]/+* males (**A–F**), from *Df(1)J5/0; P[w$^+$ TrxT-EYFP]/+* males (**G–J**) and from *Df(1)J5/ In(Y)S16*, Δ*kl-3; P[w$^+$ TrxT-EYFP]/+* (**K, L**). **A, B** Whole mount testes. No fluorescence is seen in the germ cell nuclei and in the spermatogonial cell while a prominent structured fluorescence is seen in primary spermatocytes. **C, D** In primary spermatocytes, significant amounts of TrxT-EYFP localize to nuclei and bind to *Y* chromosome loops. Note that not all thread-like structures seen in phase contrast are fluorescent. **E, F** In postmeiotic spermatids, TrxT-EYFP is localized to nuclei. **G, H** Whole mount testes from *Df(1)J5/0; P[w$^+$ TrxT-EYFP]/+* male. **I, J** In primary spermatocytes from *X/0* males TrxT-EYFP associates with the nucleolus. **K, L** In *Df(1)J5/ In(Y)S16*, Δ*kl-3; P[w$^+$ TrxT-EYFP]/+* males almost all thread-like structures seen in phase contrast are fluorescent. *Bars* represent 50 μm (**A, G**), 10 μm (**C, E, I, K**)

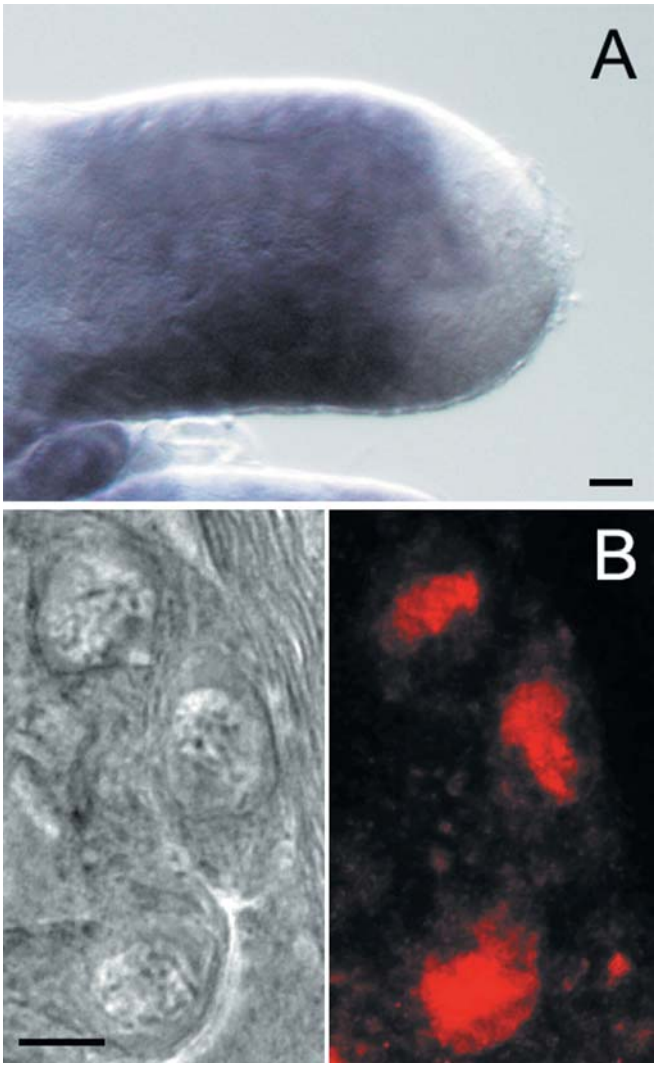

Fig. 6A, B In situ hybridization to whole testes using *TrxT* as probe (**A**). The apical tip with stem cell and spermatogonial cells remains unstained while strong expression is seen in spermatocytes. **B** Immunostaining of testes squashes shows Trx-2 protein localization in primary spermatocytes nuclei in red (*right panel*) and phase contrast (*left panel*). *Bars* represent 50 μm (**A**), 10 μm (**B**)

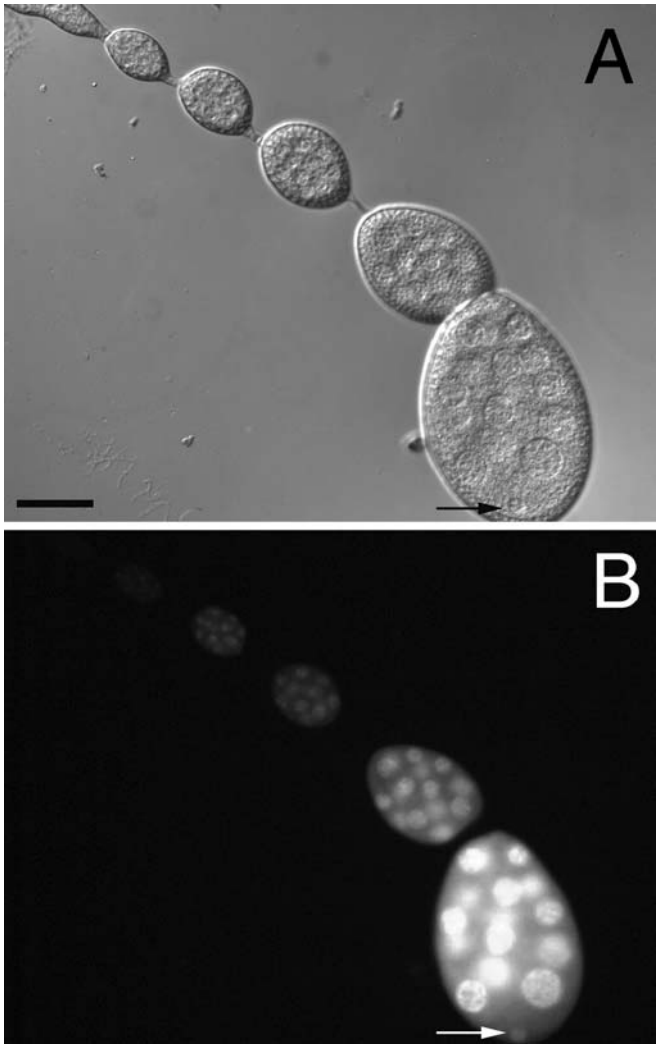

Fig. 7A, B Expression of DHD-ECFP in ovaries. The *arrows* indicate the germinal vesicle. **A** Nomarski image of an ovariole from germarium to stage S8 in oogenesis. **B** CFP fluorescence visualizes DHD localization. *Bar* represents 50 μm

this expression and the cellular localization of the DHD protein in more detail. The *P[w$^+$ dhd-ECFP]* construct does not rescue *Df(1)J5* female sterility, suggesting that the DHD-ECFP fusion protein is not fully functional. Using in situ hybridization, Salz et al. (1994) showed that *dhd* mRNA is present in the nurse cells from stage 9 of ovary development and is transported into the oocyte. Our results show that the DHD-ECFP protein is present in the nurse cells from stage S3 and throughout oogenesis (Fig. 7A, B). Furthermore, the protein is localized in the nucleus like TrxT despite the computer-predicted cytoplasmic localisation (PSORT). The DHD-ECFP protein is also detected in the germinal vesicle, i.e., the oocyte nucleus. No staining is seen in the follicle cells.

Discussion

We have identified and characterized a third *D. melanogaster* thioredoxin-encoding gene, *TrxT*. Surprisingly, *TrxT* is located 471 bp upstream of *dhd*, a female-specific thioredoxin-encoding gene. The two genes are divergently transcribed and our results show that the 471 bp separating the two genes together with the first intron in *TrxT* are sufficient for correct transcriptional regulation. Furthermore, correct expression is not dependent on an *X*-chromosomal location. This gene pair might make a good model for unraveling how closely spaced promoters are differentially regulated by a short (<500 bp) common control region. It is unclear how the gene pair has evolved. The fact that *dhd* is intronless may suggest a retrotransposition event. We find no imperfect poly(A) tail in the *dhd* sequence that would support such an hypothesis. However, there are no clear homologies

outside the coding sequences that would suggest a gene duplication event. The TrxT protein is more similar to the Trx-2 protein than to DHD. Bauer et al. (2002) have shown a functional difference between DHD and Trx-2 in that Trx-2 can be used as a reducing substrate for thioredoxin peroxidase-1. The sequence similarity of TrxT to Trx-2 suggests that it is functionally different from DHD, but this remains to be determined.

The fluorescence in the TrxT-EYFP-expressing line indicates an association of TrxT with the *Y* chromosome loops. These Y chromosome lampbrush-like loops unfold in young spermatocytes, grow throughout development and, as the chromosomes begin to condense prior to metaphase I, they disintegrate (Bonaccorsi et al. 1988). The results from the *Y* chromosome rearrangements show that the *ks-1* and *kl-5* loops are decorated by TrxT-EYFP while at least most of the late appearing loop *kl-3* remains unstained.

Several proteins have previously been shown to be associated with the *Y* chromosome or the *Y* chromosome loops in spermatocytes, e.g., dMBD2/3 a *Drosophila* homolog of the mammalian methyl-DNA binding proteins MBD2 and MBD3. dMBD2/3 forms distinct foci that disappear for the most part in *X/0* males, suggesting that they form on the activated *Y* chromosome (Marhold et al. 2002). Since dMBD2/3 has been shown to mediate transcriptional repression in tissue culture and is suggested to be a component of a *Drosophila* NuRD-like complex (Roder et al. 2000; Ballestar et al. 2001), it was proposed that the function of dMBD2/3 is to keep certain genes on the *Y* chromosome silenced during genome activation in the spermatocyte stage (Marhold et al. 2002). The putative RNA binding protein RB97D has been shown to form an RNase-sensitive association with the C-loop from the *ks-1* fertility locus (Heatwole and Haynes 1996). In contrast to TrxT, RB97D is expressed not only in testes but also at low levels during most of development. Furthermore, in the absence of *Y*, i.e., in *X/0* males, RB97D staining is diffuse in the whole nucleus while at least some of the TrxT protein is found associated with other structures. Another RNA binding protein, BOULE, also associates with the C-loop from the *ks-1* fertility factor (Cheng et al. 1998). Loss of function of *boule* has a meiotic arrest phenotype, resulting in male sterility (Castrillon et al. 1993; Eberhart et al. 1996). It has been proposed that the repetitive sequences within the loops serve as protein binding sites and thereby activate or sequester proteins required at later stages of spermatogenesis (for reviews see Hennig et al. 1989; Hackstein and Hochstenbach 1995). In line with this proposal, Cheng et al. (1998) suggested that the *ks-1* loop in this case may bind BOULE protein to be synchronously released together with other *Y* -loop bound factors as the *Y* chromosome loops disintegrate at the end of the spermatocyte growth phase. It is possible, although unlikely, that the association of TrxT with the lampbrush loops serves this function. First, in contrast to BOULE, the TrxT protein is nuclear even in the absence of *ks* and *kl* regions. Secondly, the female germline-specific DHD protein as well as the more general thioredoxin Trx-2 are also seen in the nuclei, suggesting a more general role for thioredoxin proteins in the nuclei of germline cells. Third, TrxT is not seen during meiosis but reappears as a bright signal in the postmeiotic spermatid nuclei. It is interesting to note that spermatid-specific thioredoxins have also been identified in human and mouse (Miranda-Vizuete et al. 2001; Sadek et al. 2001; Jimenez et al. 2002). In contrast to the *Drosophila* TrxT, the mammalian spermatid thioredoxins are present also in mature sperm and are mostly localized to the tail structures. At earlier spermatogonial and spermatid stages, mammalian Sptrx is not expressed (Jimenez et al. 2002).

Our results suggest that TrxT is redundant for male fertility, i.e., in laboratory conditions we fail to detect a fertility defect in *TrxT* mutant males. Redundancy is very common among thioredoxins (for reviews see Åslund and Beckwith 1999; Carmel-Harel and Storz 2000). Single mutants in the two *Saccharomyces cerevisiae* thioredoxin-encoding genes *trx1* and *trx2* have wild-type growth rate and cell morphology while *trx1 trx2* double mutants are auxotrophic for methionine, have decreased rates of DNA replication and a subsequent increase in cell size and generation time (Muller 1991). However, in certain physiological stress situations the redundancy is lost, e.g., the *trx2* single mutant is extremely sensitive to H_2O_2 (Muller 1991). Similar examples have been shown in *Escherichia coli.* Although strains carrying individual mutations in the *trxA*, *trxB* and *trxC* genes are viable and thus considered redundant, *trxA* mutants are extremely sensitive to H_2O_2 in both the exponential and stationary growth phase, and *trxB* mutants are sensitive in stationary phase (Takemoto et al. 1998; Ritz et al. 2000). Our observation that Trx-2 is localized to the nuclei in primary spermatocytes indicates that Trx-2 may fulfill the TrxT function in *TrxT* mutant males. However, it is also possible that, in line with the *E. coli* and *S. cerevisiae* systems, *TrxT* is essential, but only in certain physiological conditions, e.g., oxidative stress.

We speculate that TrxT may function as a chromatin-modulating protein. The *Y* chromosome is distinctive in being entirely heterochromatic and required only for one specific developmental pathway, i.e., spermatogenesis. The activation of the *Y* chromosome fertility factor genes is therefore highly specific and critical. Although TrxT is redundant for male fertility it may function to facilitate the activation of the *Y* chromosome in the germline. This could occur through a redox reaction that controls the activation status of a second protein or through the formation of a complex containing TrxT. In the postmeiotic spermatid nuclei, TrxT may have a similar function in preparing the chromatin for the replacement of histones with protamines that occurs during elongation of the nuclei. However, the fact that DHD protein is localized in the oocyte nucleus may suggest a more general role for thioredoxin in germ cell nuclei. Although the intracellular localization differs between TrxT and DHD on one hand and mammalian Sptrx on the other, it should be noted that

the only tissue-specific thioredoxins so far isolated in *Drosophila* and human are specific for the germline.

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