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Expression pattern of Drosophila translin and behavioral analyses of the mutant

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Abstract

Translin is an evolutionarily conserved ~27-kDa protein that binds to specific DNA and RNA sequences and has diverse cellular functions. Here, we report the cloning and characterization of the translin orthologue from the fruit fly Drosophila melanogaster. Under protein-denaturing conditions, purified Drosophila translin exists as a mixture of dimers and monomers just like human translin. In contrast to human translin, the Drosophila translin dimers do not appear to be stabilized by disulfide interactions. Drosophila translin shows a ubiquitous cytoplasmic localization in early embryonal syncytial stage, with an enhanced staining in ventral neuroblasts at later stages (8–9), which are probably at metaphase. An elevated expression was seen in several other cell types, such as cells around the tracheal pits in the embryo and oenocytes in the third instar larva. RNA in situ hybridization showed an increased expression in the ventral midline cells of the larval brain, suggesting a neuronal expression, which was corroborated by protein immunostaining. In adult flies, Drosophila translin is localized in the brain neuronal cell bodies and in early spermatocytes. Interestingly, Drosophila translin mutants exhibit an impaired motor response which is sex specific. Taken together, the multiple cellular localizations, the high neuronal expression and the attendant locomotor defect of the Drosophila translin mutant suggest that Drosophila translin may have roles in neuronal development and behavior analogous to that of mouse translin.
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Keywords: Drosophila; Translin; TB-RBP; Locomotor defect; Neuronal expression; Mutant

Introduction

Translin is an evolutionarily conserved ~27-kDa protein that binds to specific DNA and RNA sequences and has diverse cellular roles. Translin was first identified from extracts of lymphoid cell lines by its ability to bind single-stranded oligonucleotides derived from consensus sequences at chromosomal breakpoint

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junctions associated with leukemia and lymphomas (Aoki et al., 1995). Mouse translin (testis brain RNA-binding protein, TB-RBP) also regulates the expression of a variety of RNA sequences enriched in the brain and the testes, indicative of a role in translational regulation (Han et al., 1995). In neuronal dendrites, translin associates with BC1 ribonucleoprotein particles, and has been proposed to regulate dendritic translation of mRNAs in response to trans-synaptic activity (Kobayashi et al., 1998). Further, TB-RBP has also been implicated in intracellular and intercellular mRNA transport and binds to meiotic and non-coding mRNA in stages of mouse spermatogenesis (Morales et al., 1998, 2002; Cho et al., 2005). Biochemical studies have shown that human translin exists as a stable octamer in its native state and binds DNA with high affinity through its ends (Kasai et al., 1997; Aoki et al., 1999; VanLoock et al., 2001; Sengupta and Rao, 2002). These evidences suggest a role for translin in a variety of cellular processes such as DNA damage response, RNA transport and translational regulation.

Yeast two-hybrid analysis revealed translin associated factor-X (TRAX), a 33-kDa protein as translin interactor that enhances the DNA-binding affinity of translin and decreases its RNA-binding affinity (Aoki et al., 1997; Chennathukuzhi et al., 2001a). Interestingly, TRAX with a nuclear localization signal is proposed to mediate translin recruitment to the nucleus. Translin on the other hand has a nuclear export signal that is required for its exit from the nucleus (Aoki et al., 1997; Chennathukuzhi et al., 2001a; Cho et al., 2004). Translin interacts with microtubules and is associated with the centrosome in Xenopus (Wu et al., 1999; Castro et al., 2000). It has also been implicated in cell division; the levels of TB-RBP and TRAX closely parallel the proliferative state in various cell types (Ishida et al., 2002; Yang et al., 2004). Recently, it has been shown that Drosophila TRAX is stabilized by translin (Claussen et al., 2006). TB-RBP knock-out mice show spermatogenic and behavioral defects as well as an altered gene expression profile. In addition, these mice also show sex-specific differences in learning and memory tests, locomotor activity, anxiety-related behavior, and sensorimotor gating, handling-induced seizures, and alterations in monoamine neurotransmitter levels in several forebrain regions (Chennathukuzhi et al., 2003a; Stein et al., 2006).

Translin-like proteins exist in mouse, chicken, Xenopus, Drosophila, and the fission yeast Schizosaccharomyces pombe and share 99, 86, 81, 52, and 36% identities, respectively, with human translin (Wu et al., 1997; Aoki et al., 1999; Castro et al., 2000; Laufman et al., 2005; Claussen et al., 2006). However, the budding yeast Saccharomyces cerevisiae does not have a translin ortholog. The Drosophila melanogaster genome has a single putative TRANSLIN and TRAX gene in the annotated sequence (http://www.fruitfly.org/blast/). Comparative studies of human and Drosophila translin have been carried out in our laboratory (Sengupta et al., 2006). Besides a difference in their oligomerization status, human translin bound to nucleic acids whereas Drosophila translin did not. We were the first to clone and over-express Drosophila translin (Sengupta et al., 2006; GenBank accession no. AY392453) and here we have further characterized the gene, as well as the tissue distribution and the in vivo role of the protein.

Materials and methods

Materials and Drosophila stocks

All chemicals and enzymes were obtained from Sigma-Aldrich (USA), GE (USA) or Roche (Switzerland). DNA primers were custom-synthesized by MWG (India). Anti-Drosophila translin antibody was raised by Raj Biotech (India). The fluorochrome-coupled secondary antibodies were obtained from Molecular Probes (USA). The Canton-Special strain was used as wild type. The Drosophila translin (y^1 w^{67c23}; P{EPgy2}translin^{EY06981}) mutant was obtained from Bloomington stock center (Bloomington, USA) (Bellen et al., 2004). The heterozygote was made by crossing the Canton-Special wild type with the Drosophila translin mutant.

Cloning, expression and purification

The primers 5′CGCGGATCCATGTCGAACTTCGTGAACTT3′ and 5′CCCAAGCTTCTAT TCGGTTGCAGGAACAGC3′ were used to amplify the putative Drosophila translin-encoding sequence using cDNA synthesized from whole-fly mRNA. The BamHI- and HindIII-digested PCR product was cloned into His-tagged expression vector pQE30 and transformed into Escherichia coli XL1Blue competent cells. Protein expression was induced in the transformed clones with 2.0 mM IPTG, and the protein was purified as described for human translin (Sengupta and Rao, 2002). Clones were sequenced using the above-mentioned PCR primers (Genbank accession no.: AY392453) (data not shown). PCR amplification of the Drosophila translin mutant sequence was performed with primers F2 (5′CACCCAAGGCTCTGCTCCCACAAT3′), R1 (5′CCCAAGCTTCTATTCGGTTGCAGGAACAGC3′), F1 (5′CGCGGATCCATGTCGAACTTCGTGAACTT 3′), and R2 (5′CAATCATATCGCTGTCTCACTCA 3′). The genomic DNA used as a template was from homozygous Canton-Special wild type (+/+) or from the Drosophila translin mutant (−/−).

MALDI-TOF analysis

Drosophila and human translin (~15 pmole) were diluted in 10 mM Tris–HCl (pH 7.5), mixed with sinnapinic acid matrix and spotted on a stainless steel MALDI-TOF plate (target plate). After drying at room temperature (RT), the plate was loaded on a MALDI-TOF 2E series of Micromass for obtaining spectra, which was then subjected to analysis (with trypsinogen and bovine serum albumin as external calibration) using MASSLYNX program.

Western blot analysis and immunoprecipitation

About 50 flies were homogenized in 500 μl lysis buffer (20 mM HEPES (pH 7.4), 40 mM KCl, 5 mM $MgSO_4$, 10 mM EGTA, and 500 mM DTT, with protease inhibitors (10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml PMSF)) at 4 °C. The crude extract was centrifuged for 30 min at 16,750g at 4 °C, and the supernatant was used for immunoprecipitation (1 mg of total protein) and Western blot analyses (500 μg of total protein per lane). For an embryo protein extract, the embryos after 4 h of growth were de-chorionated in 50% sodium hypochlorite, washed thoroughly and processed using the same protocol that was followed for the adult flies. For immunoprecipitation, the protein extract was added to 100 μl of protein A Sepharose beads (pre-treated either with anti-Drosophila translin polyclonal antibody (150 μg) raised in rabbit or with pre-immune serum (150 μg)) at 4 °C for 60 min. The coupled antibody was incubated with the protein extract for 60 min at 4 °C and washed thoroughly with lysis buffer. The immuno-precipitate was pelleted by centrifugation and re-suspended in 20 μl buffer for Western blot analysis. Blots were probed with anti-Drosophila translin polyclonal antibody raised in rabbit (1:5000) or mouse monoclonal anti-Drosophila tubulin antibody (1:100). Detection was performed using an enhanced chemiluminescence kit (Amersham Pharmacia).

Drosophila embryo collection and immunostaining

Wild-type embryos were aged at 25 °C and collected by standard methods (Sullivan et al., 2000). They were fixed in 4% formaldehyde in PEM (100 mM PIPES, 1 mM EGTA, 2 mM $MgSO_4$, pH 6.9) saturated with heptane for 20 min at RT. The fixative was discarded and de-vitellinization was carried out in 2.0 ml chilled methanol. Embryos were re-hydrated and probed with rabbit anti-Drosophila translin (1:500). For double labeling, the embryos were probed with anti-Drosophila translin and monoclonal mouse anti-22C10 (1:10). DNA was visualized using propidium iodide. Third instar larvae were dissected to expose the larval body wall and fixed in 3.5% formaldehyde for 20 min at RT, and immunolabeled with anti-Drosophila translin and anti-endophilin (1:50) (Rikhy et al., 2002). Adult brains were dissected in phosphate-buffered saline (PBS) and fixed in 37% formaldehyde for 20 min at RT. The preparation was re-hydrated and probed with the anti-Drosophila translin polyclonal antibody and anti-Drosophila nc82 antibody (1:100). All the reactions were visualized using fluorochrome-coupled secondary antibodies and imaged on a confocal microscope (Biorad Radiance 2000), acquired on Laser Sharp 2000 and processed with Adobe Photoshop 6.0.

RNA in situ hybridization

RNA in situ hybridization was performed using standard protocols (Sullivan et al., 2000). The probe was labeled with digoxigenin UTP (Roche) by in vitro transcription from pBSKS using T3 and T7 polymerase, respectively. Third instar larvae were dissected in PBS on ice and fixed in 4% formaldehyde in PBS for 20 min at RT, washed and processed for in situ hybridization. Bound probe was visualized using anti-DIG and alkaline phosphatase-conjugated secondary antibodies with NBT-X-phosphate as substrate. The reaction was terminated by washing with PBS containing 0.1% Tween-20 (PBT) and visualized under a bright-field microscope.

Immunostaining of Drosophila testes

Preparation of testes for immunostaining was done as described by Ghosh-Roy et al. (2004). Briefly, samples were dissected in dissection buffer (50 mM phosphate, pH 6.7, 80 mM KCl, 16 mM NaCl, 5 mM $MgCl_2$) and fixed with 95% ethanol (pre-chilled at −20 °C) for 5 min followed by 4% formaldehyde in PBS for 20 min at RT. The apical end of the testes was pierced with a needle (squashed) to release the cysts onto a slide. Slides were frozen in liquid nitrogen and then transferred to chilled ethanol for 5 min. The slide was again fixed in 4% formaldehyde (prepared in PBS or PBT) for 20 min at RT and washed thrice for 10 min in PBT. Specimens were incubated in primary antibody at a dilution of 1:400 for 1 h at RT and washed 4 times with PBT followed by incubation with secondary antibody for 1 h. After washing again with PBT, slides were mounted under a coverslip with antifade medium (Vectashield). Images were acquired using an Apotome microscope and were processed using Adobe Photoshop.

Walking behavioral response by geotactic and phototactic test

The geotactic test was performed using a counter current apparatus as described (Benzer, 1967). Approximately 20–25 flies (2–3 days old) were loaded in the first tube. Flies were allowed to walk against the gravity for 7 s and then shuffled. The process was repeated giving each fly at least 5 chances to cross from one tube to another. The geotactic index was calculated using the formula $\Sigma(T-1)n/5N$, where $T =$ tube number, $n =$ number of flies in that particular tube and $N =$ total number of flies. For phototactic response, a 7-s pulse of light was given while the flies walked against the gravity and the phototactic index was calculated.

Behavioral response by Jump and Y-maze test

The ability of adult flies to respond towards various odors was tested using the Jump test (Ayyub et al., 1990). A set of 5 flies (2–3 days old) was exposed to benzaldehyde for 5 s and then tested for their behavior by scoring for the jumping event. Typically, each time a group of 4 sets of 5 flies each were tested and the percentage response calculated from the group score. The response index is determined from the sum of ten groups. Conversely, a Y-maze test was performed as described (Ayyub et al., 1990). About 100 flies (2–6 days old) were introduced into the start tube. Diluted benzaldehyde ($10^{-3.5}$ with water) was puffed for 1 min after which the total number of flies that moved into the odor tube versus the total number of flies migrating to the control tube (double-distilled water) was counted. Each batch of flies was tested four times and the response index calculated from the sum of four trials.

Results

Cloning and expression of Drosophila translin

A sequence that putatively encodes Drosophila translin (52% sequence identity with human translin) was identified in the annotated Drosophila genome. The gene has 5 exons as predicted by the Berkeley Drosophila Genome Project (BDGP) (Fig. 1A). Using gene-specific primers (F1 and R1, Fig. 1A) and an RT-PCR strategy, we amplified the cDNA from the adult fly. The Drosophila genomic DNA yielded a 944-bp PCR product whereas amplification of Drosophila cDNA yielded a 703-bp PCR product (data not shown). The partial sequence of the amplified PCR product was 100% identical to the sequence annotated by BDGP [Genbank accession no.: AY392453]. Drosophila translin was expressed from a His-tagged clone by IPTG induction, followed by protein purification as described earlier (Sengupta and Rao, 2002) (Fig. 1B).

In silico translation of this gene yielded a protein with 235 amino acid residues. Comparison with the primary sequence from various organisms using ClustalW (http://www.ch.embnet.org/software/ClustalW.html, Fig. 1C) revealed that Drosophila translin shares homology with human translin and bears two putative RNA and RNA/DNA-binding domains towards the N-terminus, and a conserved C-terminal hydrophobic region (Fig. 1C). Human translin or TB-RBP have two basic regions KRCLKAREH and RFHEH-WRFVLQR implicated in nucleic acid binding (Aoki et al., 1995, 1999; Pascal et al., 2002; Sugiura et al., 2004; Claussen et al., 2006). In Drosophila translin the corresponding regions are AACGLARKQ and RYSDHWTFITRQ. The C-terminal end of human translin has a putative GTP-binding motif – VTAGD, while in Drosophila translin it is VTMGD (Chennathukuzhi et al., 2001b). However, the C-terminal end is largely conserved in both Drosophila translin and human translin. The conserved cysteine residue at the 225th position which forms a disulfide linkage in the oligomer is replaced by alanine in Drosophila translin.

Having established the identity of the Drosophila TRANSLIN gene, we used biochemical and biophysical assays to compare its oligomerization properties with human translin (Sengupta et al., 2006). We had shown earlier that while human translin exists as a stable octamer in its native state, whereas in Drosophila translin it is perhaps a hexamer/tetramer. Analysis of Drosophila translin by SDS-PAGE under non-reducing conditions showed two bands, a predominant faster migrating band corresponding to a monomer of ~28 kDa and a faint dimer of ~57 kDa (Fig. 2B). Under similar conditions, human translin showed a predominant stable dimer of ~54 kDa and hardly any monomer (Fig. 2D). Purified human and Drosophila translin were then analyzed by MALDI-TOF (Fig. 2A and C). Drosophila translin showed a peak of ~28562 Da corresponding to a monomer, and another peak of ~57076 Da corresponding to a dimer of lower relative abundance (Fig. 2A). In contrast, under the same conditions, human translin showed peaks of comparable relative abundance of the monomer as well as the dimer (Fig. 2C). Qualitatively, the results obtained from SDS-PAGE and MALDI-TOF analyses were consistent with each other, revealing differences in the stability levels of dimers between these proteins, where the Drosophila protein dimer, unlike that of human protein, does not appear to be stabilized by disulfide interactions. Taken together, these studies suggest both Drosophila and human translin are capable of existing in part as a dimer, the latter at relatively higher level than the former, perhaps reflecting cystein (225th position) disulfide bridge involvement. The

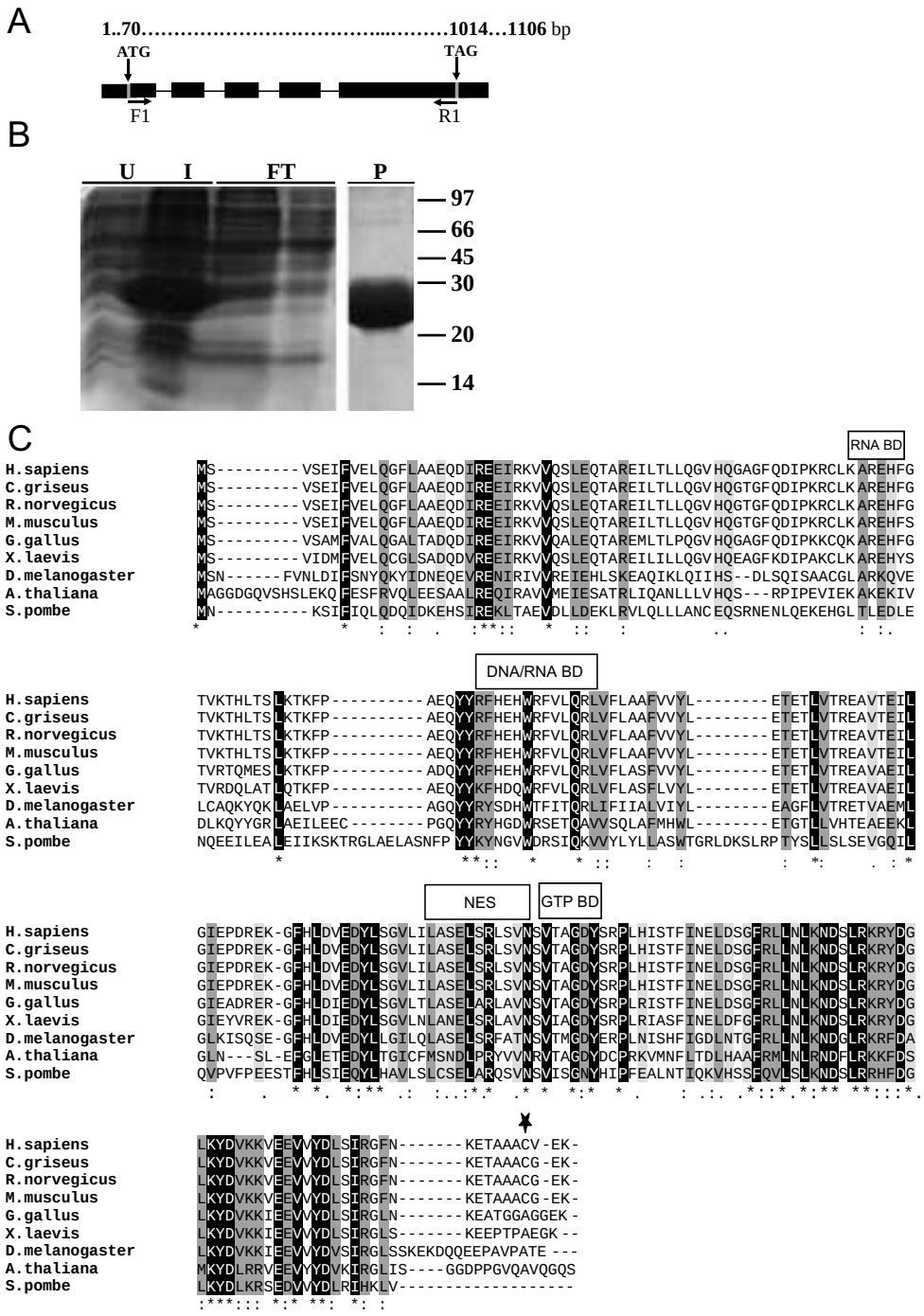

Fig. 1. Characterization of Drosophila translin: (A) gene structure of the Drosophila translin gene. The rectangular boxes indicate the exons; (B) SDS-PAGE Coomassie blue-stained gel showing expression and purification of recombinant His-tagged Drosophila translin (pQE30 system) (U: uninduced; I: induced with IPTG; FT: flow through; P: purified protein after Ni-NTA agarose affinity chromatography); and (C) Translin is conserved through evolution. Amino acid sequence alignment was performed with ClustalW. Amino acid residues in black shade are conserved residues, while the residues in dark gray show high sequence identity, and the residue in light gray high sequence similarity. BD: binding domain; NES: nuclear export signal. The conserved cystein at position 225 is indicated by a star.

underlying significance of such differences is being investigated by obtaining the crystal structure information (unpublished work). However, under reducing conditions (in the presence of DTT) both Drosophila and human translin are reduced to monomers (Fig. 2B and D). Furthermore, antibodies against Drosophila

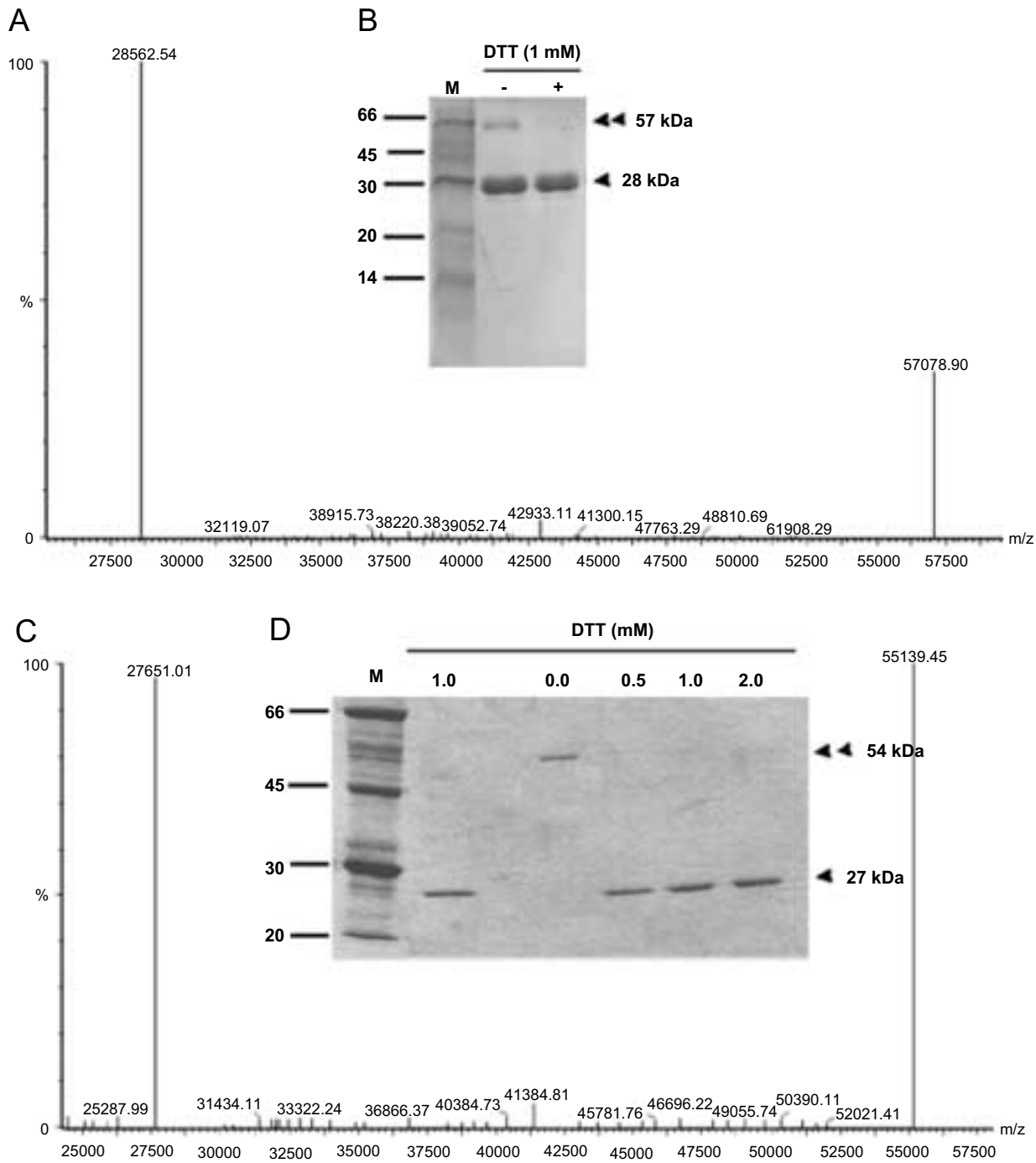

Fig. 2. Oligomerization status of Drosophila translin. MALDI-TOF was performed on Drosophila (A) and human (C) translin (monomer and dimer peaks are shown). Effect of DTT on Drosophila (B) and human (D) translin. Single and double arrowheads indicate monomer and dimer, respectively.

translin were generated to investigate its cellular localization, discussed below.

pEY06981 is a mutant of Drosophila translin

The Drosophila TRANSLIN gene is located on the second chromosome and maps to 47A11. A P-element insertion, pEY06981 in Drosophila TRANSLIN gene is located at 716 bp downstream of the start codon (Fig. 3A). The insertion site was confirmed by PCR (Fig. 3B). PCR amplicons of ~500 bp (arrowhead) and ~800 bp (star), respectively, were obtained using primers from the transposon and the Drosophila TRANSLIN gene (Fig. 3A). Homozygotic Drosophila translin mutants are viable and show no obvious morphological defects.

We separately probed Western blots of wild-type and Drosophila translin mutant embryo extracts with antibodies against full-length Drosophila translin and tubulin, the latter being used as loading control (Fig. 3C, bottom panel). In wild-type embryos and adults, the anti-Drosophila translin antibody recognized

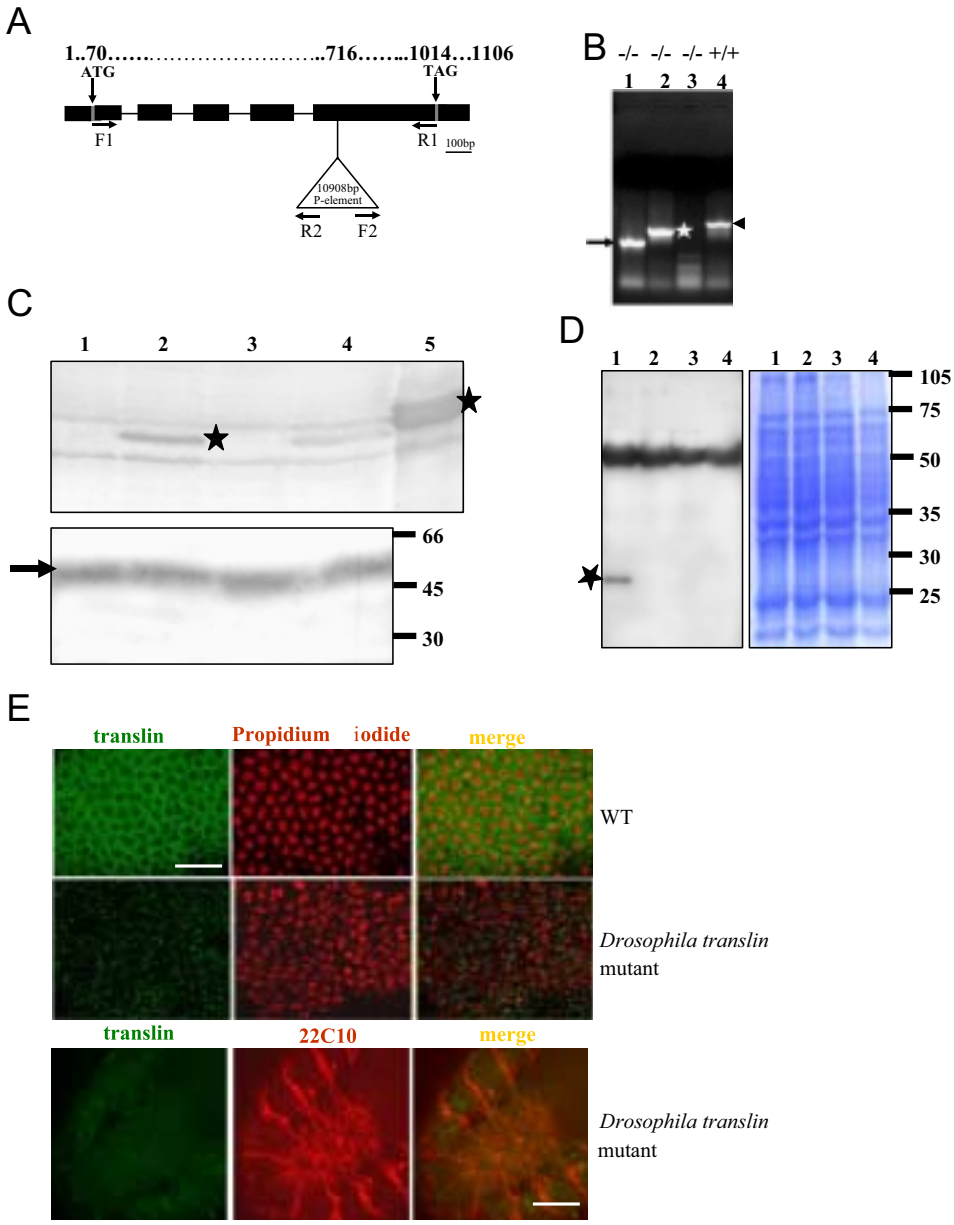

Fig. 3. Characterization of the $p^{EY06981}$ Drosophila translin mutant: (A) P-element insertion site of the Drosophila translin ($p^{EY06981}$) mutant and localization of the primer targets used for PCR amplification. (B) Confirmation of P-element insertion by PCR amplification of Drosophila translin mutant (lanes 1–3) and wild-type (lane 4) genomic DNA. Lane 1: primers F2 and R1; lane 2: primers F1 and R2; lane 3: negative control, primers F1 and R1 (as the PCR conditions did not allow 11-kb amplification), lane: 4: positive control. (C) Western blot analysis of Drosophila translin (top panel) and tubulin (bottom panel, loading control). Lane 1: Drosophila translin mutant embryo extract; lane 2: wild-type embryo extract; lane 3: Drosophila translin mutant whole-fly extract, lane 4: wild-type whole-fly extract; lane 5: recombinant Drosophila translin. (D) Immunoprecipitation of whole-fly protein extracts with rabbit anti-Drosophila translin antibody (left panel) and Coomassie blue stained gel of the total protein used for immunoprecipitation (right panel). Lane 1: wild-type protein extract with anti-Drosophila translin antibody; lane 2: wild-type protein extract with pre-immune serum; lane 3: Drosophila translin mutant protein extract with anti-Drosophila translin antibody; lane 4: Drosophila translin mutant with pre-immune serum. Drosophila translin bands are highlighted with a star. (E) Immunofluorescence analysis of Drosophila translin expression in wild-type (WT) and mutant embryos. Note the staining of the axons and dendrites by anti-22C10 antibody used as positive control for antibody accessibility in the mutant specimen. Bars: 10 μm.

a ~28-kDa polypeptide (Fig. 3C, top panel, lanes 2 and 4). Homozygous Drosophila translin mutant embryos and adults did not show a polypeptide at the corresponding molecular weight (Fig. 3C, top panel, lanes 1 and 3). We carried out immunoprecipitation of wild-type and Drosophila translin mutant extracts using pre-immune and immune sera. The rabbit polyclonal antibody specifically immunoprecipitates a 28-kDa polypeptide (indicated by a star in Fig. 3D, left panel), which was not detected in the Drosophila translin mutant; this observation was re-confirmed by immunostaining (Fig. 3E). A 4-h wild-type embryo probed with anti-Drosophila translin antibody showed a cytoplasmic staining; this was absent in the Drosophila translin mutant. In order to rule out artifacts due to antibody accessibility, we double labeled the Drosophila translin mutant embryos with the anti-22C10 antibody, which specifically stains axons and dendrites in the embryo. We observed that the Drosophila translin mutant embryos stain for 22C10 as expected but do not show detectable Drosophila translin staining, revealing that lack of translin staining in mutant embryos was due to protein under-expression rather than inaccessibility to antibody probing.

Drosophila translin is expressed ubiquitously in several tissues throughout development

In order to gain further insights into the functional role of translin in Drosophila, we extensively probed its developmental expression pattern by immunofluorescence experiments. Wild-type embryos stained with anti-Drosophila translin antibody showed ubiquitous cytoplasmic expression of Drosophila translin at certain developmental stages. Early embryonic stages (<4 h) that are syncytial revealed a uniform cytoplasmic staining pattern, very similar to that of actin staining reported earlier (Kellogg et al., 1988; Hartenstein et al., 1994) (Fig. 4A). The pre-immune serum did not show any cytoplasmic staining at the same stage, providing further evidence for the specificity of the antibody

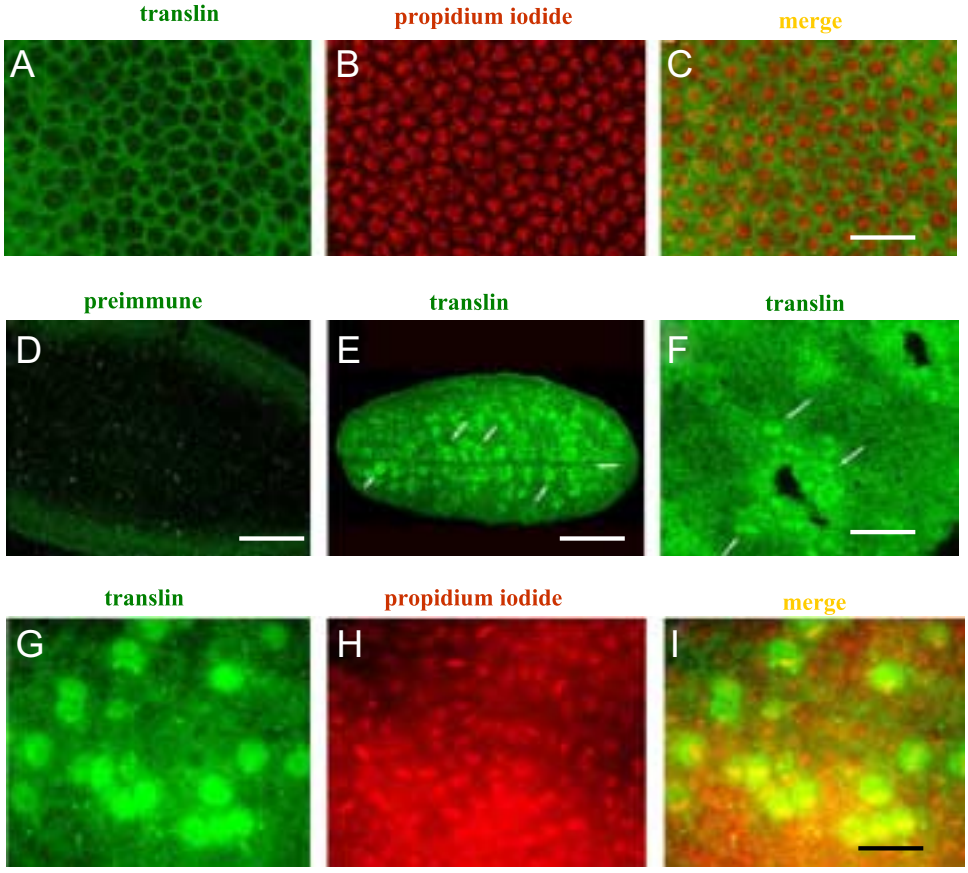

Fig. 4. Immunofluorescence analysis of Drosophila translin expression in wild-type embryonic tissues. (A–C) Region of a blastoderm embryo showing Drosophila translin expression surrounding the nuclei. (D) Embryo stained with pre-immune serum. (E) Ventral neuroblasts (arrows) at stage 8–9 show Drosophila translin enrichment. The position of the ventral furrow is indicated by a central arrow. (F) Cells around the tracheal pits (arrows) also stain with anti-Drosophila translin. (G–I) Neuroblasts. Bars: 10 μm (A–D, F–I); 100 μm (E).

(Fig. 4D). In the wild type, at embryonic stages 8–9 Drosophila translin shows an enrichment in the ventral neuroblasts as they delaminate from the ectoderm (Fig. 4E) (Urbach et al., 2003). The enrichment of Drosophila translin in neuroblasts was transient at stage 9 and was absent later. In addition, at stage 11 we also observed compact staining of Drosophila translin in cells around the primitive tracheal pits (Fig. 4F). In neuroblasts, Drosophila translin is enriched in the cells at a stage where the chromosomes appeared compactly aligned at the center of the cell. The prominently condensed chromosome organization suggests that these cells were at metaphase stage (Fig. 4G–I).

In third instar larvae, immunostaining showed Drosophila translin expression in specific ventral midline cells (Fig. 5D). This pattern of expression was also corroborated by mRNA in situ localizations at a similar developmental stage (arrow in Fig. 5B). Drosophila translin expression was also detected in the body wall of third instar larvae (Fig. 5E–G and I). The protein is highly enriched in the cytoplasm of a cluster of six cells laterally on each side of every abdominal segment; these are likely to be oenocytes (dotted regions in Fig. 5E–G and I). In addition, we detected a number of cells at an epidermal plane in each abdominal segment of the third instar larvae (arrows Fig. 5F, G and I). In order to establish the location of these cells, we double labeled dissected preparations with anti-Drosophila translin and anti-endophilin antibodies, the latter recognizing synaptic endings at the neuromuscular junction (Rikhy et al., 2002). A careful examination of 1-μm sections obtained by confocal microscopy through the double labeled neuromuscular junction preparations demonstrated that the Drosophila translin-expressing cells are below the plane of the larval musculature (Fig. 5G–I). The pattern of expression relative to endophilin staining leads us to conclude that Drosophila translin is expressed in epidermal cells in the abdominal segments. In the adult brain, Drosophila translin is expressed in a large number of neuronal cell bodies (Fig. 5J–K). This expression is not observed in Drosophila translin homozygous mutants (Fig. 5L). Again, to negate antibody accessibility artifacts, we used double labeling, in this case with anti-Drosophila translin antibody and anti-nc82 antibody, a synaptic marker which recognizes all the neuropils. We found that nc82 staining was normal in the Drosophila translin mutant (Fig. 5L–N), confirming that the Drosophila translin mutant indeed lacks the protein. In summary, Drosophila translin expression is dynamic and highly ubiquitous during embryonic, larval and adult stages, involving several tissue types; an aspect that perhaps underscores functional relevance of Drosophila translin that is still not clear.

It is well established that TB-RBP is enriched in the brain and in the testis (Wu et al., 1997). Therefore, we decided to look for Drosophila translin localization in these tissues as well. Immunostaining of Drosophila testes preparations revealed a cytoplasmic expression in early spermatocytes (Fig. 5P–R); again consistent with what has been reported for the mouse system (Wu et al., 1997).

In the light of a most recent report on Drosophila translin (Claussen et al., 2006), we were intrigued about the relationship between the translin expression pattern and the underlying putative functions. We therefore conducted the following functional assays.

Drosophila translin **mutation results in a locomotor defect**

TB-RBP knock-out mice show defects in locomotion (Chennathukuzhi et al., 2003a). In order to test whether the Drosophila mutant exhibits a similar defect, we carried out several behavioral tests. Adult Drosophila are negatively geotactic and move vertically when placed in a glass tube (Benzer, 1967). No detectable change was seen in the mutant animal when this behavior was tested for (Fig. 6A). When flies are pulsed with a strong sensory stimulus, either light or odor, they exhibit an instantaneous jump response (McKenna et al., 1989). A defective response in the Jump assay would indicate a defect either in the sensory pathway or motor pathway. We first decided to test the sensory response of the flies to light and odor. Phototactic as well as olfactory (Y-Maze) preference tests were performed on Drosophila translin and wild-type strains. The phototactic as well as the olfactory response indices of the mutant were found to be comparable to that of the wild type (Fig. 6B and C). Both wild-type and Drosophila translin mutant strains were repelled by benzaldehyde to the same extent. This suggested that the Drosophila translin mutant is not defective in the sensory pathway. We decided to use a benzaldehyde-induced jump response to test motor activity. It was found that homozygous Drosophila translin mutant females showed a significantly reduced jump response ($P < 0.0001$; t-test) as compared to the wild type and heterozygotes. The Drosophila translin homozygous mutant males also show a detectable and reproducibly small, but significant reduction in response ($P = 0.003$; t-test). Taken together, these results demonstrate that the mutation in Drosophila translin results in a reduced mobility of adult flies. This is similar to the observation in the TB-RBP knock-out mice which show an impaired motor response (Chennathukuzhi et al., 2003a). TB-RBP mutant mice also show a defect in fertility. However, no significant differences in fertility of Drosophila translin male and female mutant flies were observed as compared to the wild type (Table 1) (Claussen et al., 2006), a result that is not congruent with that observed in the mouse system.

Discussion

We had earlier reported studies on the comparison of the protein oligomerization status and nucleic acid-binding properties between human and Drosophila translin (Sengupta et al., 2006). The results had revealed stark differences between these, with Drosophila translin showing almost no nucleic acid-binding propensity, a result that appeared very surprising then, but later turned out to be in agreement with the recent

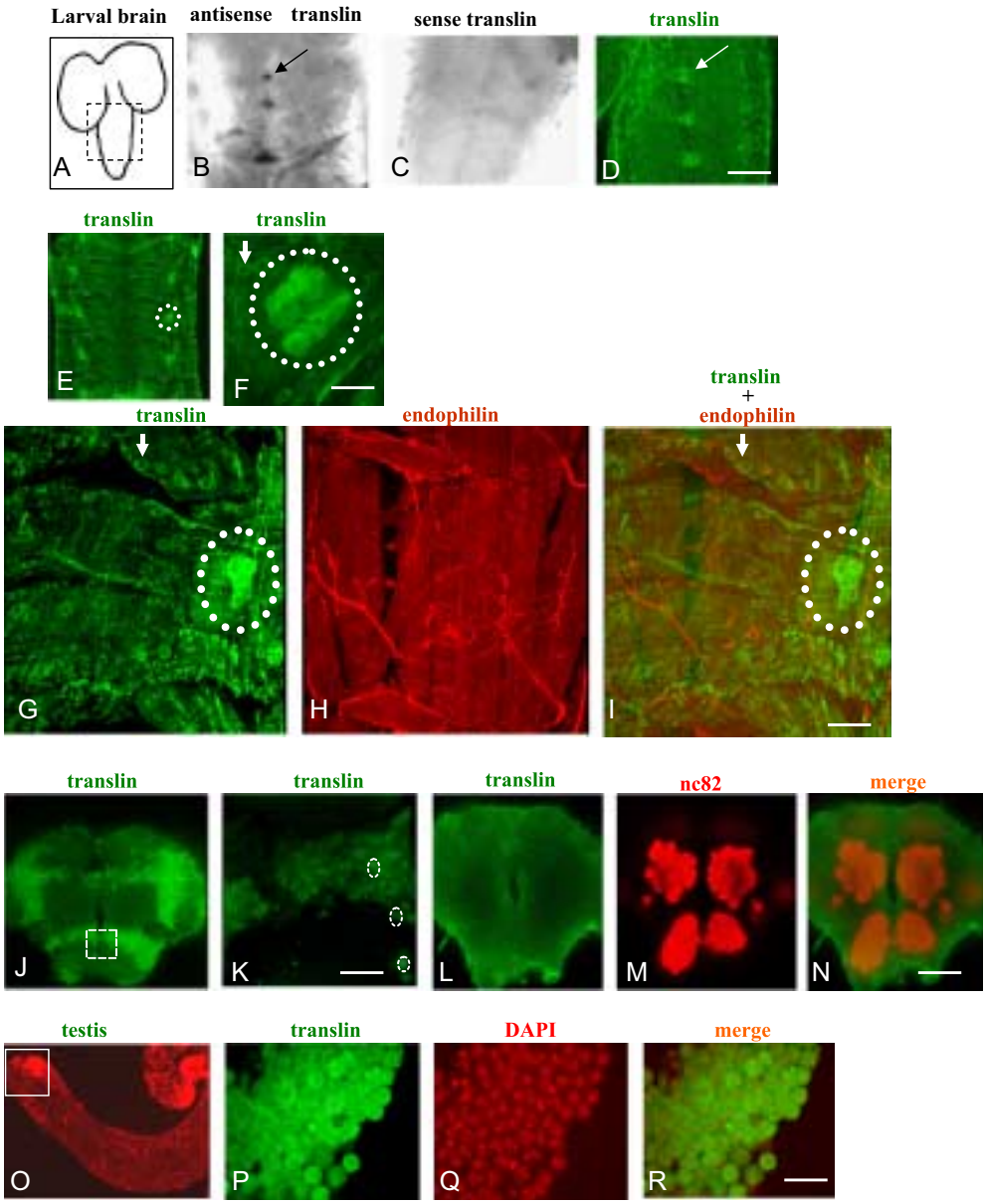

Fig. 5. Drosophila translin staining in third instar larval brain and body wall, as well as in adult brain and testis: (A) diagram of the larval brain; the box represents the magnified area in (B–D). (B, C) RNA in situ hybridization. (D) Immunostaining (1-μm confocal stacks of the entire brain (45 confocal sections)). Arrows indicate midline cells of the ventral ganglion. (E–I) Dissected abdominal segments of a third instar larval body wall. (E) A group of cells repeated in each segment (encircled by dots) were identified to be oenocytes, and is stained with anti-Drosophila translin. (F) Magnified image of the oenocyte. (G–I) Higher magnification of a single abdominal segment at a plane showing oenocytes (dotted circle) and epidermal cells (arrow) stained with anti-Drosophila translin (single projection of confocal sections 14–37) and anti-endophilin antibodies (single projection of confocal sections 1–12). (J–N) Anti-Drosophila translin staining in adult brain. (J) Wild-type adult brain showing neuronal cell bodies as observed by the projection of 1-μm confocal stacks of the entire brain (45 confocal sections). (K) Magnification of the boxed area in (J); dotted circles represent neuronal cell bodies. (L) Drosophila translin mutant adult brain showing the absence of neuronal cell body staining. (M) Anti-nc82 antibody staining of mutant brain (accessibility control). (O–R) Immunostaining of wild-type adult testis. DAPI staining pseudo-colored in red. Bars: 50 μm (B–D), 10 μm (F–I, K, P–R), 100 μm (J, L–N).

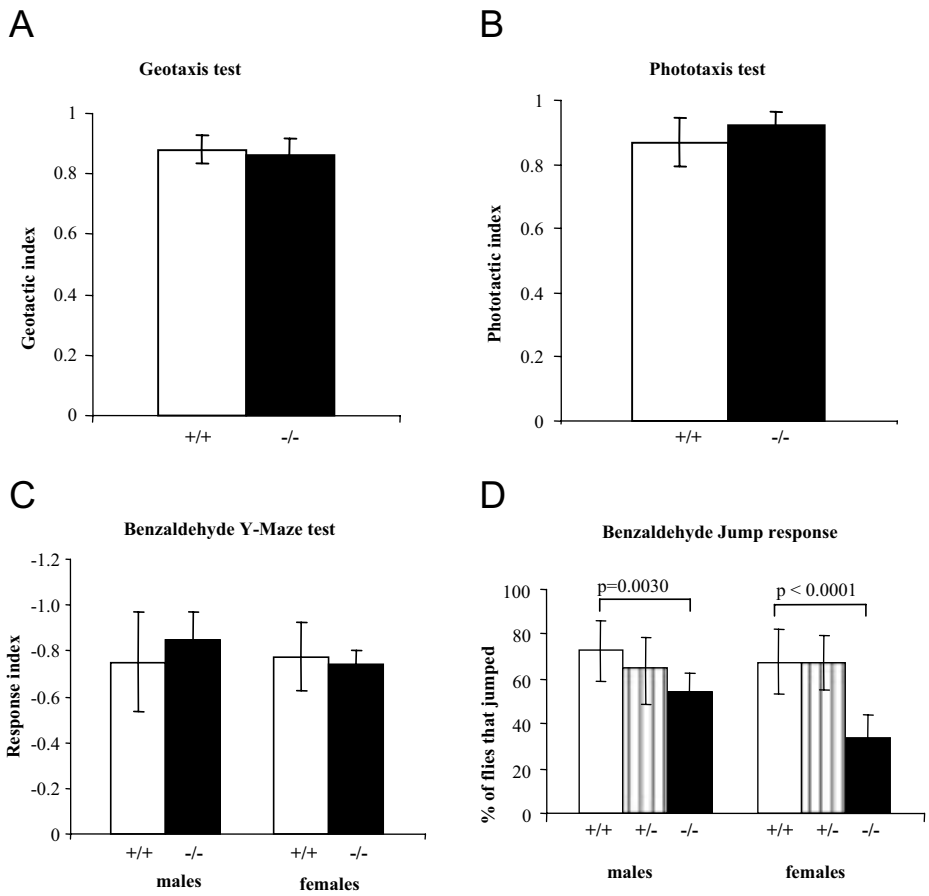

Fig. 6. Phenotypic characterization of the Drosophila translin mutant: (A) geotactic response (mean ± SE of 5 independent experiments). (B) Phototactic response (mean ± SE of 5 independent experiments). (C) Y-Maze test (mean ± SE of 5 independent experiments). (D) Jump test (mean ± SE of 10 independent experiments). +/+ wild type, +/− heterozygotes, −/− Drosophila translin mutant homozygotes.

Table 1. Drosophila translin mutant flies do not exhibit any fertility problem

Male↓ Female→	+/+	−/−
+/+	19/20 (95%)	17/20 (85%)
−/−	19/20 (95%)	19/20 (95%)

A single male was crossed to a single virgin female. After 18 days the number of vials with adult progenies were counted. A total number of 20 crosses each was set up. +/+ wild type; and −/− Drosophila translin mutant.

observation that Drosophila translin may not have any chromosomal function (Claussen et al., 2006).

Our current study highlights the importance of Drosophila translin in neuronal functions. The main observations are (1) Drosophila translin dimers, unlike human translin, do not appear to be stabilized by disulfide interactions; (2) Drosophila translin shows a ubiquitous cytoplasmic expression with enhanced levels in certain cell types at defined stages of Drosophila development; and (3) Drosophila translin homozygous mutant flies are viable and show an impaired motor response, underscoring a neuronal function of translin. These results and the implications thereof are discussed in the following sections.

Oligomerization status of Drosophila translin

Purified Drosophila translin under denaturing, non-reducing conditions exists predominantly as a monomer and a small fraction exists as a dimer (Fig. 2A and B). Under native conditions, Drosophila translin forms higher oligomers different from the human translin octamers (Sengupta et al., 2006). These differences are also reflected in the crystal structure details of both proteins (unpublished observations). Human translin under these conditions is a stable ring-shaped octamer and chicken translin is a decamer (Aoki et al., 1999; VanLoock et al., 2001; Pascal et al., 2002; Sugiura et al., 2004). A translin dimer is formed through disulfide linkages at Cys-225, and the octamer is stabilized both by conserved disulfide linkages and hydrophobic interactions at the C terminus (Wu et al., 1998; Sengupta and

Rao, 2002). However, in Drosophila translin the Cys-225 is replaced by an alanine residue, which may account for the reduced level of dimers described above. The oligomeric status of Drosophila translin is also likely to be sustained by conserved C-terminal interactions between monomers. Detailed and systematic site-directed mutagenesis efforts that "humanize" Drosophila translin followed by biochemical analyses might unveil the basis of critical differences between these two homologs.

Expression pattern of Drosophila translin and behavioral analyses of the mutant

TB-RBP/translin is largely expressed in the brain and testis. We analyzed the tissue distribution of Drosophila translin. At early embryonic stages in a multinucleate blastoderm, Drosophila translin expression is mainly in the cytoplamic segment (Fig. 4A–C). This is similar to actin and other cytoskeletal proteins (Kellogg et al., 1988; Hartenstein et al., 1994). These results are consistent with earlier studies which have demonstrated that TB-RBP co-localizes and co-immunoprecipitates with γ-actin (Wu et al., 1999). In embryonic stages 8–9, Drosophila translin appears in ventral neuroblasts. During embryogenesis, neuroblasts delaminate from the ventral neuroectoderm and subsequently divide asymmetrically to form a neuroblast and a ganglion mother cell (GMC). Division of GMCs forms the neuronal and the glial components of the brain and the ventral nerve cord (Urbach et al., 2003). Drosophila translin is enriched in neuroblasts after delamination, where it appears to be present throughout the cell (Fig. 4E). Interestingly, high levels of Drosophila translin expression were found in cells appearing to be in metaphase as judged from the compact morphology propidium iodide-stained chromatin (Fig. 4G–I). Various experiments have shown that translin expression is strongly associated with mitotically dividing cells (Ishida et al., 2002; Yang et al., 2004; Cho et al., 2004). Our results are consistent with these observations, perhaps suggesting that Drosophila translin plays a role in mitotically dividing cells. In addition, at stage 11 Drosophila translin was observed in cells around the primitive tracheal pits (Fig. 4F). The tracheal pits after morphogenesis develop into tracheal tubes. At the third instar larval stage, RNA in situ hybridization and immunostaining reveal Drosophila translin expression in segmentally repeated cells located at the midline (Fig. 5B and D). The expression pattern suggests a role for Drosophila translin in the nervous system. Supporting this speculation, immunostaining of the adult brain revealed that indeed Drosophila translin is present in a large number of neuronal cell bodies (Fig. 5J–L). A similar finding is observed with rat translin that is also expressed in neurons. However, later rat translin is translocated to the neuronal dendrites, and this process appeared to be correlated with neuronal development and differentiation events (Kobayashi et al., 1998). Our findings suggest a role for Drosophila translin in the adult nervous system. Drosophila translin expression appears enhanced in oenocytes in the abdominal segments and in cells of the epidermis (Fig. 5E–G and I). Oenocytes are a group of six specialized secretory cells that are restricted to the abdominal segments A1–A7, rich in hydrocarbon biosynthesis (Pho et al., 1996), and have been shown to synthesize sex-specific hydrocarbons (Ferveur et al., 1997).

TB-RBP and TRAX have been shown to influence each others nuclear and cytoplasmic distribution in mouse male germ cells (Cho et al., 2004). In mouse embryonic fibroblasts TRAX levels closely parallel the levels of TB-RBP throughout the cell cycle (Yang et al., 2004). TRAX mediates TB-RBP entry into the nucleus through its nuclear localization signal, whereas TB-RBP bears a nuclear export signal for the exit of TB-RBP/TRAX from the nucleus. A dynamic and transient entry and exit has been observed for TB-RBP from the nucleus to the cytoplasm that raises intriguing possibilities on its nuclear role (Cho et al., 2004). Almost complete lack of information on the expression and localization status of Drosophila TRAX renders it difficult for one to hypothesize a good model for Drosophila translin at this stage. Drosophila TRAX has more than one transcript of which at least one at protein level lacks a nuclear localization signal (Claussen et al., 2006).

Despite large gaps in our understanding of the translin/TRAX system in Drosophila, the elevated expression levels of translin in neuronal cells strongly indicate a neuronal function. The discovered motor defect in Drosophila translin mutants is consistent with this hypothesis. However, the molecular basis of this defect needs to be unraveled. Translin knock-out mice also have been reported to have sex-specific defects learning and memory, locomotor activity, anxiety-related behavior and alterations in monoamine neurotransmitter levels in several forebrain regions (Stein et al., 2006). This is similar to the defects seen with fragile X mental retardation protein (FMRP) and therefore it has been suggested that TB-RBP and FMRP may be involved in a similar function or pathway. As a Drosophila fmr1 mutant also shows locomotor and courtship behavior defects (Dockendorff et al., 2002) it is tempting to speculate that Drosophila translin might be involved in the same pathway or function like FMR1. Studying a double mutant of Drosophila translin and fmr1 would confirm our hypothesis.

The TB-RBP–mRNA complex co-immunoprecipitates with KIF17b – a neuronal isoform of neuronal kinesin KIF17 (Chennathukuzhi et al., 2003b). It would

be of interest to examine the consequences of Drosophila translin mutations in a KLP64D (Drosophila homolog of KIF17) background to obtain specific insights into its neuronal function.

Future efforts should unravel translin-interacting molecules as well as the mechanism by which Drosophila translin exerts its functions.

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