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Structure and expression patterns of *Drosophila* TULP and TUSP, members of the *tubby*-like gene family

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Abstract

Tubby is a mouse gene that may provide a model for adult-onset obesity in humans. It is a member of a four gene family in mammals that collectively encode the Tubby-like proteins (TULPs), putative transcription factors which share similar 260 amino acid 'tubby domains' at their C-termini. The mammalian genome also encodes distant relatives of TULPs, which have been called TUSPs (tubby domain superfamily proteins). We have characterized the transcription unit of the single *Drosophila TULP* homolog, analyzed the expression pattern of the *Drosophila TULP* and *TUSP* genes, and determined the evolutionary relationships between the *Drosophila* proteins and members of the tubby domain superfamily in other organisms. Interestingly, like its mammalian homologs, *Drosophila TULP* is principally expressed in the embryonic central and peripheral nervous systems. This suggests that mammalian and *Drosophila* TULPs may possess some conserved functional properties in the nervous system. The *Drosophila TUSP* gene is also expressed in the central nervous system and olfactory organ but in few other peripheral sensory organs.

Keywords: Tubby; Tubby-like proteins; Tubby domain superfamily proteins; Suppressor of cytokine signaling; WD40

1. Results

Defects in neuronal survival and function occur in mice and humans with mutations in *tubby* and other tubby-like proteins (TULPs) (Noben-Trauth et al., 1996; Kleyn et al., 1996; Stubdal et al., 2000; Ikeda et al., 2002). Mutations in mouse *tubby* cause progressive obesity in mice (Coleman and Eicher, 1990), perhaps due to abnormal function of hypothalamic cells (Guan et al., 1998), and also exhibit degeneration of cochlear cells and retinal cells (Ohlemiller et al., 1997; Ikeda et al., 2002). Mutations in the mouse or human *TULP1* genes also result in apoptotic retinal degeneration (Heckenlively et al., 1995; Ikeda et al., 1999), while mutations in mouse *TULP3* result in embryonic lethality due to neural tube defects (Ikeda et al., 2001). Mouse *tubby* is expressed in the central and peripheral nervous systems, in a complex pattern that includes the brain, retina, and other sensory neurons (North et al., 1997; Sahly et al., 1998). It was recently reported that the levels of *tubby* expression in the adult rat brain are regulated in a complex manner by thyroid hormone, which might explain the influence of thyroid hormone on body weight (Koritschoner et al., 2001). Mouse *TULP1* is expressed exclusively in photoreceptors, but there is little overlap at the cellular level with the *tubby* pattern of expression (Ikeda et al., 1999). The *TULP2* gene is primarily expressed in mouse testis (North et al., 1997), and *TULP3* is expressed nearly ubiquitously in mouse embryos and adults (Ikeda et al., 2001; Nishina et al., 1998). Although the biochemical functions of TULP proteins have long remained mysterious, there is recent evidence suggesting that they act as DNA binding transcription factors whose cytoplasmic/nuclear distribution is regulated by G-protein-coupled receptor pathways (Boggon et al., 1999; Santagata et al., 2001). The mouse Tubby protein is also phosphorylated on conserved tyrosine residues in tissue culture cells after treatment with insulin, suggesting Tubby may function as an adaptor analogous to IRS-1 (Kapeller et al., 1999).

Genes in mice and humans that encode TUSP proteins have been recently identified by their sequence similarities to the C-terminal tubby domain (Li et al., 2001). By Northern analysis, transcripts from the mouse *TUSP* gene are abundant in brain and testes, while the human *TUSP* gene appears to be transcribed in the brain, and perhaps in skeletal muscle, kidney and placenta (Li et al., 2001). There are no known mutants at present in *TUSP* genes. We wished to

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test whether the expression patterns of the *Drosophila TULP* and *TUSP* genes resembled their mammalian homologs.

Sequence searches indicate that the *Drosophila* genome contains only one member each of the *TULP* and *TUSP* gene families (Altschul et al., 1997; Myers et al., 2000). Using a genomic probe from the fly coding region, Northern blots indicate that *TULP* produces transcripts of approximately 2.6 kb, which are most abundant during 2–8 h of embryonic development (Fig. 1C). Analysis of genomic sequence, sequence from a cDNA isolated in our lab (pDTulp15B), and sequence from a cDNA isolated by the Berkeley *Drosophila* Genome Project (AY060625), indicates that *Drosophila TULP* has two promoters separated by 19.5 kb (Fig.

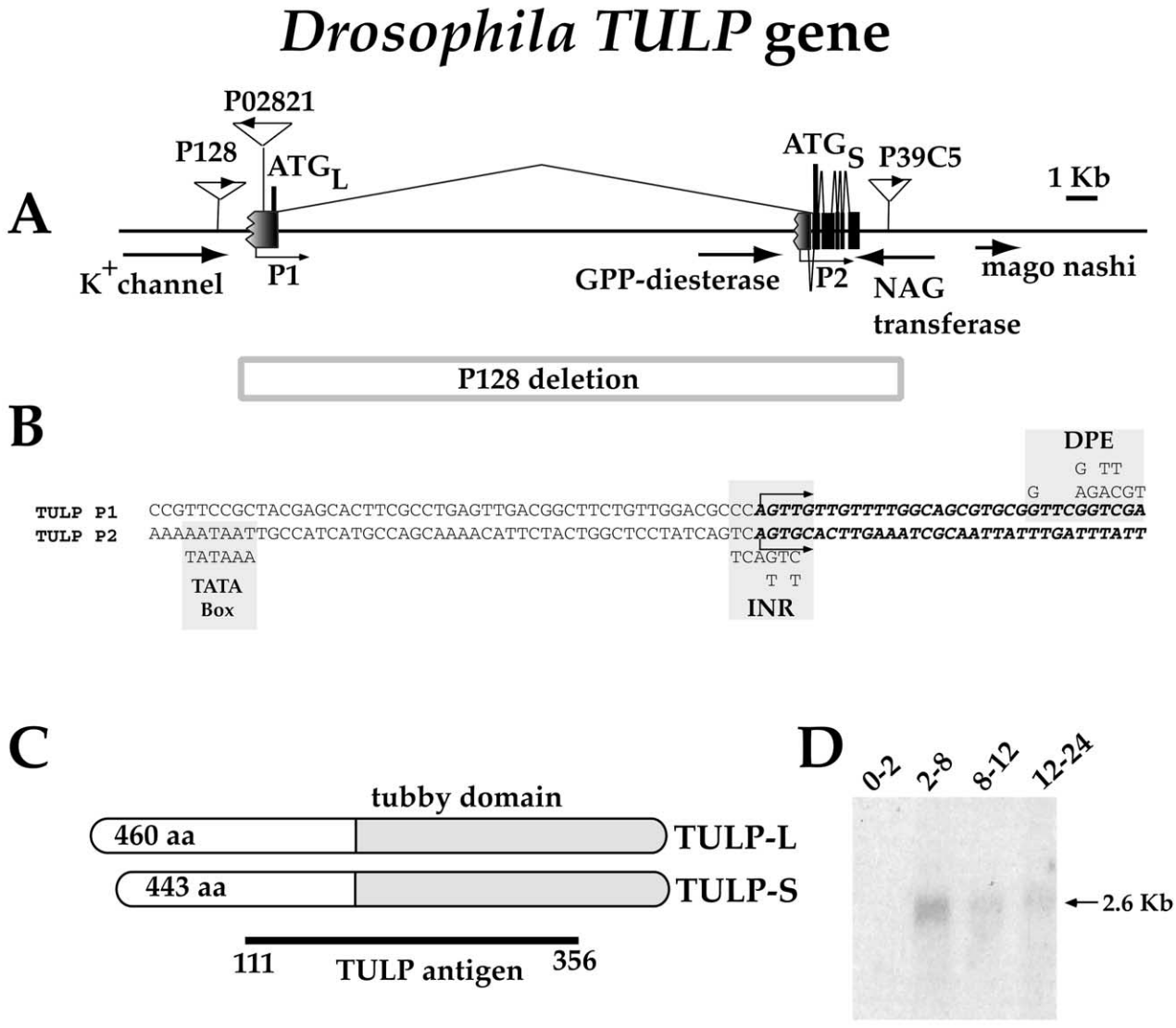
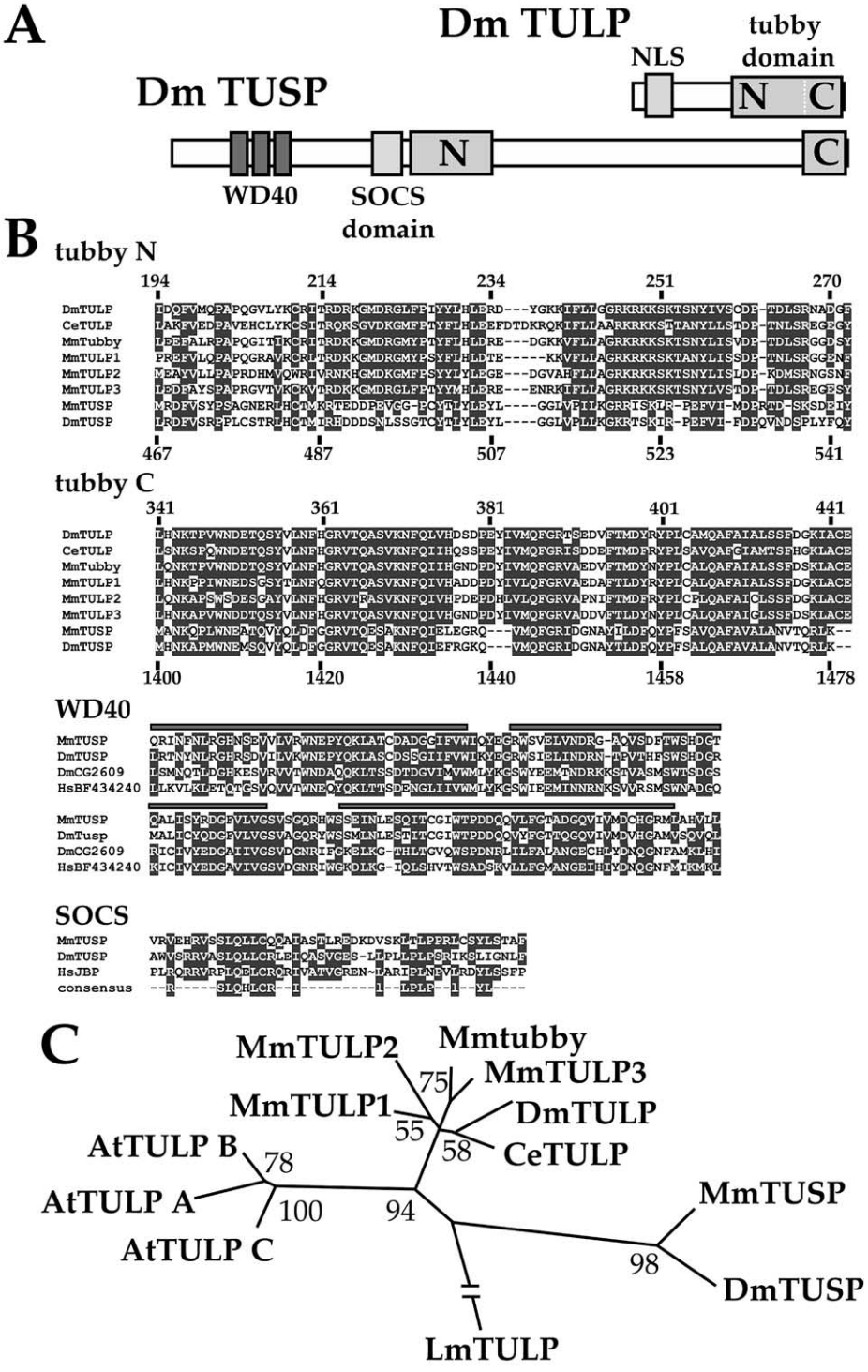


Fig. 1. Genomic organization of the *Drosophila* TULP locus. (A) A 40 kb genomic region including the *TULP* locus is represented. Black boxes represent *TULP* exons. The putative transcription start sites for the two *TULP* promoters are marked with arrows below the line and labeled P1 and P2. Bold arrows below the line represent the size, placement, and orientation of nearby genes predicted from genomic sequence (Myers et al., 2000). The position of the ATGs encoding the first methionines of the long (L) and short (S) isoforms of the TULP proteins are indicated above the line. The name, orientation, and position of three P-element insertions into the *TULP* locus are indicated with arrowed triangles. Below the schematic a gray rectangle represents the approximate breakpoints and extent of a deletion that is associated with the P128 insertion. (B) A prediction of promoter elements surrounding the transcriptional start sites inferred from two different putative full length *TULP* cDNAs (see Section 2). Sequence that corresponds to the $5'$ termini of the *TULP* cDNAs is represented in bold italics, while upstream genomic sequence is normal type. The first nucleotide of both *TULP* cDNAs corresponds to the $+1$ position of a consensus Initiator element (INR) (Kutach and Kadonaga, 2000). The *TULP* cDNA encoding the L isoform has no apparent TATA sequence $5'$ of the INR but does have a putative DPE, downstream promoter element (Kutach and Kadonaga, 2000) at the correct spacing $3'$ of the $+1$ position. The *TULP* cDNA encoding the S isoform has no apparent DPE-like sequence, but does have an apparent TATA Box. (C) A schematic representation of the TULP-L and -S protein isoforms. The proteins differ only in the 17 most N-terminal amino acids. The C-terminal tubby domain is shaded in gray. The TULP protein region used as an antigen to prepare α-TULP serum from rabbits is denoted below the schematic proteins (see Section 2). (D) A developmental Northern blot probed with a *TULP* genomic fragment (see Section 2). The numbers at the top denote the embryonic stages (in hours) of the poly A + RNA loaded in each lane.

1A). Promoter P1 initiates a transcript that encodes a 460 codon open reading frame (TULP-L protein), and promoter P2 a transcript that encodes a 443 codon open reading frame (TULP-S protein) that overlaps precisely with all but the first 17 codons of TULP-L (Fig. 1B). Comparison of the *Drosophila* TULP isoforms with other Tubby/TULP proteins reveals high levels of sequence similarity to the highly conserved C-terminal tubby domain as well as lower levels of similarity to a 70 amino acid N-terminal region (boxes and sequences in Fig. 2A, B). The core of the N-terminal homology region contains a presumptive nuclear localization signal (Nishina et al., 1998). In the C-terminal tubby domain, eight of the ten tyrosines that are potential phosphorylation sites/SH2 binding motifs (Kapeller et al., 1999), are conserved between *Drosophila* TULP and mouse Tubby.

The *Drosophila TUSP* gene was identified as the only significant match obtained when searching the *Drosophila*



genomic database with the tubby domain of the human *TUSP* sequence (Altschul et al., 1997; Li et al., 2001; Myers et al., 2000). Alignment of TUSP with Tubby/TULP proteins revealed that the tubby homology domain, which is contiguous in TULP proteins, is separated by 970 amino acids into two domains in TUSP proteins. We call these two subdomains tubby domain-N and tubby domain-C (Fig. 2A, B). In addition to the sequence similarities in their split tubby domains, TUSP proteins contain matches to suppressor of cytokine signaling (SOCS) domains and to WD40 repeats (Fig. 2A, B).

Phylogenetic analysis (Fig. 2C) supports an ecdysozoan group of TULP proteins containing the *Drosophila* and *Caenorhabditis elegans* homologs, separate from the mammalian group. Neither the fly nor the nematode protein appears to be a true homolog of a specific mammal tubby/TULP family member. Plant TULP family proteins cluster in a single group, suggesting that they arose from duplication of a single ancestral plant *TULP* gene. The TUSP proteins from all organisms cluster together forming a distantly related clade.

Whole mount in situ hybridization of *Drosophila* embryos was used to examine temporal and spatial expression of the *TULP* and *TUSP* transcripts (Fig. 3). At stage 9 *TULP* transcripts are detected in a subset of neuroblasts (Fig. 3A). By stage 12 *TULP* transcripts are found in both the CNS and PNS (Fig. 3B). In late-stage embryos, *TULP* expression persists in the CNS and PNS with more abundant expression in the antennal-maxillary sensory neurons and in bilateral groups of cells in the brain (Fig. 3C, D). *TUSP* transcripts are detectable after stage 12 in a subset of CNS cells (Fig. 3H). At later stages of embryogenesis, *TUSP* expression is also detected in the antennal-maxillary sensory neurons and in bilateral groups of cells in the brain (Fig. 3I). *Drosophila* TULP protein distribution in embryos was analyzed with a rabbit polyclonal antibody raised against a 245 amino acid region of the protein (Fig. 1C). The TULP protein is detected in a pattern identical to that of the transcript (Fig. 3E). In the chordotonal neurons, the TULP protein accumulates in both the nucleus and cytoplasm (Fig. 3J). This distribution mirrors that for mammalian Tubby protein in the hypothalamus and other regions of the mouse brain (He et al., 2000).

Determining the function of TULP and TUSP family proteins seems likely to be informative for human disease as well as for an understanding of feeding behavior and energy balance in mammals, and perhaps other animals. Here we show that the neuronal expression pattern, and nuclear/cytoplasmic distribution of the *Drosophila* TULP protein, resembles that of its mammalian homologs. This suggests that future studies on loss of function alleles of *Drosophila TULP* and *TUSP*, as well as the use of gain-of-function phenotypes in genetic suppressor screens (Simon et al., 1991), may be very useful in learning more about the metazoan genetic and physiological pathways that include the TULP and TUSP proteins.

2. Materials and methods

2.1. Drosophila TULP cDNA and northerns

The pDTulp15B *Drosophila TULP* cDNA was isolated from an 8–12 h cDNA library (Brown and Kafatos, 1988) using a radiolabeled PCR probe of 345 bp that included conserved codons of the tubby domain. The probe was synthesized using primers that amplified STS DM87D3S (Berkeley *Drosophila* Genome Project). The 345 bp genomic probe was also used to probe a Northern blot containing total RNA extracted from 0–2, 2–8, and 8–24 h embryos. RNA extraction, electrophoresis, blotting, hybridization and washes were performed as previously described (Kuziora and McGinnis, 1988).

2.2. Database searches, alignment and phylogenetic reconstruction

The BLAST algorithm (Altschul et al., 1997) was used to search protein and EST databases for homologous sequences. The ISREC PROSITE database identified nuclear localization motifs. Phylogenetic relationships

Fig. 2. Domain organization and phylogenetic relationships of TULP and TUSP proteins. (A) A comparison of *Drosophila* TULP and TUSP protein domains. *Drosophila* TULP shares an N-terminal region of homology with vertebrate Tubby and TULP s. This domain contains a putative nuclear localization signal (NLS) at its core. The C-terminus of *Drosophila* TULP contains a highly conserved tubby domain. The *Drosophila* TUSP protein also contains weaker similarity to the tubby domain, in two regions separated by ~970 amino acids. These halves are labeled with an N (amino-half) or C (carboxy-half) reflecting their position in TULP and TUSP. TUSP also contains three WD40 repeats followed by a suppressor of cytokine signaling or SOCS domain. (B) Sequence alignments of the conserved domains highlighted in panel A. *Drosophila* TULP (DmTULP; CG9398), *Caenorhabditis* TULP (CeTULP; NP_495710), *Mus* tubby (MmTubby; AAB53495), *Mus* Tulp1 (MmTULP1; AAD13757), *Mus* TULP2 (MmTULP2;NP_032833), *Mus* TULP3 (MmTULP3; O88413), *Drosophila* TUSP (DmTUSP; AAF56725), *Mus* TUSP (MmTUSP; AAF87974). The numbers above DmTULP sequence, and below the DmTUSP sequence denote the amino acid positions relative to the N-terminus of the TULP-S isoform. The three WD40 repeats are marked above the alignment with bars. The repeats in the TUSP proteins are most closely related to a family of predicted proteins containing WD40 repeats represented here by the fly (DmCG2609; AAF47556) and human (Hs; Accession number BF434240) homologs. Neither protein is predicted to contain a tubby domain. The SOCS domain of the TUSP family members are most similar in database searches to the SOCS domain from the family of JAK binding proteins (hsJBP;NP_003736). The consensus derived from the PFAM alignment of SOCS domains is shown below for reference. (C) Phylogenetic reconstruction of the relationships between Tubby/TULP proteins and TUSP proteins. Numbers at the nodes indicate bootstrap support for branching relationships that are greater than 50 on a 1–100 scale. Additional plant and protist sequences are: *Arabidopsis* TULPA (AtTULPA; AAL66970), *Arabidopsis* TULP B (AtTULPB; AAD39275), *Arabidopsis* TULP C (AtTULPC; AAL15194), and *Leishmania* TUSP (LmTUSP; from clone P1408, see Section 2).

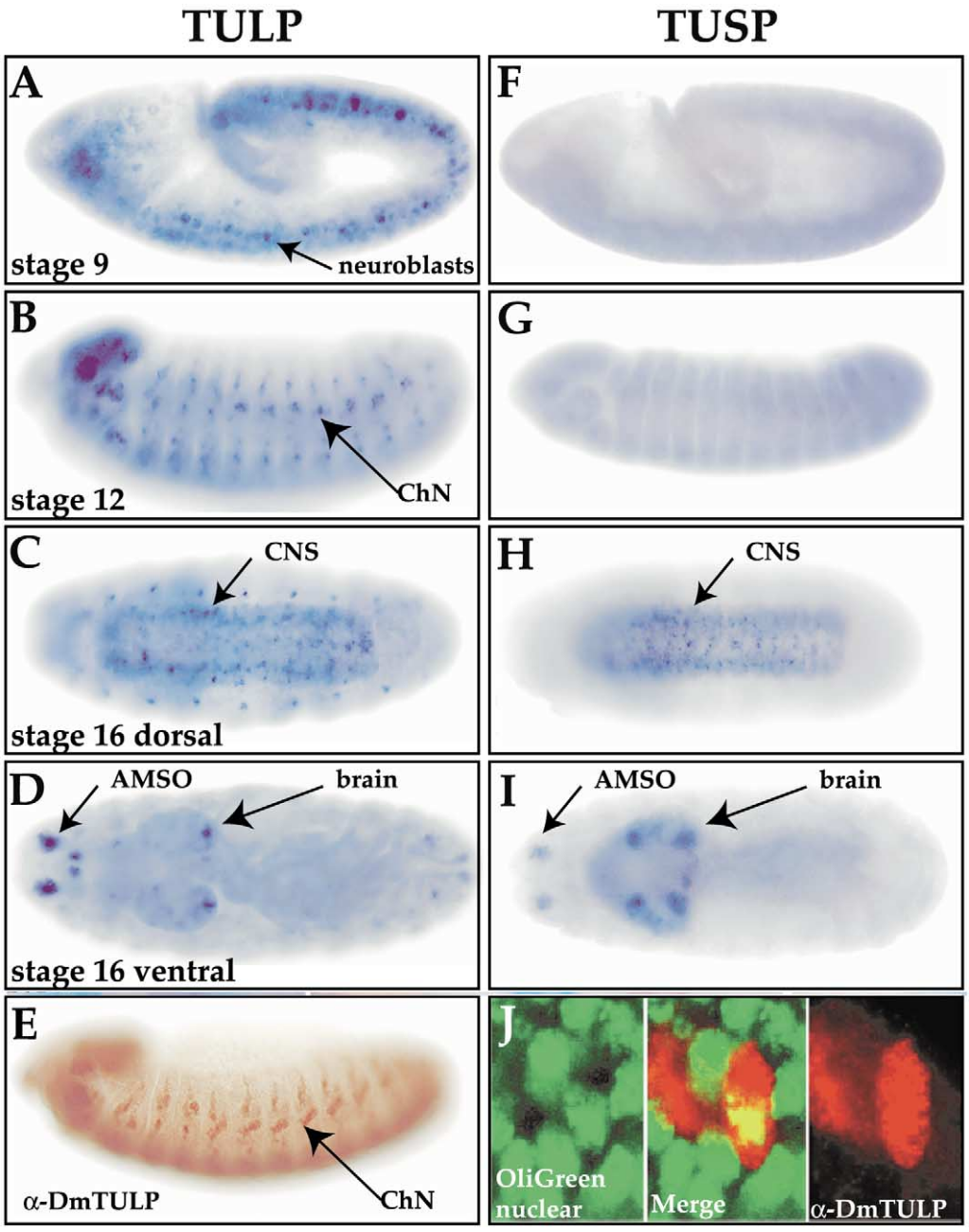


Fig. 3. *TULP* and *TUSP* RNA and protein localization in *Drosophila* embryos. (A–D) Whole mount in situ hybridizations of digoxigenin-labeled *TULP* cDNA to embryos. (A) At stage 9, *TULP* RNA is expressed in neuroblasts. (B) At stage 12, *TULP* RNA is strongly expressed in a subset of CNS and the PNS cells (e.g. chordotonal neurons, ChN). (C,D) At stage 16, *TULP* RNA expression continues in most of the CNS and PNS, but some groups of cells accumulate higher levels of RNA, e.g. in the posterior brain and the antennal-maxillary sense neurons (arrows). (F–I) Whole mount in situ hybridizations of a digoxigenin-labeled *TUSP* RT-PCR fragment to embryos (Section 2). (F,G) *TUSP* RNA is not detected in embryos prior to stage 12. (H,I) At stage 16, *TUSP* RNA is detected in a subset of neurons in the CNS, two clusters of neurons in the posterior and lateral brain and in the antennal-maxillary sense organ (AMSO). (E) Rabbit α-TULP serum was used to detect *Drosophila* TULP protein with HRP immunohistochemistry. The TULP protein is found in a pattern that corresponds to the distribution of *TULP* transcripts (compare panel (B) with (E), chordotonal neurons – ChN). (F) Sub-cellular localization of TULP was determined with deconvolution microscopy on a cluster of lateral chordotonal cells. TULP protein was detected with the rabbit α-TULP primary and an α-rabbit Texas red secondary. Oli-Green (Molecular Probes) was used as a nuclear counterstain. The TULP protein appears both nuclear, as shown by the yellow co-localization of Oli-green and Texas red, and cytoplasmic, as shown by the red fluorescence outside the nucleus.

were inferred using neighbor joining (NJ) and maximum likelihood (ML). The alignments were performed on a gap-deleted set of tubby domain sequences. Support for ML tree inference utilized quartet puzzling reliability values from 10,000 puzzling steps (analogous to bootstrap replicates). The NJ inference of tree topology and the subsequent bootstrap analysis were implemented in DAMBE (Xia and Xie, 2001). The quartet puzzling ML analysis was

performed with PUZZLE (Strimmer and von Haeseler, 1996). The NJ tree derived from the ML distance matrix was used to compute the parameters for the models of substitution and rate heterogeneity. The ML model of evolution allowed for among-site rate heterogeneity with 1 invariant and 8 γ-distributed rate categories.

Sequence data for *Leishmania* major chromosome 14 was obtained from The Sanger Institute website at http://www.sanger.ac.uk/Projects/L_major/. Sequencing of major chromosome 14 was accomplished as part of the *Leishmania* Genome Network with support by the Wellcome Trust.

2.3. Antibody staining and in situ hybridization

A histidine-tagged *Drosophila* TULP fusion protein containing amino acids 111–356 was expressed in *E. coli*, purified using glutathione-Sepharose (Pharmacia), and injected into rabbits to generate anti-*Drosophila* TULP antiserum. For staining with this antiserum, embryos were collected, dechorionated and fixed for 15 min in 4% formaldehyde. The antiserum was used at a 1:100 dilution, and detected with either goat anti-rabbit IgG antibodies conjugated to biotin (Jackson Immunoresearch) followed by HRP detection with Vector Labs's ABC system using diaminobenzidine (DAB) or FITC-labeled anti-rabbit secondary antibodies (Jackson Labs). In situ hybridizations to detect RNA transcripts were performed using a variation of the protocol described by (Tautz and Pfeifle, 1989). Digoxigenin-labeled antisense probes were made from the pDTulp6 cDNA insert, in pBluescript II KS(-), which was linearized with *Xho* I and transcribed from the T7 promoter. The TUSP Probe consisted of 1009 nucleotides of cDNA prepared from an RT-PCR reaction, the 5′ primer sequence began 356 nucleotides downstream from the initiating ATG of the TUSP open reading frame. Primer sequences are available on request.

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