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The *Drosophila* SOX-domain protein Dichaete is required for the development of the central nervous system midline

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SUMMARY

SOX-domain proteins are a class of developmentally important transcriptional regulators related to the mammalian testis determining factor SRY. In common with other SOX-domain genes, the *Drosophila Dichaete* gene has a dynamic expression profile in the developing central nervous system, including cells of the ventral midline. We find defects in the differentiation of midline glia and concomitant axonal defects in *Dichaete* mutants that are rescued by driving Dichaete expression in the midline. Since *Dichaete* is required for the correct specification or differentiation of midline glia, we have used the ventral midline as a model system to study SOX gene function in vivo and demonstrate a genetic interaction between *Dichaete* and the POU domain gene *ventral veinless*. In mammals, a protein related to Dichaete, SOX2, also interacts with POU transcription factors. The midline phenotypes of *Dichaete* mutations are rescued by expression of mouse SOX2. Our data suggest that SOX gene structure, function and interactions have been conserved during evolution.

Key words: *Drosophila, Dichaete,* SOX-domain, Midline, Nervous system

INTRODUCTION

The *Drosophila Dichaete* gene encodes a SOX-domain protein with a DNA-binding domain closely related to that of the mammalian sex determining factor SRY (Nambu and Nambu, 1996; Russell et al., 1996). SOX-domain proteins form part of a larger family of transcription factors whose DNA-binding domains (HMG-domains) are related to that of the general chromatin protein HMG-1 (Gubbay et al., 1990; Grosschedl et al., 1994). The HMG-domain binds DNA via contacts in the minor groove and induces a bend in the DNA (Bianchi et al., 1992; Giese et al., 1992). This bending property has led to the suggestion that SOX-domain proteins act as architectural components by modulating chromatin structure around transcriptional regulatory elements (Pontiggia et al., 1994; Giese et al., 1995). In addition, a number of SOX proteins appear to act as classical transcription factors, since they contain transactivation domains and can transactivate reporter genes located downstream of multimerised binding sites (van de Wetering et al., 1993; Hosking et al., 1995; Sudbeck et al., 1996). However, for other SOX proteins, most notably SOX2 and the related HMG-domain factor LEF-1, there is little evidence for direct transcriptional activity (Giese and Grosschedl, 1993; Kamachi et al., 1995). In these cases, physical interactions with other proteins, the transcription factor OCT3 in the case of SOX2 and β-catenin in the case of LEF-1, are required to modulate transcription (Ambrosetti et al., 1997; Behrens et al., 1996; van de Wetering et al., 1997). In order to understand better the function of SOX-domain proteins in the context of development, an in vivo analysis which identifies target genes and interacting partners is required to complement biochemical studies. Most SOX genes so far identified are expressed in tissue-specific and temporally regulated patterns during embryogenesis, suggesting that they may have key roles in development (Pevny and Lovell-Badge, 1997). To date, a function has been described for four SOX-domain proteins; however, little is known about their target genes or interacting partners (Sinclair et al., 1990; Foster et al., 1994; Wagner et al., 1994; Schilham et al., 1996; Nambu and Nambu, 1996; Russell et al., 1996). *Dichaete*, in common with many vertebrate SOX-domain genes, is expressed in the developing central nervous system (Uwanogho et al., 1995) and a function for *Dichaete* in neurogenesis was previously suggested (Nambu and Nambu, 1996).

The CNS of the trunk region of the *Drosophila* embryo develops from two distinct groups of cells within the neurogenic region: neuroblasts and midline precursors. Most neural cells of the ventral nerve cord originate from neuroblasts that segregate from the neuroectoderm after gastrulation (Goodman and Doe, 1993). The midline originates from a group of mesectodermal cells, specified at the cellular blastoderm by the expression of *single minded* (Crews et al., 1988; Nambu et al., 1990). Midline precursors give rise to small lineages and amongst their daughters are three pairs of midline glia (MGL) termed anterior, middle and posterior midline glia (MGA, MGM and MGP, respectively). Due to cell

death, the nomenclature and exact lineage history of the MGL is still unclear (Jacobs and Goodman, 1989; Klämbt et al., 1991; Bossing and Technau, 1994). MGL are required for the separation of the anterior and posterior commissures, the segmental nerve tracts that connect the two sides of the nerve cord. It has been suggested that commissure separation is driven by the migration of midline glia from an anterior position to a location between the commissures (Klämbt et al., 1991).

We demonstrate that Dichaete is localised in the midline of the ventral nerve cord and in the lateral neuroectoderm. In the absence of *Dichaete* function, midline glia are reduced in number and are inappropriately distributed, resulting in the fusion of commissures and the collapse of longitudinal neuropile tracts toward the midline. These phenotypes are rescued by supplying *Dichaete* function only in the midline and we have used the midline as an in vivo assay for *Dichaete* function. We show that mouse SOX2 rescues *Dichaete* midline phenotypes in *Drosophila* and demonstrate a genetic interaction between *Dichaete* and the POU-domain gene *ventral veinless*. Since vertebrate SOX2 interacts with the POU-domain factor OCT3 (Yuan et al., 1995), we suggest that SOX-OCT interactions have been conserved during evolution.

MATERIALS AND METHODS

Drosophila stocks

Drosophila stocks were maintained on standard yeasted cornmeal-agar food at 25°C. Mutant nomenclature follows FlyBase conventions (FlyBase, 1998). The following stocks were used: *Ab(3L)D*^{3}, *Df(3L)fz-GS1a* (*Df* in the text), (Russell et al., 1996), *vvl*^{zm} (de Celis et al., 1995); enhancer trap lines AA142 and X55 (Klämbt et al., 1991); GAL4 lines *sim*GAL4 (Scholz et al., 1997) and MZ1407 (J. Urban, personal communication). UAS*vvl* was a gift from M. Llimargas and J. Casanova (personal communication). New *Dichaete* alleles were generated in standard genetic screens, details of which will be published elsewhere (N. S. S. et al., unpublished data).

Molecular biology

The *D*^{r11} allele was cloned from a homozygous stock by PCR with primers flanking the coding region. Six independent clones from two separate PCR reactions were sequenced on both strands using an ABI Prism kit and automatic sequencer at the Department of Genetics sequencing facility. A base substitution (G to A at position 1234 of the cDNA sequence, X96419) generated *Eco*RII and *Ban*I restriction enzyme polymorphisms, which were used to confirm that the mutation is present on the *D*^{r11} chromosome and not the progenitor. UAS*Dichaete* was generated by inserting the full-length *Dichaete* cDNA into the *Eco*RI site of pUAST (Brand and Perrimon, 1993). Truncated *Dichaete* was generated by PCR to remove the last 75 amino acids and inserting the resulting product into the *Eco*RI and *Xba*I sites of pUAST. UAS*SOX2* was generated by cloning a 1.6 kb genomic DNA fragment containing the entire mouse SOX2-coding region (A gift from R. Lovell-Badge; Collignon et al., 1996) between the *Not*I-*Xba*I sites of pUAST. Constructs were injected into *y w* embryos using standard techniques (Karess, 1985).

Generation of anti-Dichaete antiserum

A 1131 bp *Pvu*II-*Dra*I cDNA fragment, containing the coding sequence for amino acids 11 to 382 of *Dichaete* (Russell et al., 1996) was subcloned into *Sma*I-digested pUC18. DNA from a clone where the *Pvu*II-generated end is next to the *Kpn*I site of the pUC polylinker was digested *Eco*RI and *Aat*II and the generated fragment cloned between the *Eco*RI and A*at*II sites of pGEX3X (Pharmacia). Fusion protein was purified from *E.coli* strain BL21-DE3 according to the manufacturer's recommendations. Purified glutathione-S-transferase fusion protein was used to immunise rabbits according to standard procedures (Harlow and Lane, 1988). The initial antiserum was predominately reactive against glutathione-S-transferase. However, after sequential boosts with factor-Xa cleaved and purified Dichaete protein, a high reactivity with Dichaete was achieved.

In situ hybridisation and immunohistochemistry

Embryo staging was according to Campos-Ortega and Hartenstein (1985). In situ hybridisation with a digoxigenin-labelled *argos* cDNA probe (Freeman et al., 1992) was carried out with minor modifications to the procedure of Tautz and Pfeifle (1989). Antibody stainings were carried out essentially as described (Patel, 1994). Primary antibodies were detected with biotin-conjugated secondaries and the ABC elite kit (Vectastain), with alkaline phosphatase-conjugated secondaries (Dakopatts) or with fluorescent secondaries (Jackson Immunoresearch). The following primary antibodies were used at the indicated dilutions: Rabbit anti-Dichaete 1/2000, mouse anti-β-Galactosidase 1/10000 (Cappel), mouse anti-BP102 1/100 and mouse anti-Fasciclin II 1/50 (Developmental Studies Hybridoma Bank, Iowa) and mouse anti-Slit 1/10 (a gift from D. Hartley).

RESULTS

Dynamic expression of *Dichaete* in the developing CNS

Dichaete mRNA is expressed in a dynamic pattern throughout embryogenesis. In order to study the tissue-specific and temporal aspects of *Dichaete* expression in greater detail, we generated a polyclonal antiserum against the Dichaete protein. As expected for a putative transcription factor, the protein is nuclear in most tissues; however, in some cells, particularly in the neuroectoderm, high levels are found cytoplasmically. The significance of this observation is at present unclear. The pattern of Dichaete expression is very similar to that of *Dichaete* mRNA (Russell et al., 1996, Nambu and Nambu, 1996). An early dynamic phase similar to that shown by other zygotic transcriptional regulators involved in segmentation is followed, after gastrulation, by widespread expression in the central nervous system (CNS) of the trunk and head. Dichaete is first detected in the neuroectoderm at late stage 6 to early stage 7. The expression is very dynamic and over the course of the next few hours waves of neuroblasts initiate Dichaete expression (Fig. 1B,D). By stage 16, Dichaete is restricted to two clusters of cells in each of the thoracic segments (Fig. 1H) and a single cell in each of the abdominal segments (data not shown). Dichaete is also localised in the hindgut, brain and the chordotonal organs of peripheral nervous system (Fig. 1C,E,G).

Dichaete is localised in the developing midline; it is first detected very weakly at early stage 7 (data not shown) and by stage 9 strongly labels the two rows of midline progenitors (Fig. 1B). As development proceeds, expression is lost from some midline cells (Fig. 1F). We mapped these Dichaete-expressing cells with the enhancer trap line AA142 which, from stage 12, expresses *lacZ* in the anterior midline glia (MGA) and the middle midline glia (MGM) and at much lower levels in the posterior midline glia (MGP) (Klämbt et al., 1991). At stage 12, Dichaete colocalises with β-gal from the AA142 enhancer trap in all three pairs of midline glia and, as with AA142, expression is strongest in the MGA and MGM (Fig. 2A,C). By stage 14, Dichaete is now found at high levels in MGP and at much lower

levels in MGA and MGM (Fig. 2D-F); this is in contrast to AA142, where the MGA and MGM continue to express *lacZ* at high levels, Thus, Dichaete expression in the midline is dynamically modulated as in the neuroectoderm.

Dichaete mutants have specific axonal defects

The strong segmentation phenotype of *Dichaete* null alleles hampers an assessment of the role of *Dichaete* in neurogenesis (Goodman and Doe, 1993). To circumvent this problem, we focused our analysis on the thoracic segments, since segmentation defects are largely restricted to the abdominal segments. We also generated new chemically induced *Dichaete* alleles in the hope of recovering hypomorphic mutations with reduced effects on segmentation. The D^{r11} allele is homozygous viable but is lethal over other *Dichaete* mutations. Hemizygous D^{r11} mutant embryos have infrequent segmentation defects (30% of mutant embryos) that are almost always restricted to the first and fourth abdominal segments; we never observe animals where segmentation is disrupted throughout the A-P axis. The D^{r11} mutation encodes a glycine-to-serine substitution in the second helix of the DNA-binding domain. All known SOX-domain proteins have a glycine at this position; however, other HMG-domains, for example the first HMG-domain of HMG1, have a serine at this position (Landsman and Bustin, 1993). HMG1 shows no sequence specificity but binds DNA with high affinity (Bianchi et al., 1992). Mutant D^{r11} protein tested in vitro can still bind DNA but with reduced sequence specificity (S. Oehler, personal communication) suggesting it may be defective in target site recognition. D^{r72}, a second chemically induced allele, behaves identically to the null allele D^{3} in our experiments (Russell et al., 1996) and we used them interchangeably. However, since low levels of Dichaete are detected with our antisera, D^{r72} may retain some residual *Dichaete* function.

We analysed the CNS phenotypes of *Dichaete* alleles with a monoclonal antibody that labels all the axons of the CNS (BP102). In the majority of experiments described below, we also used anti-Fasciclin II, which specifically labels longitudinal axons and found comparable defects (data not shown). In wild-type embryos, BP102 staining shows the axons arranged in two longitudinal tracts that run the length of the anterior-posterior axis of the ventral nerve cord. In each segment anterior and posterior commissures connect the longitudinals (Fig. 3A). *Dichaete* mutants show complete or partial fusion of commissures, thinning of longitudinals and a collapse of longitudinals towards the midline; 80% of hemizygous D^{3} and D^{r72} mutant embryos are severely affected throughout the nerve cord (Fig. 3C). In hemizygotes for the hypomorphic allele, D^{r11}, 80% of the mutant embryos have neuropile defects and 30% have disruptions throughout the length of the nerve cord (Fig. 3B). We believe that the neuropile defects are due to a requirement for Dichaete in the nervous system and not a consequence of defects in segmentation for three reasons. Firstly, the segmentation defects in *Dichaete* null alleles are mainly restricted to the abdominal segments, whereas the nerve cord defects are frequent in both thorax and abdomen. Secondly, in D^{r11}, segmentation defects are infrequent and restricted to one or two segments, whereas the neuropile defects are found in most mutant individuals and, in some cases, extend over all segments. Thirdly, we are able to rescue most of the axonal defects by supplying Dichaete specifically in the nervous system (see below).

Dichaete mutants have midline defects

Midline cells, especially the midline glia (MGL), are essential for normal neuropile formation (Klämbt and Goodman, 1991; Klämbt et al., 1991; Klaes et al., 1994; Sonnenfeld and Jacobs, 1994). Since Dichaete is expressed in midline progenitors and later in MGL, we examined the fate of these cells in *Dichaete* mutants. In wild-type embryos, at the end of stage 12 when the commissures are separating, 5-6 MGL in each segment strongly express *lacZ* from the AA142 enhancer trap line (Fig. 4A). An additional 2-3 weakly staining cells lie posterior to the posterior commissure, but we excluded them from our analysis since their origin is unclear and they appear to have no role in axonal patterning (Bossing and Technau, 1994; Klämbt et al., 1991). In wild-type stage 16 embryos, the AA142-positive MGL are reduced to 4 cells per segment with 65% of the labelled nuclei located dorsal to the commissures, either over the anterior commissure or over the space separating anterior and posterior commissure. The remainder of the nuclei are ventral to and between both commissures (Fig. 4C,G). To examine the fate of these cells in *Dichaete* mutants, we generated a recombinant chromosome carrying AA142 and a *Dichaete* deficiency. In null mutants at stage 16, we found an average of 2-3 AA142-positive cells per segment (n=60 segments), a clear reduction compared to wild type (Fig. 4D). The remaining cells are almost always found ventral to the commissures and only very rarely dorsaly (Fig. 4F,H). In addition to these changes in the number and dorsoventral distribution, we also found changes in the anterior-posterior distribution of MGL. In *Dichaete* mutants, the remaining MGL are more frequently located anterior to the anterior commissure than in wild type (Fig. 4D). We examined earlier stages to establish when the MGL defects first occur. At late stage 12 in D^{r72}/Df embryos, we found on average 2-3, (2.7) MGL per segment (n=36 segments; Fig. 4B). Again, the remaining cells are mostly located ventrally. In wild-type embryos, 5-6 cells are found and approximately 65% of these are located dorsal to the commissures.

As an additional MGL marker, we examined the expression of Slit protein (Rothberg et al., 1988). In wild-type stage 16 embryos, Slit is localised on the surface of MGL and shows the glia completely ensheathing the anterior and posterior commissures (Rothberg et al., 1990; Fig. 4E). In *Dichaete* null mutants, there are few slit-expressing cells at stage 16, and those remaining are mostly located ventral to the commissures. With the cell surface marked by Slit, we can clearly see that, in *Dichaete* mutants, the cell bodies of the MGL lie along the ventral surface of the commissures; they do not migrate and ensheath the commissures (Fig. 4F). We also examined the expression of *Argos* mRNA, which is expressed in MGL (Freeman et al., 1992), and find a similar reduction in MGL number (data not shown). In the midline of *Dichaete* mutant embryos, we do not detect any significant change in the number or position of the MGP, MNB and VUMs marked with the X55 enhancer trap line (Klämbt et al., 1991; data not shown), suggesting that *Dichaete* function in the midline is restricted to the MGL marked by the AA142 enhancer trap.

Expression of *Dichaete* in the midline rescues CNS defects

To test the idea that the nerve cord phenotypes of *Dichaete* mutants are directly due to defects in the midline, we attempted to rescue the thoracic nerve cord phenotypes by directing Dichaete expression in the midline using a *sim*GAL4 line (Scholz et al., 1997). In D^{r72}/Df embryos carrying AA142, *sim*GAL4 and UAS*Dichaete*, Dichaete is expressed in all midline cells from stage 10 (not shown). In the majority of mutant embryos, the axonal phenotypes are rescued (Fig. 5A).

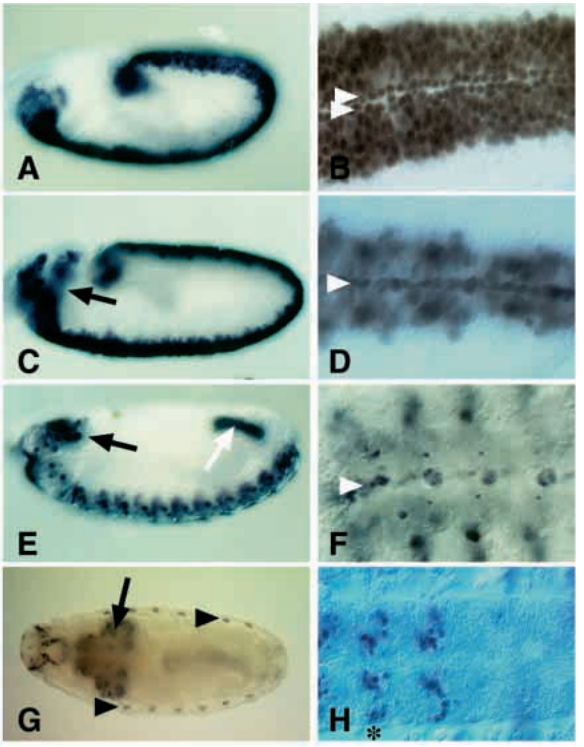

Fig. 1. Dichaete expression in the developing nervous system. Wild-type embryos stained with anti-Dichaete at stage 9 (A,B), stage 11 (C,D), stage 13 (E,F) and stage 16 (G,H). A, C and E are lateral views with dorsal to the top and anterior to the left and G is a ventral view with anterior to the left. B,D,F and H are flat preparations with anterior to the left. Midline progenitors and glia are marked with white arrowheads, the brain with black arrows and the hindgut with a white arrow. A black asterisk denotes the thoracic clusters of CNS cells and the black arrowheads the chordotonal organs of the PNS.

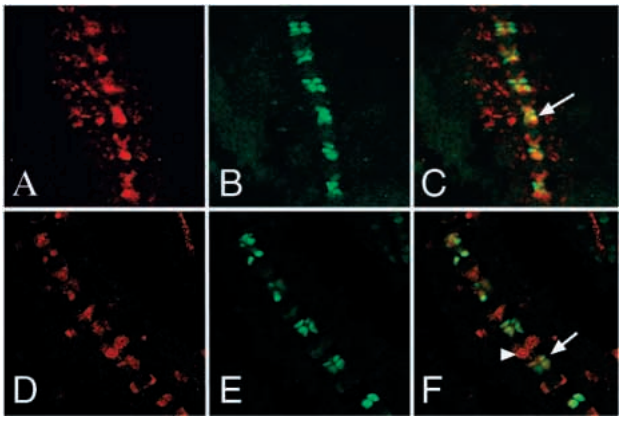

Fig. 2. Dichaete is expressed in midline glia. Flat preparations of stage 12 (A-C) and stage 14 (D-F) embryos stained with anti-Dichaete (red) and anti-β-Gal from AA142 (green) visualised by confocal microscopy. The merged images (C,F) show coexpression of Dichaete and β-Gal. White arrows denote the position of coexpressing cells and the arrowhead denotes those expressing higher levels of Dichaete. Anterior is to the top.

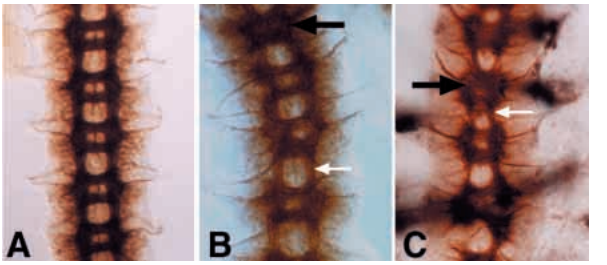

Fig. 3. Nervous system defects in *Dichaete* mutants. Flat preparations of stage 16 embryos stained with monoclonal antibody BP102. Wild type (A), D^{r11}/Df (B) and D^{r72}/Df (C). Thinning of longitudinal connectives is marked with white arrows and fusion of commissural connectives with black arrows.

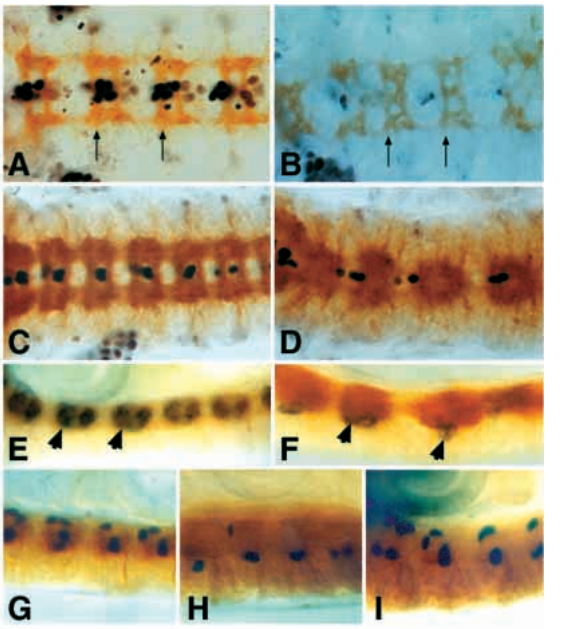

Fig. 4. *Dichaete* mutants have MGL defects. Flat preparations of thoracic segments from wild type (A,C) and *Dichaete* null mutants (B,D) carrying the AA142 enhancer trap line at stage 12 (A,B) and stage 16 (C,D). AA142-positive midline glia are in black, stained with an anti-β-Gal antibody and the structure of the neuropile is visualised with BP102 (brown). Developing anterior commissures are marked with black arrows. Lateral views of stage 16 embryos stained with BP102 (brown) and an antibody against the Slit protein (black) in wild-type (E) and *Dichaete* null embryos (F), the characteristic ensheathment of the commissures by the slit-expressing glia is absent in *Dichaete* mutants (black arrowheads). The position of the glial nuclei is also aberrant in *Dichaete* mutants. Lateral views of the thoracic segments of stage 16 embryos stained with BP102 and anti-β-Gal to reveal AA142-expressing cells, anterior to the left and dorsal to the top. In wild type (G), the glia are found both dorsal and ventral to the commissures, in *Dichaete* mutants, the few remaining glia are almost always ventral (H). The correct number and distribution of glia is restored by expressing Dichaete in the midline (I) *sim*GAL4/UAS*Dichaete; D^{r72}/Df(3L)GS1a, AA142*.

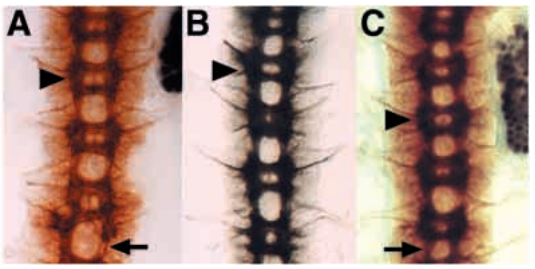

Fig. 5. Midline expression of Dichaete and mouse SOX2 restores the structure of the neuropile. Flat preparations of the thoracic segments of stage 16 embryos stained with BP102, anterior is to the top. *sim*GAL4/UAS*Dichaete*; D^{r72}/*Df* (A), *sim*GAL4/UAS*mSOX2*; D^{r72}/*Df* (B), *sim*GAL4/UAS*Dtruncated*; D^{r72}/*Df* (C). Arrowheads mark examples of rescued commissures and arrows indicate abdominal defects which may be due to defects in segmentation. See Fig. 3 for wild type and D^{r72}/*Df*.

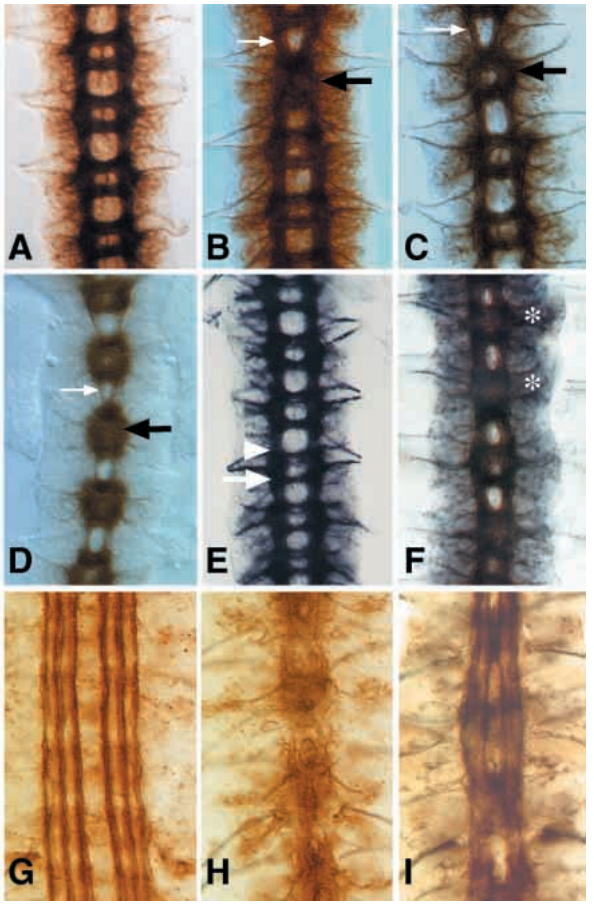

Fig. 6. *Dichaete* and *ventral veinless* show a synergistic interaction. Flat preparations of stage 16 embryos stained with BP102 in wild type (A), D^{r11}/*Df* (B), vvl^{ZM}/vvl^{ZM} (C) and D^{r11} vvl^{ZM}/D^{r11} vvl^{ZM} (D). White arrows mark thin longitudinals and black arrows mark fused or partially fused commissures. Ectopic expression of both genes outside of the midline shows synergistic effects. UAS*Dichaete*/GAL4-MZ1407 (E), the anterior commissure is frequently thicker (white arrowhead) and the posterior thinner (white arrow). UAS*Dichaete*/GAL4-MZ1407; UAS*vvl*/+ (F), the commissures appear fused (asterisk) and the nerve cord collapses towards the midline. Flat preparations of stage 16 embryos stained with anti-Fasciclin II in wild type (G), D^{r11} vvl^{ZM} /D^{r11} vvl^{ZM} (H) and UAS*Dichaete*/GAL4-MZ1407; UAS*vvl*/+ (I).

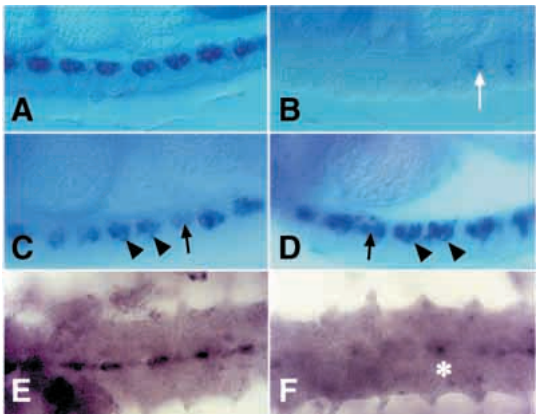

Fig. 7. Expression of *slit* and *argos* in *Dichaete* and *vvl* mutants. Anti-SLIT staining in wild type (A), Dr^{11} vvl^{ZM}/Dr^{11} vvl^{ZM} (B), vvl^{ZM}/vvl^{ZM}(C) and D^{r11}/D^{r11} (D); all embryos are lateral views at stage 16 with anterior to the left. In the double mutant, virtually all Slit-expressing cells are absent and those remaining express Slit at a very low level (white arrow). In the single mutants, Slit is expressed at lower levels in some cells (black arrows) and the morphology of remaining cells is abnormal (black arrowheads). In situ hybridisation with an *argos* cDNA probe to wild type (E) and Dr^{11} vvl^{ZM}/Dr^{11} vvl^{ZM} (F). Flat preparation of stage 16 embryos with anterior to the left, a remaining *argos*-expressing cell is marked with an asterisk.

Defects are now found in only 9% of thoracic segments and thinning of longitudinals in 17% of thoracic segments (n=100 segments), in contrast to the severe defects in 80% of thoracic segments without UAS*Dichaete*. Similarly, the number and location of AA142-positive MGL is also rescued. We found an increase from 2.3 to 3.6 cells at stage 16 (n=30 segments) with 2 cells per segment located dorsaly and 1.6 ventrally, very similar to the wild-type distribution (Fig. 4I). Ectopic expression of Dichaete in the midline of wild-type embryos does not produce any axonal phenotypes when analysed with BP102 and does not affect the number or position of MGL (not shown). Taken together, these experiments demonstrate that *Dichaete* is directly required for the development of the MGL involved in structuring the neuropile.

Evolutionary conservation of SOX gene function

Vertebrate SOX2 proteins are the closest known relatives to Dichaete. The HMG-domains of Dichaete and SOX2 are 88% identical and both proteins bind to very similar sequences (Collignon et al., 1996; S. Oehler, personal communication). To test for functional conservation, we generated transgenic flies that express the mouse *SOX2* gene under the control of the UAS system. When SOX2 is expressed under the same conditions as Dichaete in the midline of D^{r72}/*Df* mutant embryos fusion of commissures and longitudinal defects are found in only 23% of thoracic segments (n=40) (Fig. 5B). Therefore, SOX2 can efficiently substitute for Dichaete function in the midline. Outside of the DNA-binding domain the only other region of similarity between Dichaete and SOX2 is at the C terminus, this region is required for SOX2 function in a cell culture assay (Yuan et al., 1995). We generated a truncated form of Dichaete, which lacks the C-terminal 75 amino acids, and find that truncated Dichaete driven by *sim*GAL4 efficiently rescues the midline phenotypes (Fig. 5C). Thus, in this respect, SOX2 and

Dichaete do not appear to behave similarly. However, we found that, in the wing hinge, where ectopic expression of wild-type Dichaete induces structural deletions, truncated Dichaete is not functional (S.R., unpublished observations). This suggests that the C-terminal domain has a context-dependent function that is not required in the midline.

A genetic interaction between *Dichaete* and *ventral veinless*

Midline glia have been extensively studied at the cellular and molecular level, and many mutations have been isolated that affect their development (Rothberg et al., 1988; Klämbt and Goodman, 1991; Klämbt et al., 1991; Klaes et al., 1994; Sonnenfeld and Jacobs, 1994). Our findings allow us to use the midline phenotype to assay for genes that genetically interact with *Dichaete* and for potential downstream genes. Since SOX2 can rescue *Dichaete* mutant phenotypes and is known to interact with the POU-domain transcription factor OCT-3 (Ambrosetti et al., 1997), we tested for a genetic interaction between *Dichaete* and the *Drosophila* POU-domain gene *ventral veinless*. *vvl* is expressed in the midline and is required for correct MGL development (Anderson et al., 1995; de Celis et al., 1995). In embryos homozygous for the vvl^{ZM} allele, which has reduced but detectable levels of Vvl, anterior and posterior commissures fail to separate correctly, the longitudinals are thinner and there are regions where they collapse towards the midline (Fig. 6C). As with D^{r11} (Fig. 6B), only a small number of neuromeres are affected. In D^{r11} *vvl* double mutants, however, all phenotypes are far more pronounced and occur in almost every hemisegment (Fig. 6D) demonstrating a strong synergistic effect on the development of the nerve cord. Staining with anti-Fasciclin II shows that most of the longitudinal axons cross the midline many times with a *roundabout*-like phenotype (Fig. 6H; Kidd et al., 1998). We analysed the midline of the double mutant embryos with anti-Slit and found very few cells at stage 16. The few remaining cells stain very weakly and are found ventral to the commissures (Fig. 7B). Similar results are obtained with *Argos* mRNA (Fig. 7F). In single D^{r11} and *vvl* mutants, Slit expression is only weakly affected. However, glial cells with reduced expression and aberrant morphology are found (Fig. 7C,D).

To support the contention that *Dichaete* and *vvl* interact, we examined the consequences of ectopic expression of Dichaete and Vvl using a GAL4 line, MZ1407, which is expressed in many segregating neuroblasts and their progeny but not in the midline (data not shown, J. Urban, personal communication; Sweeney et al., 1995). Ectopic expression of Vvl alone does not disrupt the neuropile as seen with BP102. Expression of Dichaete alone causes weak defects in the commissures, mainly thinning of posterior commissures and thickening of the anterior commissure (Fig. 6E). When Dichaete and Vvl are expressed together, however, the neuropile phenotypes are far more severe. The longitudinals collapse towards the midline throughout the neuropile and, in some segments, commissures appear fused (Fig. 6F). In this case, unlike the D^{r11} *vvl* double mutant, anti-Fasciclin II staining shows collapse of the longitudinals toward the midline but they do not cross the midline. Taken together, these data suggest that, as in the mouse, SOX and POU domain transcription factors interact to regulate the expression of target genes.

DISCUSSION

The *Drosophila* SOX-domain gene *Dichaete* is required for the correct development of the CNS and this function is independent of an earlier requirement for *Dichaete* during blastoderm segmentation. By using a newly generated hypomorphic allele and directed transgene expression, we show that *Dichaete* function is necessary for the correct development of the midline glia of the CNS. Many vertebrate SOX genes are expressed widely in the developing CNS (Pevny and Lovell-Badge, 1997). For example, in the chick, both *SOX2* and the related *SOX3* gene are expressed in the floor plate, the vertebrate homologue of the midline (Uwangogho et al., 1995). Similarly, in the mouse, *SOX1*, *SOX2* and *SOX3* are all expressed in overlapping domains and appear to have a degree of functional redundancy in the CNS (Pevny et al., 1998). The CNS-specific phenotypes found in *Dichaete* mutants permit a genetic analysis of the role of SOX-domain genes in CNS morphogenesis and we have used this system to provide in vivo evidence for a conserved SOX-POU interaction.

Evolutionary conservation of SOX gene function

The HMG-domain of Dichaete is 88% identical to that of vertebrate SOX2, and the ability of mouse SOX2 to rescue the neuropile phenotype of *Dichaete* mutants suggests that the sequence conservation is reflected in functional conservation. The HMG-domain of SOX proteins induces a strong bend in DNA upon binding (Ferrari et al., 1992; Giese et al., 1992) and it has been suggested that the role of some of these transcription factors could be mainly architectural. In this context, it is not surprising that Dichaete function can be provided by SOX2, since they have virtually identical HMG-domains and they bind to very similar sequences (S. Oehler, personal communication). Outside of the DNA-binding domain, there is almost no similarity between Dichaete and SOX2. Towards the C terminus of SOX2, there is a 30 amino acid region that is reported to function as a context-dependent activation domain (Yuan et al., 1995). Although there is a region of limited similarity in the Dichaete protein, we find that elimination of this domain from Dichaete does not affect its ability to function in the midline. Interestingly, we find that it does affect Dichaete function when assayed in other tissues, notably the wing imaginal disc (S. R., unpublished data), supporting the suggestion that some SOX-domain proteins have context-dependent activation domains (Giese and Grosschedl, 1993).

In tissue culture and in vitro experiments, SOX2 has been shown to interact with the POU-domain protein OCT-3 (Yuan et al., 1995). Our demonstration of a genetic interaction between *Dichaete* and the POU-domain gene *ventral veinless* suggests that SOX-POU interactions have been conserved during evolution. SOX2 and OCT-3 interact via their DNA-binding domains (Ambrosetti et al., 1997) and it is possible that this type of interaction also occurs between Dichaete and Vvl. In this respect, the hypomorphic allele, D^{r11}, is of interest since the mutation is associated with an amino acid substitution in the DNA-binding domain that appears to reduce DNA-binding specificity. It is possible that the change in the DNA-binding domain could also affect the proposed interaction with Vvl. While we favour the interpretation that Dichaete and Vvl act together to activate expression of target genes, the synergistic effect of *Dichaete* and *vvl* mutations on midline

development could, in principal, be explained in different ways. It is possible that both genes have partially redundant functions in the midline or that they operate on different, but converging pathways. Since we find that ectopic expression of Vvl only shows an effect when it is co-expressed with Dichaete outside the midline, this possibility is considered to be unlikely. Similarly we have no evidence to suggest that either gene is required for the expression of the other in the midline (N. S. S., unpublished observations).

Dichaete is required for midline development.

The rescue of the axonal defects observed in *Dichaete* null alleles by targeted expression of Dichaete in the midline demonstrates that these phenotypes are a consequence of a direct function of Dichaete in the midline and not a secondary effect resulting from segmentation defects. In the wild-type embryo, Dichaete is initially expressed in all cells of the midline and later becomes restricted to the midline glia. In *Dichaete* mutants, we find defects only in the MGL and not in other midline cells, such as the VUM neurones and the MNB and its support cells, suggesting that the axonal defects are a consequence of defects only in MGL. Using independent markers, AA142, Slit and *Argos*, we can reliably analyse MGL from the end of stage 12 where we find 2-3 MGL in *Dichaete* mutants as opposed to the 5-6 cells found in wild-type embryos. Thus Dichaete is required early for the formation or survival of the correct number of MGL. Interestingly, the phenotypes reported for a hypomorphic *slit* allele that has reduced Slit expression, include early loss of MGL and commissure fusion and are very similar to those observed in *Dichaete* mutants (Rothberg et al., 1990). This observation raises the possibility that *slit* is a target gene for Dichaete. As we describe, Slit expression is severely reduced in D^{r11} vvl^{ZM} double mutants at stage 16 and, in the single mutants, we find variability in Slit expression within individual embryos. Earlier, at stage 13, in both *Dichaete* null and D^{r11} vvl^{ZM} double mutants, we find fewer Slit-expressing cells and those remaining have variable levels (data not shown). It is possible that the early defects that we observe in the MGL of *Dichaete* mutants reflect a reduction in *slit* expression. Since early *slit* expression is not eliminated in any of the mutant conditions that we examined, it is probable that other transcription factors, such as Single minded, must be sufficient to initiate *slit* expression (Nambu et al., 1990). Thus Dichaete may have a role in maintaining *slit* expression. When we ectopically express Dichaete and/or Vvl outside of the midline, we do not find ectopic expression of Slit; this result supports our view that these transcription factors are not sufficient for the initiation of *slit* transcription outside the midline.

Our analysis also indicates that those MGL that remain in *Dichaete* mutants are abnormal. In wild-type embryos, the MGL are clustered anterior to the commissures prior to commissure separation, but after the commissures separate the nuclei are found between the commissures. The MGL do not migrate correctly in *Dichaete* mutants since they are more frequently found in anterior positions with respect to the commissures. Similar defects in the dorsoventral positioning of MGL are also observed since they are frequently found ventral to the commissures and fail to ensheath the commissural axons as in the wild type. The fusion of commissures and defects in the longitudinal connectives found in *Dichaete* mutant embryos may be a consequence of the defects in MGL migration and commissure ensheathment. In *pointed*, *Star* and *spi* mutants, the correct number of MGL develop, but they fail to migrate correctly. These mutations are associated with a fusion of commissures, indicating that the migration of midline glia is required for correct commissure separation (Klämbt, 1993; Klämbt et al., 1991). However, we can not eliminate the possibility that the early reduction in MGL contributes to the phenotype. Thus, it is possible that *Dichaete* is required at an early stage in the development or specification of MGL and also later for the correct behaviour of surviving glial cells. At present, it is not clear if these functions are separable, one regulating glial number before commissure formation and a second regulating the properties of glial cells later.

Note. Supplementary figures containing data not shown are available at our web site: http://www.gen.cam.ac.uk/~sr120/index2.html

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