Toutatis, a TIP5-related protein, positively regulates Pannier function during *Drosophila* neural development

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Summary

The GATA factor Pannier (Pnr) activates proneural expression through binding to a remote enhancer of the *achaete-scute* (*ac-sc*) complex. Chip associates both with Pnr and with the (Ac-Sc)-Daughterless heterodimer bound to the *ac-sc* promoters to give a proneural complex that facilitates enhancer-promoter communication during development. Using a yeast two-hybrid screening, we have identified Toutatis (Tou), which physically interacts with both Pnr and Chip. Loss-of-function and gain-of-function experiments indicate that Tou cooperates with Pnr and Chip during neural development. Tou shares functional domains with chromatin remodelling proteins, including TIP5 (termination factor TTFI-interacting protein 5) of NoRC (nucleolar remodelling complex), which mediates repression of RNA polymerase 1 transcription. In contrast, Tou acts positively to activate proneural gene expression. Moreover, we show that Iswi associates with Tou, Pnr and Chip, and is also required during Pnr-driven neural development. The results suggest that Tou and Iswi may belong to a complex that directly regulates the activity of Pnr and Chip during enhancer-promoter communication, possibly through chromatin remodelling.

Key words: Toutatis, Pnr, Chip, Iswi, Neural development, *achaete-scute*, Enhancer-promoter communication, *Drosophila melanogaster*

Introduction

Transcriptional activation of many developmentally regulated genes is mediated by proteins binding to enhancers scattered over the genome, raising the question on how long-range activation is restricted to the relevant target promoter (Bulger and Groudine, 1999; Dorsett, 1999). Numerous studies have highlighted the essential role of boundaries, which maintain domains independent of their surrounding (West et al., 2002).

The patterning of the large sensory bristles (macrochaetae) on the thorax of *Drosophila melanogaster* is a powerful model to study how enhancers communicate with promoters during regulation of gene expression. Each macrochaeta derives from a precursor cell selected from a group of equivalent *ac-sc*-expressing cells, the proneural cluster (Gomez-Skarmeta et al., 2003). *ac* and *sc* encode basic helix-loop-helix proteins (bHLH) that heterodimerize with Daughterless (Da) to activate expression of downstream genes required for neural fate. Transcription of *ac* and *sc* in the different sites of the imaginal disc is initiated by enhancers of the *ac-sc* complex (Gomez-Skarmeta et al., 1995; Garcia-Garcia et al., 1999) and the expression is maintained throughout development by autoregulation mediated by the (Ac-Sc)-Da heterodimers binding to E boxes within the *ac-sc* promoters (Martinez and Modolell, 1991; Van Doren et al., 1991; Van Doren et al., 1992). Each enhancer interacts with specific transcription factors that are expressed in broader domains than the proneural clusters and define the bristle prepattern (Gomez-Skarmeta et al., 2003). Thus, the GATA factor Pannier (Pnr) (Ramain et al., 1993; Heitzler et al., 1996) binds to the dorsocentral (DC) enhancer (Garcia-Garcia et al., 1999) and activates proneural expression to promote development of DC sensory organs. The *Drosophila* LIM-domain-binding protein 1 (Ldb1), Chip (Morcillo et al., 1997) physically interacts both with Pnr and the (Ac-Sc)-Da heterodimer to give a multiprotein proneural complex which facilitates the enhancer-promoter communication (Dorsett, 1999; Ramain et al., 2000).

Chromatin plays a crucial role in control of eukaryotic gene expression and is a highly dynamic structure at promoters (Näär et al., 2001). In *Drosophila*, the polycomb (Pc) group and the trithorax (Trx) group proteins are chromatin components that maintain stable states of gene expression and are involved in various complexes (Simon and Tamkun, 2002). The Pc group proteins are required to maintain repression of homeotic genes such as *Ultrabithorax*, presumably by inducing a repressive chromatin structure. Members of the Trx group were identified by their ability to suppress dominant Polycomb phenotypes. We recently provided evidence that enhancer-promoter communication during Pnr-driven proneural development is negatively regulated by the Brahma (Brm) chromatin remodelling complex (Heitzler et al., 2003; Treisman et al., 1997; Collins et al., 1999), homologous to the yeast SWI/SNF complex.

Here, we present Toutatis (Tou), a protein that associates both with Pnr and Chip and that positively regulates activity of the proneural complex encompassing Pnr and Chip during enhancer-promoter communication. Tou has been previously identified in a genetic screen for dominant modifiers of the extra-sex-combs phenotype displayed by mutant of

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polyhomeotic (*ph*), a member of the Pc group in *Drosophila* (Fauvarque et al., 2001). Tou shares functional domains with Acf1, a subunit of both the human and *Drosophila* ACF (ATP-utilizing chromatin assembly and remodelling factor) and CHRAC (chromatin accessibility complex) (Ito et al., 1999), and with TIP5 of NoRC (nucleolar remodelling complex) (Strohner et al., 2001). Hence, Tou regulates activity of the proneural complex during enhancer-promoter communication, possibly through chromatin remodelling. Moreover, we show that Iswi (Deuring et al., 2000), a highly conserved member of the SWI2/SNF2 family of ATPases, is also necessary for activation of *ac-sc* and neural development. Since Iswi is shown to physically interact with Tou, Pnr and Chip, we suggest that a complex encompassing Tou and Iswi directly regulates activity of the proneural complex during enhancer-promoter communication, possibly through chromatin remodelling.

Materials and methods

Yeast two-hybrid system

Sequences encoding the DNA binding domain (DBD) of Pnr (Arg141-Thr280) were PCR amplified and inserted in frame with the LexA DBD (LexA^{DBD}Pnr^{DBD}) into pBTM116. The resulting fusion protein was used as bait to screen an embryonic cDNA expression library made into pASV4 carrying the VP16 activation domain (VP16^{AD}) (Beackstead et al., 2001). Yeast two-hybrid screening followed the method of Le Douarin et al. (Le Douarin et al., 2001).

β-Galactosidase assays on transformants of the L40 yeast strain were carried out as described previously (Seipel et al., 1992). VP16^{AD} and LexA^{DBD} fusion proteins expressed throughout the current study did not activate the β-galactosidase reporter in L40 cells. Expression of LexA and VP16 fusion proteins were analysed by western blot using anti-LexA- or anti-VP16-specific antibodies.

Plasmid constructions

Sequences encoding the Tou domains (TouA: Thr546-Met1415, TouB: Met1-Ala600, TouC: Thr546-Glu1120, TouD: Ala1089-Met1415, TouE: Pro1303-Iso1866, TouF: Pro1826-Val2086, TouG: Asp2059-Asn2615, TouH: Pro2446-Leu2857, TouI: His2831-Ser3116, TouJ: Thr546-Glu953, TouK: Leu954-Glu1120, TouL: Ala1089-Pro1238, TouM: Gly1239-Asn1307 and TouN: Pro1308-Met1415) were PCR amplified and inserted in frame with VP16^{AD} into pASV4. The fragment encoding TouA was also inserted into pXJB, which allows expression in transfected Cos cells of tagged proteins carrying the B epitope of the oestrogen receptor at their N terminus (Ramain et al., 2000). The expression vector pXJPnr, encoding the truncated wild-type Pnr has been described previously (Haenlin et al., 1997). The expression vector encoding the full-length Chip carrying a N-terminal Flag epitope (F-Chip) has also been described previously (Ramain et al., 2000). pXJF-Iswi encodes a full-length Iswi carrying an N-terminal Flag epitope. The expression vectors encoding the N-terminal domain of Chip (B10-N^{T}Chip) and the C-terminal domain of Chip (B10-C^{T}Chip) carrying at their N terminus the B epitope of the oestrogen receptor were described by Ramain et al. (Ramain et al., 2000). Sequences encoding the N^{T} Chip, the C^{T} Chip and Iswi were also inserted in frame with the LexA^{DBD} into pBTM116.

Fly stocks and genetics

The following mutant stocks were used: *pnr*^{D1}, *pnr*^{VX1} (Ramain et al., 1993), *Chip*^{E} (Ramain et al., 2000) and *osa*^{616} (Treisman et al., 1997). *tou*^{1} and *tou*^{2} (Fauvarque et al., 2001). *tou*$^{E44.1}$ was generated by imprecise excision of *EY08961* (Bellen et al., 2004). Misexpression of full-length transgenes was achieved using the UAS/Gal4 system, using the following stocks: EP622, an EP target element from the collection of P. Rorth (Rorth, 1996) allowing overexpression of *tou*, *UAS-Iswi*^{K159R} (Deuring et al., 2000). Thirty hemithoraces were examined for each of the genotypes presented in Fig. 2. For each genotype, the phenotype was found to be remarkably similar from fly to fly.

DNA tranfections in Cos cells, immunoprecipitations

Cell transfections, protein extracts preparations, immunoprecipitations and western blot analysis follow the method of Haenlin et al. (Haenlin et al., 1997).

GST pull-down assays

The method of Ramain et al. (Ramain et al., 2000) was used for GST pull-down assays.

Staining for β-galactosidase activity

Wing discs were stained using the method of Cubadda et al. (Cubadda et al., 1997). A101 contains a *lacZ* gene inserted into the *neuralized* locus (Boulianne et al., 1991) and is found by specific staining in all sensory organs precursors (Huang et al., 1991). The transgenic strain *DC-aclacZ* has been described previously (Garcia-Garcia et al., 1999). In Fig. 6, each staining was for one hour at 22°C and a representative imaginal disc is shown for each genotype. The stainings were done at different stages of the study but they were performed using the same batch of reagents. In addition, control stainings of imaginal discs from wild-type larvae were included in each experiment to allow comparisons between the different genotypes.

Results

Toutatis physically interacts with Pannier

Previous experiments in vertebrates (Tsang et al., 1997) and in *Drosophila* (Haenlin et al., 1997; Ramain et al., 2000) have demonstrated that the function of GATA factors during development is regulated by dimerization of their DNA binding domain (DBD) with cofactors. Hence, we used the yeast two-hybrid system to identify Pnr-interacting proteins and we used a bait in which the DBD of Pnr was fused to the DBD of LexA. A screen of 10^6 primary transformants of a *Drosophila* embryonic cDNA expression library yielded 35 potential positive clones. Among the different clones, we identified a domain of Tou, designated as TouA (Fig. 1B). Tou is predicted to encode a 3116 amino acids sequence protein that contains a MBD domain, a DDT domain, a WAKZ motif, two PHD zinc fingers and a C-terminal bromodomain (Fig. 1). Tou shares these functional domains with members of the WAL family of chromosomal proteins, including human TIP-5 (Strohner et al., 2001), *Drosophila* Acf1 (Ito et al., 1999) and human WSTF (Williams syndrome transcription factor) (Lu et al., 1998). This observation suggests that these proteins may have related functions.

Toutatis function is required for neural development

Pnr promotes development of DC sensory organs (Garcia-Garcia et al., 1999; Ramain et al., 2000). Tou physically interacts with Pnr in yeast and *tou* mRNA was found to be ubiquitously expressed in the wing imaginal disc (data not shown). *tou* mRNA is expressed in the wing pouch, in agreement with the wing defects associated with *tou* alleles (Fauvarque et al., 2001) and in the dorsal-most region of the thorax, covering the site of appearance of the DC bristles and the domain of *pnr* expression. Hence, we asked whether Tou is also required during development of DC sensory organs.

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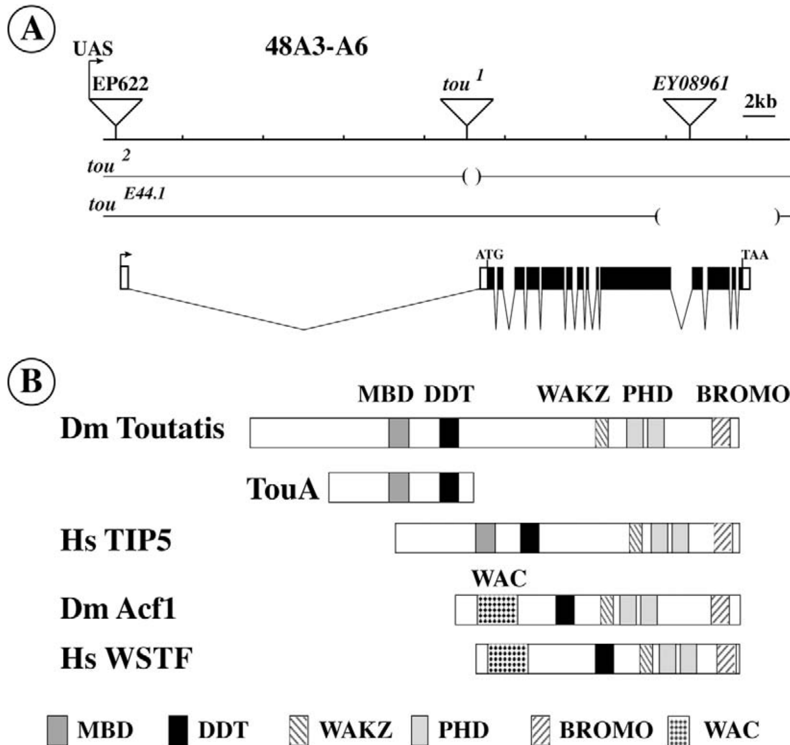

Fig. 1. Tou displays homology with members of the WAL family of chromatin remodelling factors. (A) Physical map of the *tou* locus. Structure of the *tou* gene and locations of the transposon insertions in *EP662*, tou^{1} and *EY08961* are shown. tou^{2} and $tou^{E44.1}$ were generated by imprecise excision of tou^{1} and *EY08961*, respectively. The transposon in *EP622* contains UAS sequences that allow overexpression of *tou* with the UAS/Gal4 system. Noncoding and coding regions of the *tou* transcript are depicted as open and filled boxes, respectively. (B) Tou shares functional domains with proteins expressed in *Drosophila* (Dm Acf1) and in human (Hs TIP5; Hs WSTF) and involved in chromatin remodelling. Tou carries a MBD domain (methyl-CpG binding domain), a DDT domain (after DNA binding homeobox and different transcription factors), a WAKZ motif (WSTF/Acf1/KIAA0314/ZK783.4), two PHD fingers (plant homeodomain) and a C-terminal bromodomain (BROMO). TouA, isolated during the yeast two-hybrid screening, contains the MBD and DDT domains. Dm Acf1 and Hs WSTF lack the MBD but display a N-terminal WAC motif (WSTF; Acf1; cbp146).

tou^{1} corresponds to a PlacW transposon insertion within the large intron of *tou* at 836 bases pairs (bp) upstream of the second exon of *tou* that contains the ATG translational start (Fig. 1A) (Fauvarque et al., 2001). tou^{2} was generated by imprecise excision of the tou^{1} insertion, in which 200 bp of the intron was replaced by a 7 kb sequence from the P-element. Oregon wild-type flies have 2.00 DC bristles per heminotum (Fig. 2A) We found that both tou^{1} and tou^{2} give rise to homozygous escapers lacking DC bristles (Fig. 2B) (1.92±0.05 and 1.4±0.05 DC bristles/heminotum, respectively). It suggests that *tou* is necessary for DC neural development. tou^{1} and tou^{2} are associated with molecular lesions affecting intronic sequences and display an abnormal bristle phenotype, suggesting that tou^{1} and tou^{2} disrupt functioning of an important regulatory element. Accordingly, the level of *tou* expression is strongly reduced in tou^{2} thoracic discs (data not shown), suggesting that tou^{2} is likely to be a loss of function allele. This hypothesis is reinforced by the fact that the loss of DC bristles is more severe for tou^{1}*/Df(2R)en-SFX31* and tou^{2}*/Df(2R)en-SFX31* (1.23±0.09 and 1.15±0.06 DC bristles/heminotum, respectively) than for tou^{1} and tou^{2} (1.92±0.05 and 1.4±0.05 DC bristles/heminotum, respectively). *Df(2R)en-SFX31* is a deficiency that spans *tou* and other genes between 48A1 and 48B5. Moreover, the requirement for *tou* to promote DC neural development is further demonstrated by analysis of $tou^{E44.1}$. $tou^{E44.1}$ was generated by imprecise excision of the transposon insertion in *EY08961* (Fig. 1A) (Bellen et al., 2004) and encodes a mutant protein lacking the C terminus, containing the PHD fingers and the bromodomain. Since the $tou^{E44.1}$ flies lack DC bristles (1.57±0.13 DC bristles per heminotum) and because PHD fingers and the bromodomain are believed to play important functions, we conclude that $tou^{E44.1}$ is a loss-of-function allele and that Tou is required to promote neural development.

We next investigated genetic interactions between the *pnr* and *tou* alleles. We made use of tou^{1} and tou^{2}, which are loss-of-function alleles (Fig. 1) and addressed whether they interact with pnr^{D1}. pnr^{D1} encodes a mutant protein carrying a single point mutation in the DBD that disrupts interaction with the Ush antagonist (Cubadda et al., 1997; Haenlin et al., 1997). Hence, Pnr^{D1} constitutively activates *ac-sc* at the DC site, leading to excess DC sensory organs (Ramain et al., 1993; Haenlin et al., 1997). pnr^{D1}/+ flies have 3.34±0.1 DC bristles per heminotum (Fig. 2C) and we observed that this excess is suppressed when *tou* function is simultaneously reduced. For example, tou^{2}*;* pnr^{D1}/+ flies have 2.17±0.08 DC bristles per heminotum (Fig. 2D). Conversely, pnr^{VX1} encodes a truncated protein, lacking the domain of interaction with Chip, which is required for enhancer-promoter communication during *ac-sc* expression and development of DC sensory organs (Ramain et al., 2000). Hence, pnr^{VX1} displays a loss of DC bristles (1.86±0.03 DC bristles/heminotum) and this loss is aggravated when *tou* function is simultaneously reduced. tou^{2}/+; pnr^{VX1}/+ flies display 1.57±0.05 DC bristles/heminotum. $Chip^{E}$ carries a single point mutation that disrupts the enhancer-promoter communication (Ramain et al., 2000) and is associated with reduced proneural expression at the DC site and loss of sensory organs (1.6±0.07 DC bristles/heminotum) (Fig. 2E). The loss of DC bristles was accentuated when *tou* function was simultaneously reduced as in $tou^{2}Chip^{E}$ flies, which display 1.11±0.05 DC organs/heminotum (Fig. 2F). These observations indicate that Tou probably cooperates with Pnr and its Chip cofactor during development of the DC bristles.

We also analysed the effects of overexpressed *tou* on neural development. We made use of the UAS/Gal4 system and *tou*

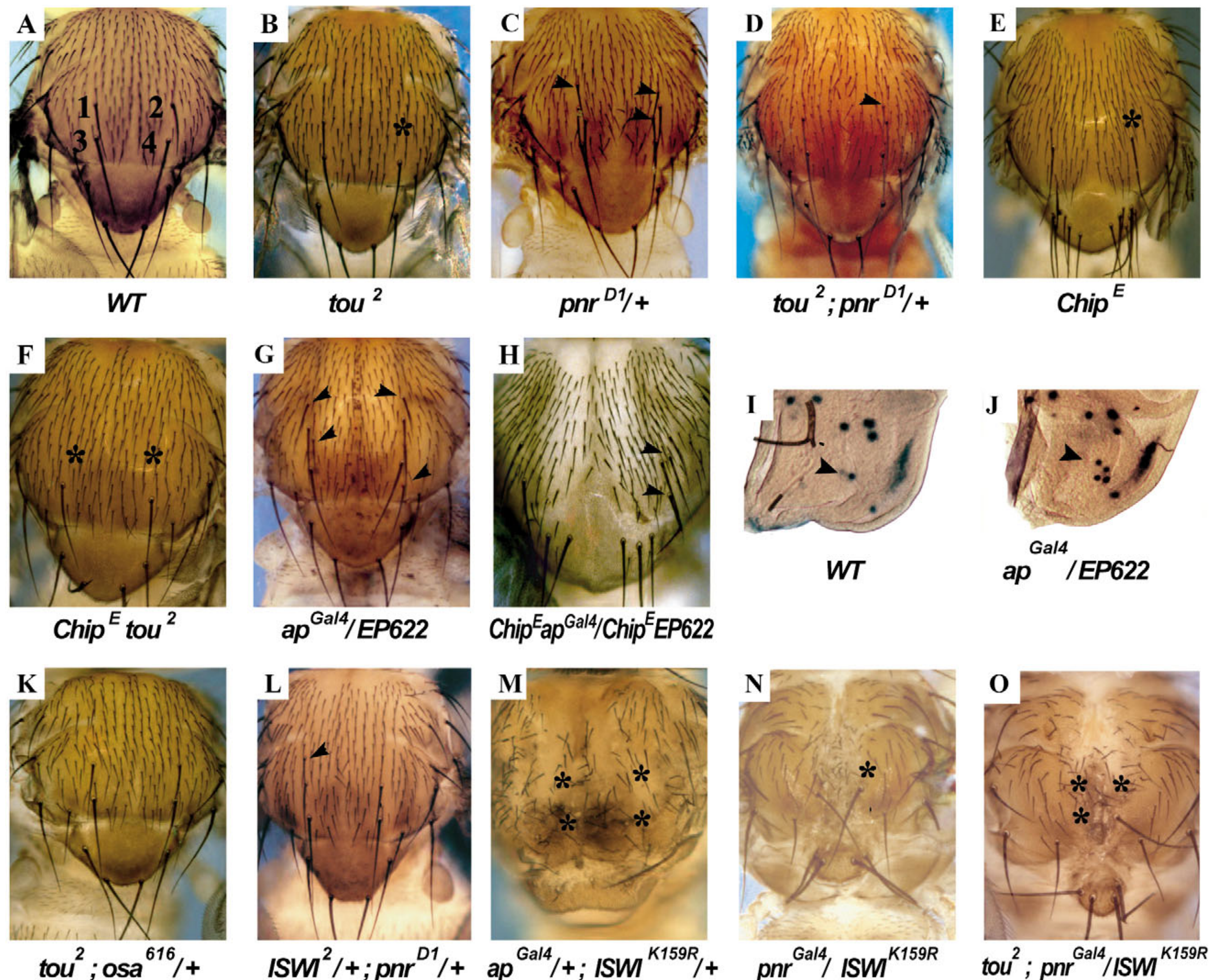

Fig. 2. *tou* mutants genetically interact with *pnr* and *Chip* and exhibit loss of DC bristles. (A) Wild-type flies have four DC bristles (1-4). (B) Loss of function *tou* alleles [tou^{1}, $tou^{E44.1}$ (not shown), tou^{2}] results in phenotypes similar to *pnr* mutants, which lack DC bristles (asterisk). (C,D) tou^{2} behave as dominant suppressor (tou^{2}; pnr^{D1}/+) of the excess of DC bristles (arrowheads) observed with pnr^{D1} encoding a constitutive activator of *ac-sc* (pnr^{D1}/+). (E,F) tou^{2} behave as dominant enhancer ($Chip^{E}$ tou^{2}) of the loss of DC bristles associated with $Chip^{E}$. (G) In contrast, overexpressed *tou* in the domain of *apterous* expression (ap^{Gal4}/*EP622*) leads to an excess of DC bristles (arrowheads) associated with extra DC precursors in the imaginal disc (J), as revealed by A101 staining, in comparison with staining of wild-type disc (I). (H) Overexpressed *tou* suppresses the loss of DC bristles associated with $Chip^{E}$ ($ap^{Gal4}Chip^{E}$/$EP622Chip^{E}$) and produces extra sensory organs (arrowheads). (K) *tou* and *osa* are antagonistic during neural development. Lowering the dosage of Osa suppresses the loss of DC bristles characteristic of tou^{2} (tou^{2}; osa^{616}/+). (L) Reducing Iswi function ($Iswi^{2}$/+; pnr^{D1}/+) also suppresses excess DC bristles associated with pnr^{D1}. (M) Overexpression of the dominant-negative Iswi (ap^{Gal4}/$Iswi^{K159R}$) in the domain of *ap* expression leads to loss of multiple sensory organs, including the DC bristles (asterisks). (N,O) The loss of DC bristles (asterisks) resulting from overexpressed $Iswi^{K159R}$ in the domain of *pnr* expression (pnr^{Gal4}/$Iswi^{K159R}$) is aggravated when tou function is simultaneously reduced (tou^{2}; pnr^{Gal4}/$Iswi^{K159R}$), reinforcing the hypothesis that Tou and Iswi could be subunits of a multiprotein complex regulating neural development.

was overexpressed in the dorsal compartment of the disc *Gal4-*ap^{MD544} (Rincon-Limas et al., 1999). *Gal4-*ap^{MD544}/*EP622* flies have excess DC sensory organs (2.78±0.12 DC bristles/heminotum) (Fig. 2G). Moreover, the loss of DC bristles associated with $Chip^{E}$ is suppressed when Tou is overexpressed (Fig. 2H). Indeed, $Chip^{E}$ ap^{Gal4}/$Chip^{E}$ *EP622* flies have 2.23±0.08 DC bristles/heminotum.

The development of extra DC sensory organs, revealed by phenotypic analysis, was compared with the positions of DC bristle precursors detected with a *lacZ* insert, A101, in the *neuralized* gene that exhibits staining in all sensory organs. In *EP622/Gal4-*ap^{MD544}, additional DC precursors are seen that lead to excess DC bristles (Fig. 2I,J). Hence, we conclude that Tou cooperates with Pnr and Chip during neural development.

Toutatis interacts both with the DBD and the C terminus of Pannier

TouA physically interacts with the DBD of Pnr in yeast. Since the C terminus of Pnr also mediates physical interactions with cofactors (Ramain et al., 2000), we then investigated whether

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TouA interacts with the C terminus of Pnr in yeast (Fig. 3A). Expression vectors encoding either the C terminus of Pnr fused to the LexA DBD ($LexA^{DBD}Pnr^{CT}$) or the unfused LexA DBD ($LexA^{DBD}$) were introduced into the L40 yeast strain together with the vector for the unfused VP16 activation domain ($VP16^{AD}$) or for TouA fused to $VP16^{AD}$ ($VP16^{AD}TouA$). L40

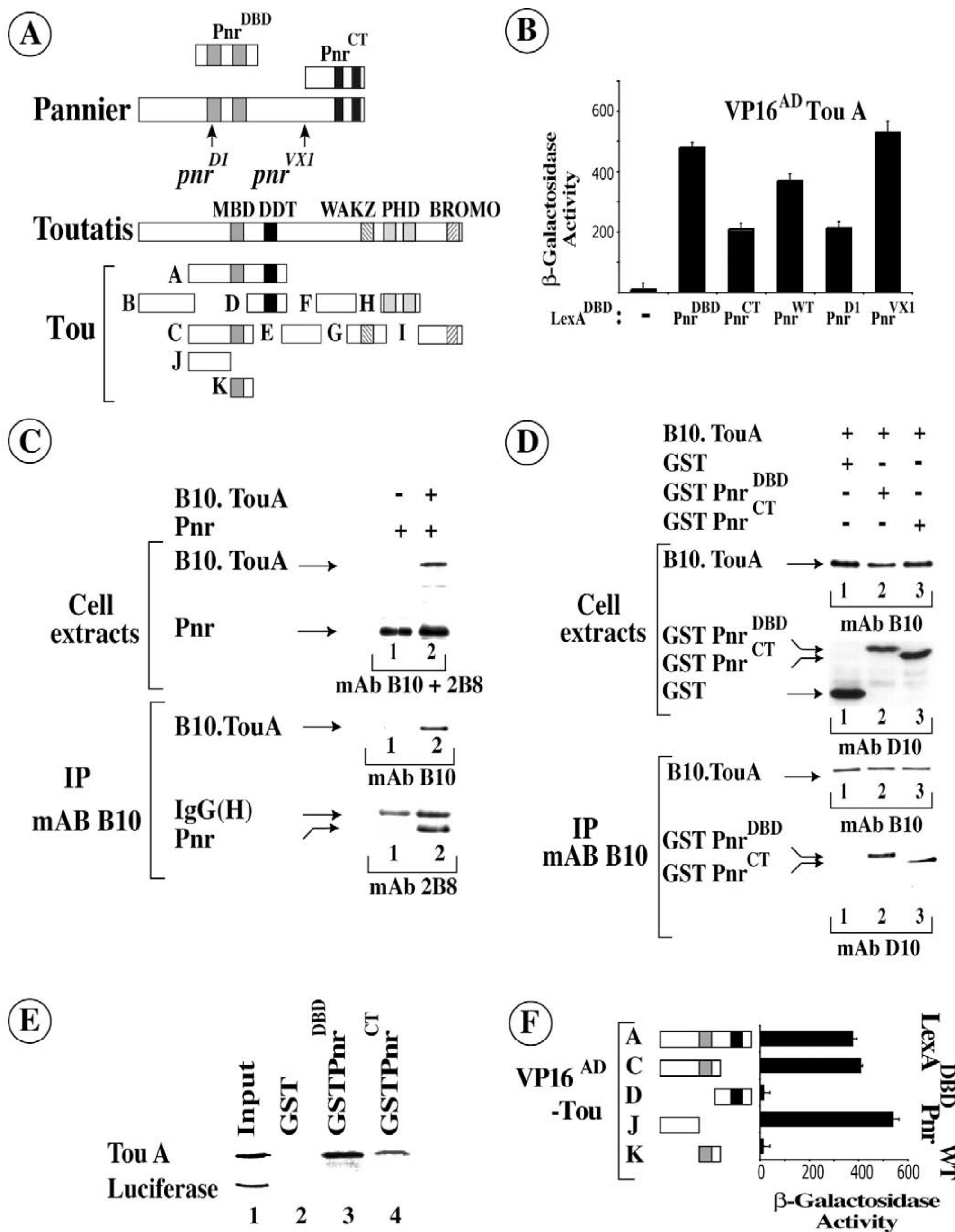

Fig. 3. Tou physically interacts with Pnr. (A) Structural features of the Pnr and Tou proteins used in the present study. The DNA binding domain of Pnr (Pnr^{DBD}), containing the two zinc fingers (grey boxes) and the C-terminal domain (Pnr^{CT}) containing the two amphipathic α-helices (black boxes). The mutations associated with pnr^{D1} and with pnr^{VX1} are localized within the N-terminal zinc finger and in the N terminus of the helices respectively. A schematic drawing of the Tou domains used throughout this study (TouA to TouK). The functional domains of Tou are schematized as in Fig. 1. (B) Tou physically interacts with Pnr in yeast, through both the DBD and the C-terminus of Pnr. Expression vectors for unfused $LexA^{DBD}$(–) or $LexA^{DBD}Pnr^{DBD}$, $LexA^{DBD}Pnr^{CT}$, $LexA^{DBD}Pnr^{WT}$, $LexA^{DBD}Pnr^{D1}$, $LexA^{DBD}Pnr^{VX1}$ were introduced into L40 cells together with the unfused $VP16^{AD}$ (not shown) or $VP16^{AD}TouA$. Protein extracts made from cultured L40 transformants were assayed for β-galactosidase activity (expressed in nanomoles of substrate/mn/mg of protein). Values (±10%) are the averages of three independent experiments. (C,D) Tou physically interacts with (C) full-length Pnr, (D) the DBD of Pnr and the C terminus (CT) of Pnr in transfected cells. In each case, an immunoblot of a representative set of transfected cell extracts is shown. The transfected expression vectors are shown at the top of the panels. The B10 monoclonal antibody used to immunoprecipitate the extracts is shown on the left of the panels. The antibodies used to reveal the blots are indicated at the bottom. Pnr is recognized by the 2B8 antibody, $GSTPnr^{DBD}$ and $GSTPnr^{CT}$ are detected by the D10 antibody and the B10-tagged TouA is recognized by the B10 antibody. The locations of the proteins including the immunoglobulin heavy chain [IgG(H)] are indicated at the sides. (E) Tou directly interacts both with the DBD and the CT of Pnr in vitro. (E) Autoradiographs of SDS-PAGE gels from representative affinity chromatography experiments performed with GST control beads (lane 2), $GSTPnr^{DBD}$ beads (lane 3) and $GSTPnr^{CT}$ beads (lane 4) and in vitro translated ^{35}S proteins as indicated on the left. One-tenth of the ^{35}S input is shown in lane 1. Luciferase is used as a negative input. Experiments were performed three times and, with all proteins except luciferase, 50-fold more protein bound to $GSTPnr^{DBD}$ and $GSTPnr^{CT}$ than to GST control. (F) The N terminus of the MBD domain of Tou mediates interaction with Pnr. Expression vectors for unfused $LexA^{DBD}$ (not shown) or $LexA^{DBD}Pnr^{WT}$ were introduced in L40 cells together with the unfused $VP16^{AD}$ (not shown) or $VP16^{AD}TouA$, $VP16^{AD}TouC$, $VP16^{AD}TouD$, $VP16^{AD}TouJ$ or $VP16^{AD}TouK$. Protein extracts made from cultured L40 transformants were assayed for β-galactosidase activities (expressed in nanomoles of substrate/mn/mg of protein). Values (±10%) are the averages of three independent experiments.

cells contain a *lacZ* reporter driven by eight LexA binding sites. Protein extracts were prepared from L40 transformants grown in liquid medium and were assayed for β-galactosidase activity (Fig. 3B). No reporter activity was observed in extracts made from yeast expressing $LexA^{DBD}Pnr^{CT}/VP16^{AD}$, $LexA^{DBD}/VP16^{AD}TouA$ or $LexA^{DBD}/VP16^{AD}$. In contrast, a robust activation of the reporter was detected with yeast expressing $LexA^{DBD}Pnr^{CT}/VP16^{AD}TouA$, indicating that TouA associates with the C terminus of Pnr (Fig. 3B). Then, both the DBD and the C terminus of Pnr physically interact with TouA. Accordingly, we found that TouA interacts with wild-type Pnr (Pnr^{WT}) in which both the DBD and the C terminus of Pnr are present and with Pnr^{VX1}, a truncated Pnr lacking the C terminus (Fig. 3B). TouA also associates with Pnr^{D1}, a mutated Pnr in which the structure of the DBD is probably disrupted since a coordinating cysteine of the N terminal zinc finger has been replaced by a tyrosine (Fig. 3B).

We then analysed whether TouA interacts with Pnr in a cultured cell line. We performed immunoprecipitations of protein extracts made from Cos cells transfected with expression vectors for TouA and either Pnr (Fig. 3C) or GST fusion proteins containing the DBD of Pnr ($GSTPnr^{DBD}$) or the C terminus of Pnr ($GSTPnr^{CT}$) (Fig. 3D). We found that Pnr, $GSTPnr^{DBD}$ and $GSTPnr^{CT}$ coimmunoprecipitate with TouA (Fig. 3C,D). Finally, we also tested the abilities of in vitro-translated ^{35}S-labelled TouA to bind to $GSTPnr^{DBD}$ or $GSTPnr^{CT}$ attached to glutathione-bearing beads. TouA interacts both with the DBD of Pnr and the C terminus of Pnr (Fig. 3E). As expected, TouA did not bind GST control beads, whereas $GSTPnr^{DBD}$ and $GSTPnr^{CT}$ did not bind the negative luciferase input.

To investigate the interactions between Tou and Pnr in more detail, we constructed several expression vectors encoding in yeast contiguous domains of Tou (Fig. 3A,F; TouB to TouK). The various segments of Tou were fused with $VP16^{AD}$ and introduced into yeast together with unfused $LexA^{DBD}$ or $LexA^{DBD}Pnr^{WT}$. We observed activation of the reporter only in yeast expressing $LexA^{DBD}Pnr^{WT}/VP16^{AD}TouC$ and in yeast expressing $LexA^{DBD}Pnr^{WT}/VP16^{AD}TouJ$, showing that the amino terminus of the MBD domain of Tou mediates physical interactions with Pnr in yeast.

Toutatis also interacts with Chip

Chip is an essential cofactor of Pnr (Ramain et al., 2000) since it facilitates enhancer/promoter communication necessary for *ac-sc* expression during Pnr-driven neural development. Since TouA associates with Pnr in yeast, we made use of the yeast two-hybrid system to ask whether TouA also associates with Chip. Chip was fused to $LexA^{DBD}$ and assayed for interaction with $VP16^{AD}TouA$. We observed a strong increase of β-galactosidase activity in extracts made from yeast expressing $LexA^{DBD}Chip/VP16^{AD}TouA$ above the background level seen with extracts made from yeast expressing $LexA^{DBD}/VP16^{AD}TouA$ or $LexA^{DBD}Chip/VP16^{AD}$ (Fig. 4B; data not shown), indicating that TouA associates with Chip (Fig. 4B).

Sequence comparison between the Ldb proteins from various species reveals two conserved functional domains, involved in protein-protein interactions (Matthews and Visvader, 2003). Thus, Chip (Fig. 4A) contains a N-terminal homodimerization domain, also involved in interactions with Pnr (Ramain et al., 2000) and Osa (Heitzler et al., 2003) and a C-terminal LIM interacting domain (LID) mediating heterodimerization with LIM-homeodomain proteins (LIM-HD) (Fernandez-Funez et al., 1998) and basic helix-loop-helix proteins (bHLH) (Ramain et al., 2000).

We next examined the interaction between TouA and Chip. Assays for β-galactosidase activity revealed that the interaction between TouA and Chip is mediated by the N-terminal homodimerization domain (Fig. 4B). We also investigated whether TouA can interact with Chip in a cultured cell line by immunoprecipitating proteins extracts of Cos cells transfected with expression vectors encoding TouA and Chip, the N-terminal domain of Chip (N^T Chip) or the C-terminal domain of Chip (C^T Chip) (Fig. 4C). We found that TouA physically interacts with Chip and the interaction is mediated by the N-terminal domain of Chip. Finally, we further found that in vitro translated ^{35}S-labelled TouA interacted with GST Chip attached to glutathione-bearing beads (Fig. 4D) but did not bind GST control beads, whereas GST Chip did not bind the negative luciferase input.

We next investigated the interaction between Tou and Chip in more detail. The segments of Tou (Fig. 3A: TouB to TouI; Fig. 4E: TouL to TouN) were fused with the $VP16^{AD}$ and introduced into yeast together with unfused $LexA^{DBD}$ or $LexA^{DBD}Chip$. We found that only TouD containing the DDT domain associates with Chip (Fig. 4E). Moreover, the physical interaction was shown to be mediated by the DDT domain itself (Fig. 4E; TouM).

Tou functionally cooperates with Pnr and Chip during neural development. Since Tou physically interacts with Pnr and Chip, we then asked whether a ternary complex containing Tou, Pnr and Chip can exist in living cells. We performed double immunoprecipitations of extracts made from Cos cells containing Pnr, Tou and Chip (Fig. 5). The extract was first immunoprecipitated with the M2 antibody recognizing the flagged TouA (Fig. 5B; IP 1) and the precipitated proteins were recovered by elution with the Flag peptide. The eluate was then immunoprecipitated with the B10 antibody (Fig. 5B: IP 2) directed against the tagged Chip. We observed that Pnr coimmunoprecipitates with Chip and TouA (Fig. 5B), indicating that the trimer Chip-Tou-Pnr can be formed. Moreover, this suggests that Chip and Pnr act together to recruit Tou and to target its activity to the *ac-sc* promoter sequences**.** This observation is reminiscent to what was previously observed on the role of Osa during neural development (Heitzler et al., 2003). Osa belongs to the Brm complex and it was suggested that Pnr and Chip cooperate to recruit Osa and to target activity of the Brm complex to the *ac-sc* promoter sequences, leading to negative regulation of enhancer-promoter communication. However, Tou and Osa display antagonistic activities during regulation of *ac-sc* and may probably define distinct chromatin remodelling complexes since the loss of DC bristles associated with reduced *tou* function (tou^2 flies have 1.4±0.05 DC bristles/heminotum) is suppressed by lowering the dosage of *osa*. Indeed, tou^2*;* osa^{616}/+ flies have 1.97±0.07 DC bristles/heminotum (Fig. 2K).

Toutatis is required to activate proneural expression

To investigate whether the interactions between Pnr, Chip and Tou function in vivo, we examined effects of both loss-o-

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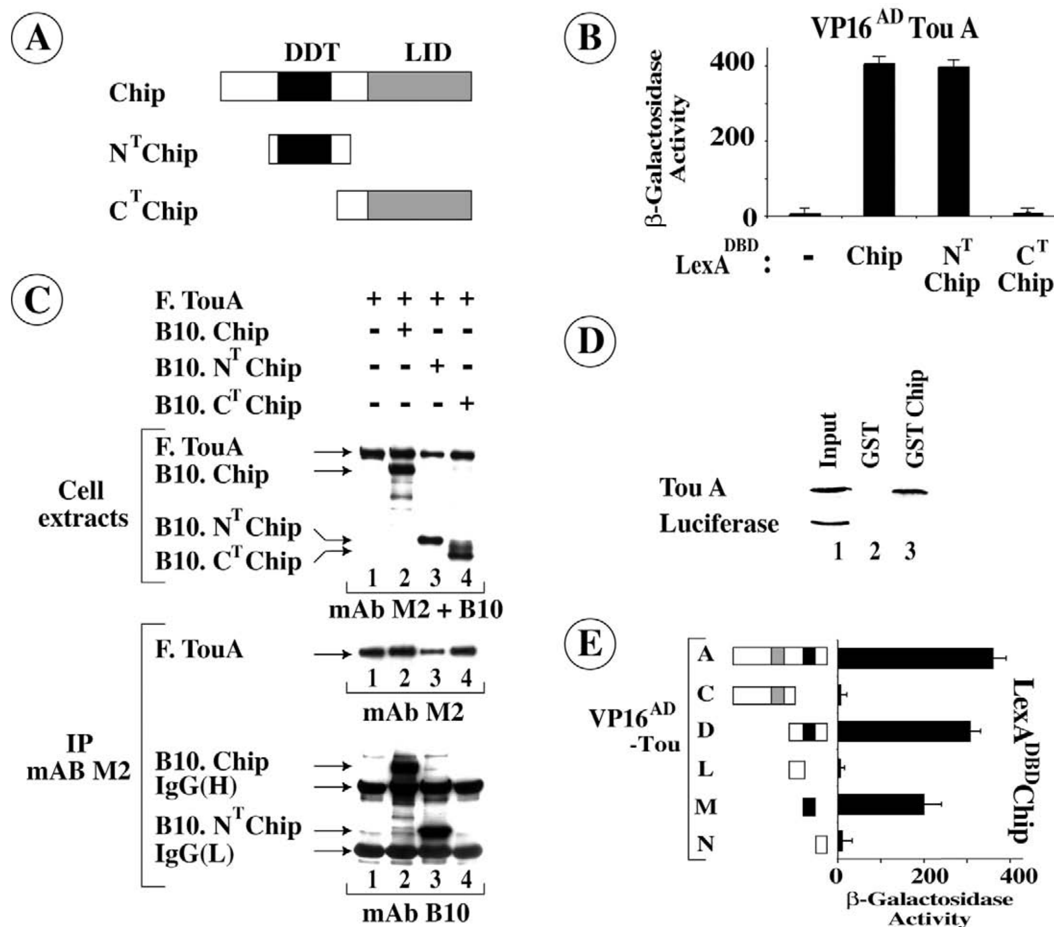

Fig. 4. Tou physically interacts with Chip. (A) Structural features of the Chip proteins used in this study: the N-terminal homodimerization domain (DDT) of Chip (N^TChip; black box) and the C-terminal LIM-interacting domain (LID) of Chip (C^TChip; grey box). (B) Tou interacts with Chip in yeast through the N-terminal homodimerization domain. Expression vectors encoding the unfused $LexA^{DBD}$ (–) or the $LexA^{DBD}$Chip, the $LexA^{DBD}N^T$Chip, the $LexA^{DBD}C^T$Chip were introduced in L40 cells together with the unfused $VP16^{AD}$ (not shown) or $VP16^{AD}$TouA. Protein extracts made from L40 transformants, grown in liquid medium, were assayed for β-galactosidase activities, which are expressed as in Fig. 3B. (C) Tou interacts with Chip in transfected cells. The layout is as in Fig. 3C. The flagged Chip is detected with the M2 antibody whereas the B10-tagged proteins (B10-Chip, B10- N^TChip, B10-C^TChip) are recognized by the B10 antibody. (D) Tou directly interacts with Chip in vitro. (D) Autoradiographs of SDS-PAGE gels from representative affinity chromatography experiments performed with GST control beads (lane 2) and GST Chip beads (lane 3) and in vitro translated ^{35}S proteins as indicated on the left. One-tenth of the ^{35}S input is shown in lane 1. Luciferase was used as a negative input. Experiments were performed three times and 50-fold more ^{35}S labelled TouA bound to GST Chip than to GST control. (E) The DDT domain of Tou mediates interaction with Chip. Expression vectors encoding the unfused $LexA^{DBD}$ (not shown) or the $LexA^{DBD}$Chip were introduced into L40 cells together with the unfused $VP16^{AD}$ (not shown) or the $VP16^{AD}$TouA, $VP16^{AD}$TouC, $VP16^{AD}$TouD, VP 16^{AD}TouL, $VP16^{AD}$TouM or $VP16^{AD}$TouN. Protein extracts made from L40 transformants, grown in liquid medium, were assayed for β-galactosidase activities (expressed as in B).

function and gain-of-function of *tou* on expression of a *lacZ* reporter driven by a minimal *ac* promoter fused to the DC enhancer [transgenic line *DC:ac-lacZ* (Garcia-Garcia et al., 1999)]. We found that expression is strongly impaired in tou^2 flies and in $tou^{E44.1}$ flies (Fig. 6B,C), in agreement with the lack of DC bristles. In contrast, overexpressed Tou (ap^{Gal4}/*EP622*) leads to increased *lacZ* expression at the DC site (Fig. 6G,H), consistent with the excess of DC bristles (Fig. 2D). The excess of DC bristles in pnr^{D1} correlates with expanded *ac-sc* expression at the DC site (Fig. 6D). Since loss of *tou* function suppresses the excess DC bristles associated with pnr^{D1}, we addressed the consequence of loss of *tou* function on expression of the *lacZ* reporter in pnr^{D1}/+ flies. We found that expanded *lacZ* expression associated with pnr^{D1} is decreased when *tou* function is simultaneously reduced (Fig. 6A,D,E). We conclude that Pnr and Tou cooperate during neural development and regulate *ac-sc* expression through the DC enhancer.

Iswi cooperates with Toutatis during neural development

The primary structure of Tou reveals that, among the members of the WAL family of chromatin remodelling factors, it is most closely related to TIP5 (Fig. 1). The fact that TIP5 associates in vivo with SNF2h, the mammalian homologue of *Drosophila* Iswi (Strohner et al., 2001) prompted us to investigate whether Iswi associates with Tou and plays a role during neural development.

We first investigated genetic interactions between *Iswi* and *pnr*. We made use of *Iswi*^{1} and *Iswi*^{2}, which are both characterized by a point mutation that introduces a premature stop codon and are consequently predicted to encode truncated Iswi. However, *Iswi*^{1} and *Iswi*^{2} behave as null alleles since truncated Iswi^{1} and Iswi^{2} are not detected in western analyses, suggesting that the C terminus is required to stabilize Iswi (Deuring et al., 2000). The *Iswi*^{2}/+ flies are wild type (2.00 DC bristle/heminotum) whereas the *pnr*^{D1}/+ flies have excess DC bristles (3.34±0.1 DC bristle/heminotum). However, this excess is lowered when *Iswi* function is simultaneously reduced [*Iswi*^{2}/+; *pnr*^{D1}/+ flies display 2.84±0.18 DC bristles/heminotum] (Fig. 2C,L. Conversely, the lack of DC bristles associated with *pnr*^{VX1} (1.86±0.03 DC bristles/heminotum) is aggravated when *Iswi* function is simultaneously reduced (1.61±0.07 DC bristles/heminotum). We also observed that the loss of DC bristles associated with *Chip*^{E} (1.6±0.07 DC bristles/heminotum) is accentuated when Iswi function is simultaneously reduced (1.29±0.07 DC bristles/heminotum) (data not shown). Thus, loss-of-function *Iswi* alleles behave like loss-of-function *tou* alleles, implying that Iswi is also required during neural development and suggesting that Iswi and Tou may act as subunits of a multiprotein complex.

It is not possible to generate flies lacking both maternal and zygotic *Iswi* function (Deuring et al., 2000). Hence, to assess the consequences of the loss of *Iswi* function on neural development we performed overexpression experiments of a dominant negative Iswi. We used the UAS-Iswi^{K159R} line (Deuring et al., 2000) which allows overexpression of a dominant negative Iswi through the UAS/Gal4 system. A conserved lysine in the ATP-binding site of the ATPase domain is replaced with an arginine. This K159R substitution eliminates ATPase and chromatin-remodelling activities of Iswi in vitro, but does not affect the stability of Iswi and its incorporation into high molecular mass complexes. Hence Iswi^{K159R} behaves as a strong dominant negative protein.

Overexpression of Iswi^{K159R} in the precursor cells using the *sca*^{Gal4} driver provokes a loss of multiple sensory bristles (Deuring et al., 2000) and it has been proposed that Iswi has a late function during neural development, essential for either viability or division of the precursor cells. We then induced widespread expression of Iswi^{K159R} in the dorsal compartment of the imaginal wing disc using *Gal4-ap*^{MD544}. Overexpression of Iswi^{K159R} is therefore induced early during development and results in a lack of sensory organs, including a frequent loss of DC bristles (Fig. 2M).

Tou and Iswi promote DC development and could be subunits of a multiprotein complex. However, we can also hypothesize that Tou can substitute for Iswi function and vice versa. To address this issue, we overexpressed Iswi^{K159R} in either a wild-type genetic background or in conditions of loss of *tou* function. Overexpressed Iswi^{K159R} in the domain of *pnr* expression reduces DC bristles (1.56±0.11 bristle/heminotum) (Fig. 2N). The loss of DC bristles is aggravated when the *tou* function is simultaneously reduced. Indeed, *tou*^{2}/+; *pnr*^{Gal4}/*UASIswi*^{K159R} and *tou*^{2}; *pnr*^{Gal4}/*UASIswi*^{K159R} flies have 1.14±0.12 bristle/heminotum (data not shown) and 0.75±0.11 bristle/heminotum (Fig. 2O), respectively. This observation reinforces the hypothesis that Tou and Iswi could be subunits of a complex during neural development.

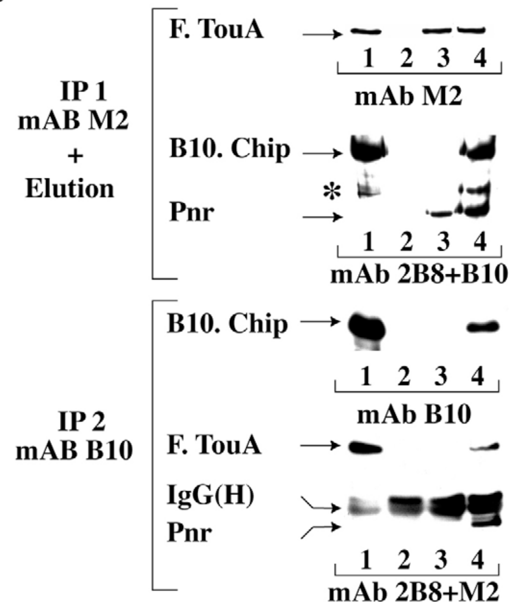

Fig. 5. The trimer Pnr-Chip-Tou can exist in living cells. The ternary complex is revealed by double-immunoprecipitation of protein extracts made from transfected Cos cells. (A) Immunoblot of a representative set of protein extracts. The transfected expression vectors are shown at the top. The asterisk denotes an artifactual band. (B) Double-immunoprecipitation of the protein extracts described in panel A. The M2 (IP 1) and B10 (IP 2) mouse antibodies are used to immunoprecipitate the flagged TouA domain (F.TouA) and the tagged full-lenght Chip (B10.Chip), respectively. They are shown on the left of the panels. After immunoprecipitation with the M2 antibody, the selected proteins were recovered by elution with the Flag peptide (IP 1 mAB M2+Elution). The antibodies used to reveal the blots are indicated at the bottom of the panels. Pnr, the F-TouA domain and the B10-Chip are recognized by the 2B8, the M2 and the B10 antibody, respectively. The locations of the proteins, including the IgG(H) of the B10 antibody, are indicated by arrows.

We next investigated whether overexpressed Iswi^{K159R} affects the activity of the DC enhancer. We found that overexpression of Iswi^{K159R} leads to (Fig. 6I) reduced expression of the *lacZ* reporter driven by the *ac* minimal promoter fused to the DC enhancer (line *DC: aclacZ*) (Garcia-Garcia et al., 1999). We next examined the consequences of loss of *Iswi* function associated with the *Iswi*^{1}/*Iswi*^{2} transheterozygous combination, which dies during late larval

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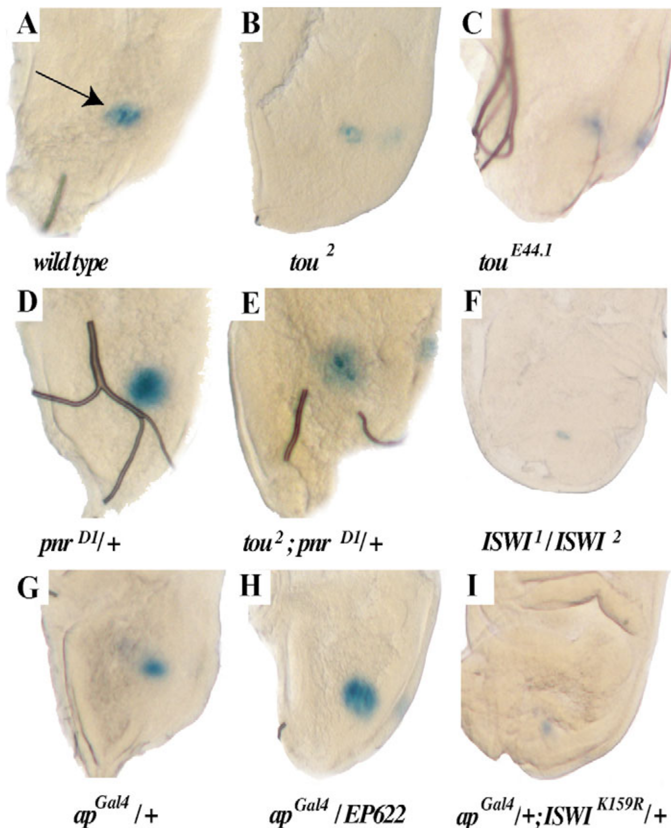

Fig. 6. Tou regulates proneural expression through the DC-specific enhancer of the *ac-sc* complex. Late third instar thoracic imaginal discs show *lacZ* expression at the DC site (Garcia-Garcia et al., 1999). In each case, the reaction was left for one hour at 22°C. (A) Wild-type pattern with *lacZ* expression in the DC area (arrow) (w^{1118}/+; *DC: ac-lacZ*/+). (B,C) Decrease of *tou* function results in a reduction in *lacZ* activity at the DC site (B: tou^2; *DC: ac-lacZ*/+; C: $tou^{E44.1}$; *DC: ac-lacZ*/+). (D,E) pnr^{D1} behave as constitutive activator of proneural expression (pnr^{D1}/*DC: ac-lacZ*/+) and causes increased *lacZ* activity. Reducing *tou* function in pnr^{D1} suppresses excess *lacZ* activity at the DC site (tou^2/tou^2; pnr^{D1}/*DC: ac-lacZ*/+), demonstrating that Pnr and Tou regulate *ac-sc* expression through the DC enhancer. (F) Reduced *Iswi* function leads to a severe decrease of *lacZ* activity at the DC site ($Iswi^1/Iswi^2$; *DC: ac-lacZ*/+). (G,H,I) Overexpressed Tou activates *lacZ* expression driven by the DC enhancer (ap^{Gal4}/*EP622*; *DC: ac-lacZ*/+) whereas overexpression of the dominant-negative Iswi ($Iswi^{K159R}$) is associated with a decrease in *lacZ* activity ($ap^{Gal4}/Iswi^{K159R}$; *DC: aclacZ*/+).

stages (Deuring et al., 2000). We observed that loss of *Iswi* function leads to decreased *lacZ* expression (Fig. 6F). These observations indicate that Iswi is also necessary for activation of *ac-sc* expression at the DC site, although we cannot rule out the possibility that this interaction is indirect.

Since Tou, Pnr, Chip and Iswi are required to activate proneural expression at the DC site, we next investigated whether Iswi can interact with Tou, Pnr and Chip. We found that Iswi can interact with Tou in both yeast and Cos cells, in agreement with previous reports showing that Iswi can interact with Tou-related proteins in insect and in mouse cells (Fyodorov et al., 2002; Strohner et al., 2001). An expression vector encoding $LexA^{DBD}$Iswi was introduced in yeast together with a vector for the unfused $VP16^{AD}$ or $VP16^{AD}$ fused to one of the various segments of Tou (Fig. 3A, TouA to TouI). Measurement of β-galactosidase activity revealed that only protein extracts made from yeast expressing $LexA^{DBD}$Iswi and either $VP16^{AD}$TouA or $VP16^{AD}$TouC display activity above background levels (Fig. 7A). Hence, TouA associates in yeast with Pnr, Chip and Iswi. Finally, immunoprecipitation of protein extracts revealed that TouA and Iswi also associate in transfected Cos cells (Fig. 7B).

We next addressed whether Iswi can interact with Chip and Pnr, by testing the abilities of in vitro translated ^{35}S-labelled Iswi to bind to GST-Chip attached to glutathione-bearing beads. We found that Iswi associates with full-length Chip. Similarly, we observed that Iswi can also associate both with the DBD and the C terminus of Pnr (Fig. 7C,D). Since Pnr and Chip directly regulate *ac-sc* expression at the DC site, our findings suggest that Iswi and Tou may belong to a complex, which, in vivo, regulates the activity of the proneural complex during enhancer-promoter communication, possibly through chromatin remodelling.

Discussion

Regulation of GATA factors by dimerization

Transcriptional activity of GATA factors during development is regulated by dimerization with cofactors. This feature is illustrated in vertebrates by the function of GATA-1 during haematopoiesis (Cantor and Orkin, 2002) and in *Drosophila* by the role of Pnr during neural development. GATA factors carry a DBD containing a C-terminal zinc finger responsible for DNA-binding and a N-terminal zinc finger that stabilizes the interaction. In addition to binding DNA, the DBD makes critical interactions with proteins that modulate activity of the GATA factors (Cantor and Orkin, 2002). There is considerable evidence that these factors function as multiprotein complexes. Thus, Wadman and colleagues (Wadman et al., 1997) have identified an erythroid complex formed between the bHLH SCL/E2A, LMO2, GATA1 and Ldb1. Functional analyses of the role of *Drosophila* Pnr during neural development have shown that Pnr also contains a C-terminal domain mediating interaction with the *Drosophila* Ldb, Chip (Ramain et al., 2000). Chip assists in the formation of a proneural complex containing Pnr, Chip and the (Ac-Sc)-Da heterodimer bound to the *ac-sc* promoters and facilitates enhancer-promoter communication (Bulger and Groudine, 1999; Dorsett, 1999) required for activation of *ac-sc* and neural development. The *Drosophila* complex and the vertebrate complex (Wadman et al., 1997) contain related transcription factors, illustrating how the mechanisms of regulation of GATA factors by dimerization are remarkably conserved in a variety of species.

The activity of the *Drosophila* complex is antagonized by dimerization with various transcription factors. Pnr associates with U-shaped (Ush), a protein structurally related to Friend of Gata, since they both contain multitype zinc fingers (Cubbada et al., 1997; Haenlin et al., 1997; Tsang et al., 1997). Moreover, regulation of GATA factors by Ush and Fog appears remarkably conserved during evolution since they contain nine zinc fingers, four of which (zfs1, 5, 6 and 9) mediate interaction with the N-terminal zinc finger of the DBD of GATA factors (Fox et al., 1999). The activity of the proneural

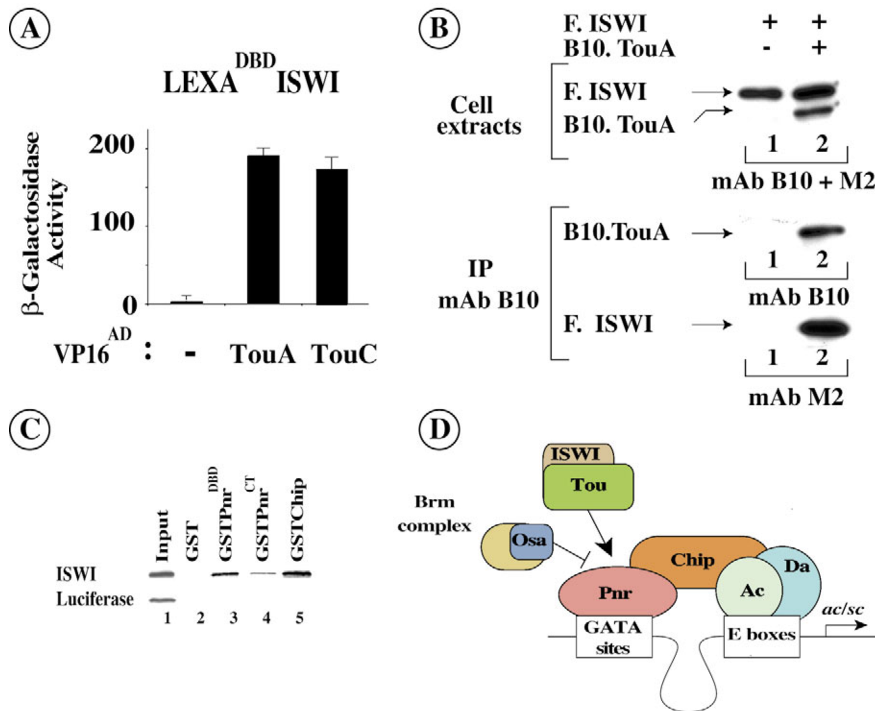

Fig. 7. Iswi associates with Tou, Pnr and Chip and directly regulates *ac-sc* expression in vivo. (A) Iswi interacts with Tou in yeast. Expression vectors encoding the unfused LexA^{DBD} or the LexA^{DBD}Iswi were introduced into L40 cells together with the VP16^{AD} (data not shown) or the VP16^{AD}TouA. Protein extracts were made from L40 transformants, grown in liquid medium, and assayed for β-galactosidase activity (expressed as in Fig. 3B). (B) Iswi interacts with Tou in transfected cells. The transfected expression vectors are shown at the top of the panels. The layout is as in Fig. 3C. F-Iswi and B10-TouA are recognized by the M2 antibody and B10 antibody, respectively. (C) Iswi directly interacts with Pnr and Chip in vitro. (C) Autoradiographs of SDS-PAGE gels from representative affinity chromatography experiments performed with GST control beads (lane 2), GST DBD Pnr (lane 3), GST C^{T} Pnr (lane 4), GST Chip beads (lane 5) and in vitro translated ^{35}S proteins as indicated on the left. One-tenth of the ^{35}S input is shown in lane 1. Luciferase is used as a negative input. (D) Model on how Tou, Iswi and the Brm complex regulate activity of the proneural complex during enhancer-promoter communication at the *ac-sc* locus. Tou and Iswi positively regulate proneural expression and may belong to a complex with antagonistic activity to that of the Brm complex (Heitzler et al., 2003). The complex containing Tou and Iswi regulates activity of Pnr and Chip during enhancer-promoter communication, possibly through chromatin remodelling.

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complex is also antagonized by dimerization of Chip with the LIM-HD Apterous and by dimerization of the proneural bHLH Ac-Sc with the bHLH Extra macrochaetae (Emc). Chip is an essential cofactor for Pnr and Apterous and neural development relies on a competition between Pnr and Ap to associate with the common Chip cofactor (Ramain et al., 2000). Emc lacks the basic domain required for DNA binding and it is believed that it sequesters Ac and Sc and prevents them from associating with Da to give the (Ac-Sc)-Da heterodimer required for activation of proneural expression (Ellis et al., 1990; Garrell and Modolell, 1990).

Our recent study in *Drosophila* has shown that the function of the proneural complex is also regulated by the Brm chromatin remodelling complex. Thus, Osa dimerizes with both Pnr and Chip, leading to recruitment of the Brm complex that regulates enhancer-promoter communication (Heitzler et al., 2003). Our present study describes Tou, a protein that associates with Iswi and that regulates activity of the proneural complex during enhancer-promoter communication, possibly through chromatin remodelling.

Does Toutatis regulate proneural expression through chromatin remodelling?

Enhancer-promoter communication is of fundamental importance, as in most cases the activities of enhancers play a determining role in turning on and off specific genes in a temporally and spatially regulated manner. Models to explain long range activation of gene expression invoke enhancer-promoter communication either through protein-protein interactions resulting in formation of loops, the free sliding of proteins recruited by a remote enhancer along DNA or the establishment of modified chromatin structure by facilitator factors which generate a chain of higher order complexes along the chromatin fibre (Bulger and Goudine, 1999; Dorsett, 1999).

In *Drosophila*, Chip was postulated to be a such facilitator required both for activity of a wing specific enhancer of the *cut* locus (Morcillo et al., 1996; Morcillo et al., 1997) and for activity of the DC enhancer of the *ac-sc* complex (Ramain et al., 2000). Enhancer-promoter communication at the *ac-sc* complex is negatively regulated by the Brm complex whose activity is targeted to the *ac-sc* promoter sequences through dimerization of the Osa subunit with both Pnr and Chip (Heitzler et al., 2003). The Brm complex is thought to remodel chromatin in a way that represses transcription.

We describe, here, the role of Tou and Iswi which may act together as subunits of a multiprotein complex to positively regulate activity of Pnr and Chip during enhancer-promoter communication. Tou and Iswi therefore display opposite activity to that of the Brm complex, raising questions about their molecular function during neural development. Tou shares essential functional domains with members of the WAL family of chromatin remodelling proteins, including Acf1 of ACF and CHRAC (Ito et al., 1999) and also TIP5 of NoRC (nucleolar remodelling complex), involved in repression of the rDNA (Santoro et al., 2002). Importantly, Acf1 and TIP5 associate in vivo with Iswi (Ito et al., 1999; Strohner et al., 2001), showing that Iswi can mediate both activation and repression of gene expression. Tou positively regulates Pnr/Chip function during the period of *ac-sc* expression in neural development, and it associates with Iswi. Since Iswi also positively regulates Pnr/Chip function, we hypothesize that a complex encompassing Tou and Iswi acts during long-range activation of proneural expression, possibly through chromatin remodelling. Further studies will help to resolve this issue.

Interestingly, Chip and Pnr seem to play similar roles both during recruitment of the Brm complex and recruitment of Tou and Iswi, since they dimerize with Osa, Tou and Iswi. In addition, Pnr and Chip apparently cooperate to strengthen the physical association with Osa and Tou. However, Osa, on the one hand, and Tou and Iswi, on the other, display antagonistic activities during neural development. Since they are ubiquitously expressed, accurate regulation of *ac-sc* expression would require a strict control of the stoichiometry between Osa, Tou and Iswi. It remains to be investigated whether the functional antagonism between Osa and Tou/Iswi relies on a molecular competition for association with Pnr and Chip. Determination of this would require a complete molecular definition of the putative complex encompassing Tou and Iswi, together with a full understanding of how this complex and the Brm complex molecularly interact with the proneural complex to regulate enhancer-promoter communication during development.

Iswi is directly required for activation of proneural expression

Biochemical analysis of Iswi and Iswi-containing complexes, together with genetic studies of Iswi and associated proteins in flies and in budding yeast, has revealed roles for Iswi in a wide variety of nuclear processes, including transcriptional regulation, chromosome organization and DNA replication (Corona and Tamkun, 2004). Accordingly, Iswi was found to be a subunit of various complexes, including NURF (nucleosome remodelling factor) (Badenhorst et al., 2002), ACF and CHRAC. Iswi-containing complexes were primarily recognized as factors that facilitate in vitro transcription from chromatin templates (Mizuguchi et al., 1997; Mizuguchi et al., 2001; Okada et al., 1998). However, genetic analysis in *Drosophila* and in *Saccharomyces cerevisiae* have provided evidence that Iswi-containing complexes are involved in both transcriptional activation and repression in vivo. For example, immunostaining of *Drosophila* polytene chromosomes of salivary glands showed that Iswi is associated with hundreds of euchromatic sites in a pattern that is non-overlapping with RNA polymerase II (Deuring et al., 2000). It suggests that Iswi may play a general role in transcriptional repression. In contrast, it was also demonstrated that expression of *engrailed* and *Ultrabithorax* are severely compromised in *Iswi*-mutant *Drosophila* larvae (Deuring et al., 2000). Recent studies have also shown that a mouse Iswi-containing complex, NoRC, plays an essential role during repression of transcription of the rDNA locus by RNA polymerase I (Strohner et al., 2001; Santoro et al., 2002; Zhou et al., 2002). Here, we present Tou, a protein that is structurally related to the TIP5 subunit of NoRC (Santoro et al., 2002). Tou positively regulates enhancer-promoter communication during Pnr-driven proneural development and its activity is targeted to the *ac-sc* promoter sequences through dimerization with Pnr and Chip. We also provide evidence that Iswi is required during neural development. Overexpression of $Iswi^{K159R}$ in the precursor cells of the sensory organs using the sca^{Gal4} driver (Deuring et al., 2000) leads to flies lacking multiple bristles, suggesting that Iswi functions late during neural development, essential for either cell viability or division of the precursor cell. Using the $Iswi^{1}/Iswi^{2}$ transheterozygous combination and individuals overexpressing $Iswi^{K159R}$ in earlier stages of development and in less restricted patterns, we show that Iswi also regulates *ac-sc* expression. Interestingly, the regulation is probably direct since Iswi associates with the transcription factors Pnr and Chip, known to promote *ac-sc* expression at the DC site (Garcia-Garcia et al., 1999; Ramain et al., 2000). Since Iswi interacts with Tou, we propose that Tou and Iswi may positively regulate activity of Pnr and Chip during enhancer-promoter communication, possibly as subunits of a multiprotein complex involved in chromatin remodelling.

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