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Role of Dpp signalling in prepattern formation of the dorsocentral mechanosensory organ in *Drosophila melanogaster*

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SUMMARY

A proneural cluster of dorsocentral bristles forms adjacent to the dorsal side of *wg*-expressing cells in the notum region of the wing imaginal disc. It has been shown that *wg* activity is required for these structures to form. However, the restriction of this proneural cluster to the dorsal posterior side of the *wg* expression domain in the anterior compartment of the wing imaginal disc has suggested that Wg signalling itself is insufficient to establish the dorsocentral proneural cluster. Some factor(s) from the posterior side must participate in this action in cooperation with Wg signalling. We have examined the role of Dpp signalling in dorsocentral bristle formation by either ectopically activating or conditionally reducing Dpp signalling. Ubiquitous activation of Dpp signalling in the notum region of the wing imaginal disc induced additional dorsocentral proneural cluster all along the dorsal side of the *wg* expression domain, and altered *wg* expression. Conditional loss-of-function of Dpp signalling during disc development resulted in the inhibition of dorsocentral proneural cluster formation and expansion of the *wg* expression domain. These results suggest that Dpp signalling has two indispensable roles in dorsocentral bristle formation: induction of the dorsocentral proneural cluster in cooperation with Wg signalling and restriction of the *wg* expression domain in the notum region of the wing imaginal disc.

Key words: *Drosophila* development, Imaginal disc, *decapentaplegic*, *wingless*, Sensory organ, Pattern formation

INTRODUCTION

Two types of sensory organs, large bristles (macrochaetes) and small bristles (microchaetes), develop in fixed numbers at constant positions on the dorsal part of the mesothorax (called notum) of *Drosophila melanogaster*. Prepattern formation of macrochaetes on the notum has provided an ideal model system for studying two-dimensional patterning. The accurate positioning of the macrochaetes is established during the third larval to early pupal stage within the epithelial sheets of the notum region of the wing imaginal discs (Hartenstein and Posakony, 1989; Huang et al., 1991). For convenience, we will refer to this region of the wing disc as the 'thoracic disc' to distinguish it from the wing pouch region. Initially, in the thoracic disc, a group of cells called a proneural cluster, characterized by the expression of the proneural genes *achaete* (*ac*) and *scute* (*sc*), are formed around the positions where macrochaetes will form (Cubas et al., 1991; Skeath and Carroll, 1991). Next, one or a few sensory mother cells (SMCs) are singled out from the proneural cluster, and each SMC subsequently undergoes two rounds of cell division forming four progeny cells that differentiate into the components of a sensory bristle (Hartenstein and Posakony, 1989; Huang et al., 1991). Thus, precise positioning of the macrochaete on the notum depends on the complex expression pattern of the *ac* and *sc* genes in the thoracic disc. *ac* and *sc* encode transcription factors of the basic helix-loop-helix family that confer upon cells the ability to become SMCs (Cabrera et al., 1987; Gonzalez et al., 1989). The removal of specific proneural clusters by *achaete-scute complex* (*ASC*) mutations leads to the absence of the corresponding SMCs and macrochaetes (Cubas et al., 1991; Gomez-Skarmeta et al., 1995).

The complex expression pattern of *ac* and *sc* is controlled through the action of enhancer-like *cis*-regulatory elements (Gomez-Skarmeta et al., 1995; Leyns et al., 1996; Ruiz-Gomez and Ghysen, 1993; Ruiz-Gomez and Modolell, 1987). These elements are presumed to respond to a 'prepattern' established by local specific combinations of factors, as first postulated by Stern (1954). The products of prepattern genes would be expected to be distributed asymmetrically in the thoracic disc and they control *ac* and *sc* expression at both transcriptional and post-transcriptional levels (reviewed by Jan and Jan, 1990; Simpson, 1996). The existence of a specific *cis*-regulatory element for individual proneural clusters has suggested that different combinations of prepattern genes promote the complex expression pattern of proneural genes (Gomez-Skarmeta et al., 1995).

The constituents of prepattern genes are largely unknown,

however, several candidate genes have been reported. Three genes residing at the *iroquois* (*iro*) locus, *araucan* (*ara*), *caupolican* (*caup*) and *mirror (mirr)*, encode a novel family of homeoproteins. These genes are expressed in the lateral half of the thoracic disc and affect proneural cluster formation in this region (Dambly-Chaudiere and Leyns, 1992; Gomez-Skarmeta et al., 1996; Gomez-Skarmeta and Modolell, 1996; Kehl et al., 1998; Leyns et al., 1996). Another possible candidate is *pannier* (*pnr*) which encodes a protein belonging to the GATA family of transcription factors (Cubadda et al., 1997; Heitzler et al., 1996; Ramain et al., 1993). Loss-of-function mutations of the *pnr* gene fail to form the proneural clusters in the dorsal side of the thoracic disc (Cubadda et al., 1997; Haenlin et al., 1997). Recently, Haenlin et al. reported that the transcriptional activity of Pnr is regulated negatively by a novel zinc finger protein, U-shaped (Ush). They suggested that the products of *pnr* and *ush* cooperate in the regulation of *ac* and *sc* expression in a specific proneural cluster, the dorsocentral proneural cluster.

Post-transcriptional regulation of proneural gene products also could contribute to prepatterning of the macrochaetes. *extramacrochaetae* (*emc*) is genetically described as an *ASC* repressor and encodes a helix-loop-helix protein devoid of a basic domain (Botas et al., 1982; Ellis et al., 1990; Van Doren et al., 1991). The Emc protein is thought to form a heterodimer with the HLH proteins encoded by the *ASC* and/or *daughterless*, thereby altering or interfering with their activity (Botas et al., 1982; Ellis et al., 1990; Van Doren et al., 1991).

Besides transcriptional regulators, morphogen gradients generated by secreted proteins could also be involved in macrochaete prepattern formation. *wingless* (*wg*), *hedgehog* (*hh*) and *decapentaplegic* (*dpp*) have been shown to generate positional information within imaginal discs (Basler and Struhl, 1994; Ingham and Fietz, 1995; Lecuit et al., 1996; Nellen et al., 1996; Neumann and Cohen, 1997; Tabata and Kornberg, 1994; Zecca et al., 1996). However, there are a few reports regarding the involvement of these secreted factors in prepattern formation of the macrochaetes. *wg* is expressed in a stripe of cells along the A/P axis in the thoracic disc (Baker, 1988; Phillips and Whittle, 1993, and also see Fig. 1-B). It has been shown that *wg* is required for the development of a subset of proneural clusters which appear in or immediately adjacent to the *wg*-expressing cells (Couso et al., 1994; Phillips and Whittle, 1993). On the other hand, ectopic activation of either Hh or Dpp signalling in the wing disc results in the induction of SMCs at numerous ectopic positions in the wing disc (Mullor et al., 1997). These results suggest that *dpp*, *hh* and *wg* participate in the prepattern formation of the macrochaetes on the notum.

Here, we have focused on whether Dpp, as a morphogen gradient, plays a part in the prepatterning of the macrochaetes. Experiments using both gain-of-function and conditional loss-of-function mutants revealed that Dpp signalling participates in this process in two major ways. One is induction of the proneural cluster in cooperation with Wg signalling and the other is restriction of the *wg* expression domain in the thoracic disc.

MATERIALS AND METHODS

Fly strains

Flies were raised on standard *Drosophila* medium at 25°C. The mutants and transgenic flies used in this work are as follows. *hs-GAL4* and *tsh-GAL4* driver lines (Shiga et al., 1996). *neur-lacZ* (*A101*), *wg-lacZ* (*17en40*), *emc-lacZ* (*emc*^{4218}), *DC enhancer fragment-3.7sc-lacZ* (*DC-lacZ*), *ac-lacZ, 3.7sc-lacZ* (Ghysen and O'Kane, 1989; Gomez-Skarmeta et al., 1995; Huang et al., 1991; Kassis et al., 1992; Van Doren et al., 1992; Wilson et al., 1989). *wg*^{Sp1} is a dominant allele (Neumann and Cohen, 1996), and *wg*^{IL114} is a temperature sensitive allele of *wg* (Nusslein-Volhard and Wieschaus, 1980). In flies of genotype *wg*^{Sp1}/*wg*^{IL114}, aDC bristles are constantly missing at 25°C. *w; wg*^{IL114} *tsh-GAL4/SM6a-TM6B* flies were crossed with *w; wg*^{Sp1}*; UAS-tkv*/SM6a-TM6B.* Pharate adult of genotype *w; wg*^{IL114} *tsh-GAL4/wg*^{Sp1}*; UAS-tkv*/+* flies can be distinguished from their 'Tubby' sibs. *punt*^{P1}/*st punt*^{135} *e* flies are viable with no phenotypes at 18°C but are lethal at or above 25°C (Letsou et al., 1995; Simin et al., 1998; Theisen et al., 1996). *w; 3.7 sc-lacZ/SM1; st punt*^{135} *e/TM6B* flies were crossed with *w; punt*^{P1}*/TM6B* and raised at 18°C until late second larval instar. Then the temperature was shifted to 29°C, using a water bath to ensure temperature constancy. Larvae at late wandering to white pupal stage were dissected and wing discs were recovered. Larvae of the *w; 3.7 sc-lacZ/+; punt*^{P1}/*st punt*^{135} *e* genotype can be distinguished from their 'Tubby' sibs. *Tb*$^{-}$ flies, whose genotypes were either *w; 3.7 sc-lacZ/+; punt*^{P1}*/TM6B or w; 3.7 sc-lacZ/+; st punt*^{135} *e/TM6B*, were used as wild-type control.

Plasmid constructions and fly transformations

A point mutation in the *tkv* cDNA (Okano et al., 1994), changing a glutamine residue (position 199) to aspartic acid was generated by PCR using mutagenic primers. It has been reported that the same amino acid substitution in Tkv results in the constitutive activation of this receptor (Hoodless et al., 1996; Nellen et al., 1996). A *Not*I-*Xho*I fragment containing the constitutively active version of *tkv* (*tkv**) cDNA was subcloned from pBluescript II KS^{-} into pUAST (Brand and Perrimon, 1993). The cDNA containing the entire *punt* ORF (Ruberte et al., 1995) was also subcloned into pUAST at the appropriate restriction sites. P-element-mediated transformation was performed using standard procedures (Rubin and Spradling, 1982; Spradling and Rubin, 1982).

Mosaic expression and conditional overexpression of *tkv** and *punt*

In the AyGAL4 construct, a FLP-out cassette containing the *hsp70* termination signals flanking the *yellow*$^{+}$ gene, flanked in turn by two FRT sites, is inserted between the *Act5C* promoter and the *GAL4* gene (Ito et al., 1997). *w; AyGAL4 UAS-GFP*^{T2} were crossed to flies of the genotype *y w hs-flp; UAS-tkv**. The resulting progeny were subjected to a heat shock (20 minutes at 37°C) during the first larval instar. *tkv** expression mosaics were monitored by GFP fluorescence. Flies of the genotype *hs-GAL4: UAS-tkv** (or *UAS-punt)* were subjected to two heat shocks at 37°C for 30 minutes separated by a 1 hour recovery at 25°C during the second to third instar larval stage, and then aged at 25°C. White pupae were collected every two hours. The heat shock time indicates the period from the beginning of the first heat shock to the pupal collection, referred to as hours 'before puparium formation' (BPF). Each dot in Fig. 1G represents an average of more than 20 animals.

Imaginal discs staining

The discs were fixed with 3.7% formaldehyde in PBS for 30 minutes at room temperature. After several washes, the discs were incubated with primary antibodies diluted in PBS containing 0.3% Triton X-100 and 10% normal goat serum (blocking solution) at 4°C overnight. After washing several times in PBS containing 0.3% Triton X-100 (PBT), discs were incubated for 2 hours at room temperature with secondary antibodies diluted in the blocking solution. After several washes in PBT, discs were mounted on the slide glass with GEL MOUNT^{TM} (Biomeda). Confocal fluorescent images were obtained

using Zeiss LSM410 or LSM510 microscopes. Antibodies were diluted as follows: anti-Wg (1:5; gift from S. Cohen); anti-β-galactosidase rabbit polyclonal antibody (1:500; Cappel); anti-rabbit IgG LRSC-conjugated (1:100; Jackson); anti-mouse IgG FITC-conjugated (1: 100; Jackson).

RESULTS

Ectopic activation of Dpp signalling induces extra macrochaete formation on the notum

Fig. 1A shows the wild-type macrochaete pattern of the notum. An anterior-dorsocentral bristle (aDC) and a posterior-dorsocentral bristle (pDC) are formed along the anterior/posterior (A/P) axis on the notum. It has been shown that *wg* activity is necessary for the formation of both aDC and pDC (Couso et al., 1994; Phillips and Whittle, 1993). However, these SMCs are not induced all along the *wg* expression domain, but induced only adjacent to the dorsal posterior side of the *wg* expression domain in the anterior compartment of the thoracic disc (Fig. 1B, we will refer to the two sides of the *wg* expression domain as the 'dorsal side', where the dorsocentral SMCs are formed, and the 'lateral side', for the opposite side). This suggests that Wg signalling alone is insufficient to induce SMCs of aDC and pDC, and that another factor(s) which resides on the dorsal posterior side of the thoracic disc is also required for inducing these SMCs. One candidate factor is Dpp. In the thoracic disc, *dpp* is induced in a stripe of cells located posterior to the dorsocentral SMCs (Fig. 1B). This expression pattern and the property of Dpp as a morphogen suggests that Dpp signalling may also participate in prepattern formation of the macrochaetes on the notum.

First, we attempted to ectopically activate Dpp signalling in the thoracic disc during larval development using the GAL4-UAS system (Brand and Perrimon, 1993). It has been shown that ectopic expression of either Dpp type-II receptor Punt, or type-I receptor Thick veins (Tkv) in which glutamine residue 199 is replaced with aspartic acid (Tkv*), activates Dpp signalling in a ligand independent manner (Hoodless et al.,

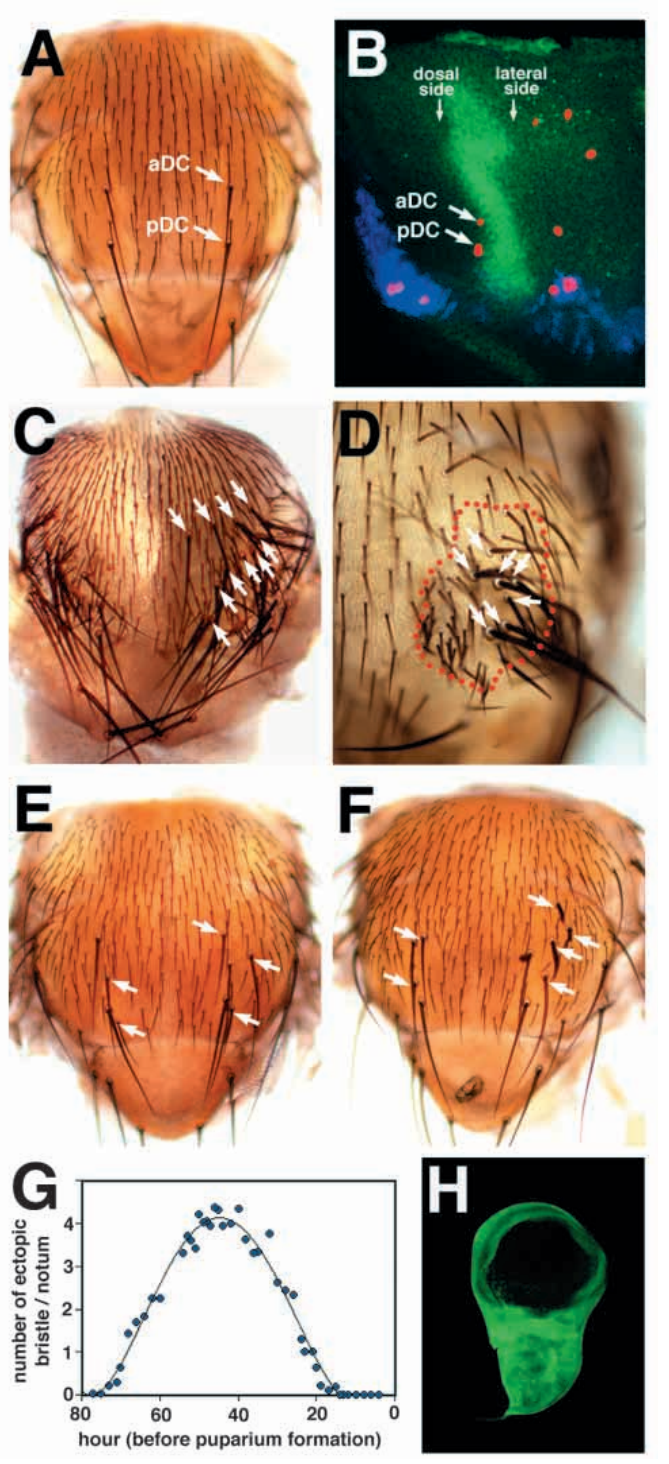

Fig. 1. Effect of ectopic Dpp signalling on the bristle pattern of the notum. (A) Two dorsocentral bristles, an anterior dorsocentral bristle (aDC) and a posterior dorsocentral bristle (pDC) of the wild-type notum are indicated by arrows. (B) The notum part of the wing disc (thoracic disc) from a wild-type late 3rd instar larva is shown with anterior up and dorsal to the left. Positions of sensory mother cells (SMCs) are visualized with *neur-lacZ* expression (red), the *wg* expression domain with anti-Wg antibody (green) and the *dpp* expression domain with GFP expression by dpp^{blk}-*GAL4* at 18°C (blue). Sensory mother cells (SMCs) of aDC and pDC (indicated by arrows) are located adjacent to the dorsal posterior side of the *wg* expression domain. These SMCs are also close to the *dpp* expression domain, however, pDC always forms at some distance apart from the *dpp* expression domain. (C-F) Notal macrochaete phenotypes seen after ectopic Dpp signal activation. (C) Pharate adult *UAS-tkv*: tsh-GAL4* notum. Overexpression of the constitutively active form of the type-I Dpp receptor (Tkv*) in the notal region causes a drastic change in the macrochaete pattern. 8-15 macrochaetes per heminotum are induced on the dorsolateral region of the notum. Some of the ectopically induced macrochaetes in this region are indicated by arrows. (D) Clone of ectopic *tkv** expression induced by the Ay-GAL4 system (Ito et al., 1997). Boundaries of the mosaic are outlined with a red dotted line. Additional macrochaetes (indicated by arrows) are induced only within the clone. Mild activation of Dpp signalling by overexpression of either *tkv** (E) or Dpp type-II receptor *punt* (F) during early third larval stage (40 hours BPF) using *hs-GAL4* driver induces ectopic macrochaetes. (G) The average number of ectopically induced macrochaetes per notum of *UAS-tkv*: hs-GAL4* flies is plotted against heat shock time (BPF). On average, about four ectopic macrochaetes are induced by heat shocks of around 50-35 hours BPF (early to mid third instar larval stage). The GAL4 expression pattern of *tsh-GAL4* is shown in H (*tsh-GAL4/UAS-GFP*^{T2}).

1996; Nellen et al., 1996). We have tested several GAL4 drivers which promote GAL4 expression in the thoracic disc. Overexpression of either *tkv** or *punt* (data not shown) using *tsh-GAL4* driver (Shiga et al., 1996; the GAL4 expression pattern in the wing disc is also shown in Fig. 1H) alters the macrochaete pattern on the notum (Fig. 1C). More than seven macrochaetes (per heminotum) are ectopically induced in the dorsolateral region (but not in the most dorsal region) of the notum. Ectopic macrochaetes seem to be induced cell-autonomously, only within the *tkv** expressing mosaic clones (Fig. 1D). To look for a correlation between the timing of ectopic Dpp signalling and macrochaete induction, *tkv** was induced for a short time period at different stages during larval development using *hs-GAL4* driver. Ectopic expression of either *tkv** (Fig. 1E) or *punt* (Fig. 1F) induces extra macrochaetes without significant notum morphology change. Fig. 1G shows the number of additional macrochaetes induced by *tkv** near the endogenous dorsocentral bristles (dorsocentral region) at different heat shock timings. A heat shock treatment around 45 hours before puparium formation (BPF) significantly induces additional macrochaetes, about four extra macrochaetes on average per dorsocentral region of the notum. A time lag of several hours between heat shock initiation and Tkv* protein expression should exist due to the indirect induction of the transgene via heat induced GAL4 proteins. Considering this, the effective period of ectopic Dpp signalling seems to be near the beginning of endogenous proneural gene expression in the thoracic disc (Cubas et al., 1991). These results suggest that Dpp signalling participates in the prepatterning of the macrochaetes, presumably in the transcriptional activation of proneural genes in the thoracic disc.

Ectopic Dpp signalling induces additional SMCs and suppresses *wg* expression in the thoracic disc

To investigate more precisely the positioning of ectopically formed macrochaetes, we observed the locations of SMCs in the thoracic discs. Ubiquitous *tkv** expression in the thoracic disc using *tsh-GAL4* induces numerous ectopic SMCs (Fig. 2B). Ten to fifteen ectopic SMCs formed along the dorsal side of the *wg* expression domain and also several SMCs formed lateral to the *wg* expression domain. This asymmetric induction suggests that Wg signalling is necessary for the induction of extra SMCs by ectopic Dpp signalling. Thoracic discs of *UAS-tkv*: tsh-GAL4* are expanded along the A/P axis, presumably due to over proliferation of the cells in the wing disc. Ectopic activation of Dpp signalling also alters the *wg* expression. In the wild-type thoracic disc, *wg* is expressed in a stripe of cells with a smooth boundary (Fig. 2A). In the *UAS-tkv*: tsh-GAL4* disc, *wg*-expressing cells exist within a narrow stripe and occasionally appear as small patches (Fig. 2B). Weak expression of *tkv** using *hs-GAL4* driver also induces additional SMCs and repression of the *wg* expression (Fig. 2C). This level of ectopic Dpp signalling induces additional SMCs only on the posterior side of the anterior compartment near the endogenous Dpp source (Fig. 2C). Relatively higher levels of *tkv** expression, in the 2×*UAS-tkv*: hs-GAL4* disc, induces ectopic SMCs more anteriorly (Fig. 2D). These results indicate that high levels of Dpp signalling activity are necessary to induce SMCs. Low levels of ectopic Dpp signalling could recruit cells, which have already received sub-threshold levels of endogenous Dpp signalling, to form additional SMCs. On the other hand, even in the 1×*UAS-tkv*: hs-GAL4* discs, reduction of *wg* expression was observed not only near the endogenous Dpp source but also around the most anterior region of the *wg* expression domain (Fig. 2C). Thus, low levels of Dpp signalling appear to be sufficient to repress *wg* expression.

Together, these results suggest that Dpp signalling has two important roles for macrochaete formation, one is induction of SMCs in cooperation with Wg signalling and the other is restriction of *wg* expression. A difference should exist between the threshold level of Dpp signalling required for SMC induction and that required for repression of *wg* expression.

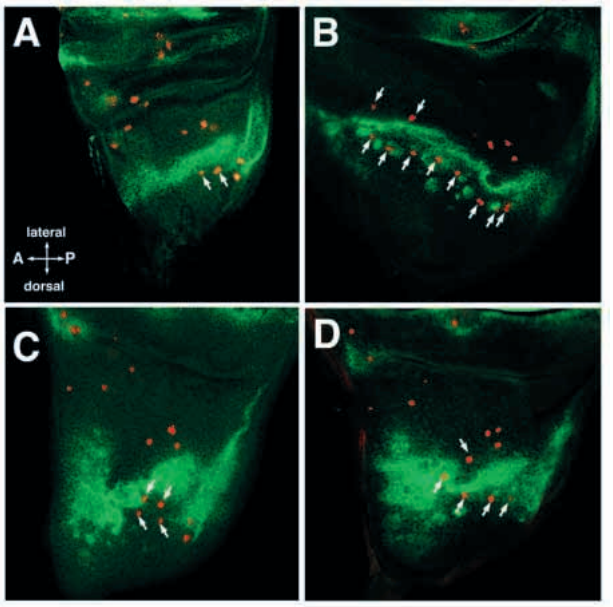

Fig. 2. Ectopic Dpp signalling affects the pattern of sensory mother cells (SMCs) and *wg* expression. Thoracic discs from late third instar larvae of wild type (A), *UAS-tkv*: tsh-GAL4* (B), *UAS-tkv*: hs-GAL4* (C) and *2×UAS-tkv*: hs-GAL4* (2 copies of transgenes) (D) with heat shock at 40 hours BPF are labeled with *neur-lacZ* to visualize SMC position (red) and anti-Wg antibody (green). Confocal microscopic images are shown with anterior left and dorsal down. (A) *wg* is expressed in a stripe of cells along the A/P axis with a smooth boundary. Two dorsocentral SMCs are indicated by arrows. (B,C) Ectopic Dpp signalling induces additional SMCs, indicated by arrows, and also affects the *wg* expression pattern. (B) *UAS-tkv*: tsh-GAL4* thoracic disc showing induction of the SMCs along both sides of the *wg* stripe. The *wg* expression domain becomes narrow and its boundary is irregular. Small patches of *wg*-expressing cells stand alone from the domain. (C) Weak activation of Dpp signalling in the *UAS-tkv*: hs-GAL4* disc induces additional SMCs only near the endogenous dorsocentral bristles. (D) Stronger Dpp signalling obtained with two copies of the transgene induces SMCs more anteriorly. On the other hand, *wg* repression is observed not only near the A/P boundary but also in the most anterior region even in the discs expressing only one copy of *tkv** (C).

Ectopic Dpp signalling induces dorsocentral proneural cluster formation all along the dorsal side of the *wg*-expressing domain

In the wild-type thoracic disc, *ac* expression associated with

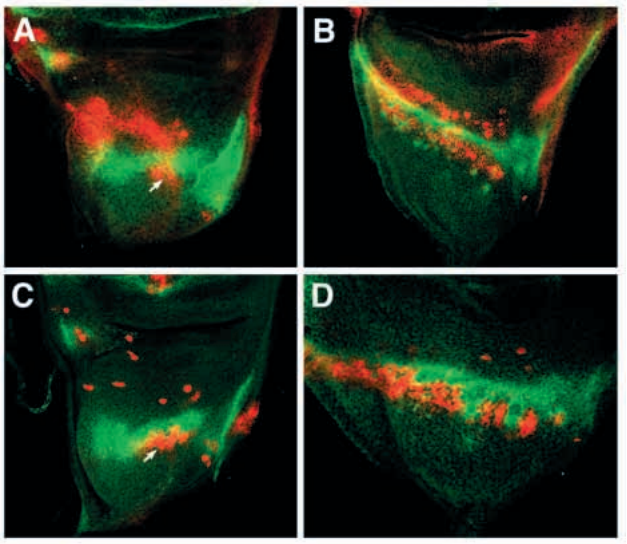

Fig. 3. Induction of the dorsocentral proneural cluster by ectopic Dpp signalling. Late third instar thoracic discs of wild-type (A,C) and *UAS-tkv*: tsh-GAL4* (B,D). *wg* expression (green) and proneural clusters (red) are monitored with anti-Wg antibody and either *ac-lacZ* reporter (A,B) or *DC-lacZ* reporter (C,D) (Gomez-Skarmeta et al., 1995). *ac-lacZ* expression localizes in the proneural clusters and high levels of accumulation are observed in some SMCs at this stage. The dorsocentral proneural cluster is indicated by an arrow (A). In the *UAS-tkv*: tsh-GAL4* discs, *ac-lacZ* expression is altered and broad expressions are seen on both sides of the *wg* expression domain (B). The dorsocentral proneural cluster is also monitored using the *DC-lacZ* reporter whose expression is selectively localized in the dorsocentral proneural cluster (indicated by an arrow in C). This reporter also contains the SMC enhancer and expresses β-galactosidase in all SMCs (C). Additional dorsocentral proneural clusters are observed all along the dorsal side of the *wg* expression stripe (D). *DC-lacZ* expression is likely to be complementary to the *wg* expression. The discs are shown with anterior left and dorsal down.

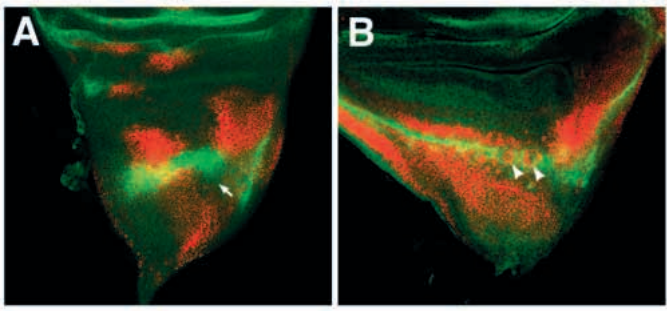

Fig. 4. Expression of the *ASC* negative regulator, *extramacrochaetae* (*emc*) seems not to be suppressed by ectopic Dpp signalling. (A,B) Expression of *wg* (green) and *emc-lacZ* (red). (A) The *emc* expression pattern is rather complex in the wild-type thoracic disc. Relatively high levels of *emc-lacZ* expression are observed in the central region of the anterior compartment and in most of the posterior compartment of the disc. An endogenous dorsocentral proneural cluster is formed in the region where no or little *emc* expression is observed (arrow). (B) High levels of *emc-lacZ* expression are observed even in a *UAS-tkv*: tsh-GAL4* disc. Ectopic dorsocentral proneural clusters are formed in the region where *emc* is highly expressed (compare with Fig. 3D). Expression of *emc-lacZ* and *wg* appears mostly to be complementary rather than overlapping. Ectopic *emc-lacZ* expression is occasionally observed in the punctuate *wg* repression area (indicated by arrowheads). The discs are shown with anterior left and dorsal down.

the dorsocentral proneural cluster appears only adjacent to the dorsal posterior side of the *wg*-expressing cells (Fig. 3A). Ectopic Dpp signalling using *tsh-GAL4* driver abnormally extends *ac* expression to the anterior end of the thoracic disc (Fig. 3B). Dorsocentral specific proneural gene expression was also monitored using *DC-lacZ* reporter (Gomez-Skarmeta et al., 1995). This reporter line selectively expresses β-galactosidase in the dorsocentral proneural cluster in the thoracic disc (Fig. 3C and Gomez-Skarmeta et al., 1995)**.** As this reporter contains an SMC enhancer, it also expresses β-galactosidase in all of the SMCs in the wing disc (Culi and Modolell, 1998). β-Galactosidase expression in the proneural cluster can easily be distinguished from that in SMCs based on the shape and intensity of expression. In the *UAS-tkv*: tsh-GAL4* discs, *DC-lacZ* proneural expression extends to the anterior edge of the thoracic disc, however, it appears only on the dorsal side of the *wg* expression domain (Fig. 3D). This result indicates that ectopically induced SMCs on the dorsal side of the *wg* expression domain are SMCs of the dorsocentral bristles. Ectopic *DC-lacZ* expression was observed only near the *wg* expression domain. This expression is likely to complement the *wg* expression domain (Fig. 3D). These results seem to indicate that Wg signalling is required for dorsocentral proneural cluster formation, but that only those cells which do not express *wg* have the potential to become dorsocentral proneural cells.

On the other hand, *ac-lacZ* expression lateral to the *wg* expression domain corresponds to the expression of another reporter line, *6.0-0.0 kb enhancer fragment-3.7 sc-lacZ* (Gomez-Skarmeta et al., 1995) (data not shown). The latter reporter expression reflects the locations of several proneural clusters in the thoracic disc (aNP, aPA, tr1 and tr2). It has been shown that *wg* activity is not required for the formation of these proneural clusters (Phillips and Whittle, 1993). Dpp signalling appears to cooperate with other factor(s) to induce several *wg* independent proneural clusters.

Ectopic expression of *tkv** does not affect *emc* expression

Anterior expansion of the dorsocentral proneural cluster in the presence of ectopic Dpp signalling could result from alteration of the *ASC* modulator(s). Emc is a helix-loop-helix protein that lacks a transcriptional activator domain (Botas et al., 1982; Ellis et al., 1990; Van Doren et al., 1991). Emc protein appears to suppress the formation of Ac-Da and/or Sc-Da complexes and inhibit their transcriptional activities. The loss-of-function *emc* mutation results in the appearance of some additional bristles near the endogenous ones. This phenotype is similar to that of the flies expressing low levels of *tkv** as shown in Fig. 1E. We observed *emc* expression using an *emc-lacZ* reporter in *UAS-tkv*: tsh-GAL4* discs. *emc* expression is retained even in the presence of ectopic Dpp signalling (Fig. 4B). This result indicates that *emc* expression is independent of Dpp signalling. Importantly, ectopic proneural clusters and SMCs are induced even in the *emc*

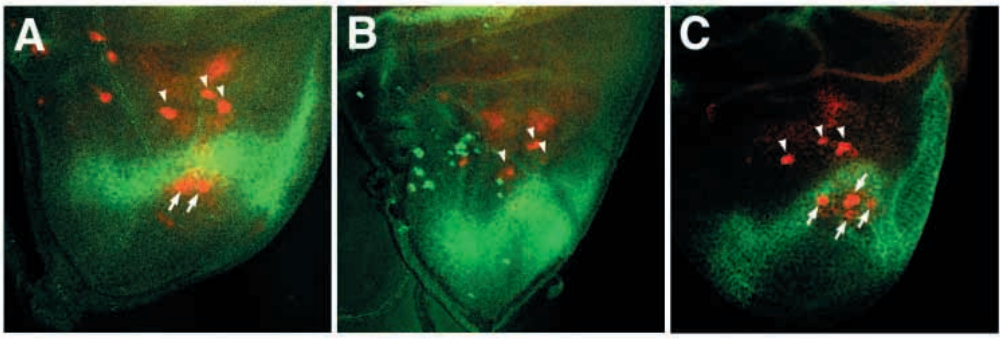

Fig. 5. Reduction of Dpp signalling activity leads to ectopic *wg* expression and inhibition of dorsocentral SMC formation. (A-C) Thoracic discs in the late third larval stage labeled with anti-Wg antibody (green) and 3.7 *sc-lacZ* expression (red) are shown. High levels of *sc-lacZ* expression localize in SMCs at this stage. (A) Positions of the dorsocentral SMCs in the wild-type disc are indicated with arrows. (B) A $punt^{P1}/punt^{135}$ (*punt-ts*) thoracic disc from larvae which were transferred from 18°C to the nonpermissive temperature (29°C) about 48 hours BPF; we referred to this mutant as 'severe *punt-ts*'. In the severe *punt-ts* mutant disc, *wg* expression expands to the dorsal edge of the disc. SMCs lateral to the *wg* expression domain still exist in the mutant discs (indicated with arrowheads in B and C). However, the dorsocentral SMCs are no longer observed within the expanded *wg* expression domain. The severe *punt-ts* mutant disc is smaller than that of wild type. (C) A thoracic disc of a *punt-ts* mutant which was shifted to a mild heat condition (at 25°C); we referred to this as 'mild *punt-ts*'. In the mild *punt-ts* disc, the *wg* expression domain is slightly expanded toward the dorsal edge and additional SMCs are formed (arrows). The discs are shown with anterior left and dorsal down.

expressing region (Fig. 4B, compare with Figs 2B and 3B). Therefore it is possible that ectopically activated Dpp signalling causes the induction of proneural genes at high levels and that these activities could overcome the inhibitory effects of the Emc protein.

Endogenous Dpp signalling is required for dorsocentral proneural cluster induction and repression of *wg* expression

Dpp signalling is important for induction and proliferation of imaginal discs (Lecuit et al., 1996; Nellen et al., 1996). To minimize the activity of *dpp* in early morphogenesis of imaginal discs (and to focus on the induction of the proneural clusters), we used conditional loss-of-function Dpp signalling mutants. Some allelic combinations of the *punt* mutations exhibit temperature sensitivity for Dpp signalling (Letsou et al., 1995; Simin et al., 1998; Theisen et al., 1996). $punt^{P1}/punt^{135}$ (termed *punt-ts*) is permissive at 18°C and non permissive at 29°C. *punt-ts* flies were cultured at 18°C and transferred to 29°C at the second to early third larval instar stage. We monitored the position of SMCs and *wg* expression in the *punt-ts* discs. In this condition, *wg* expression expanded to the dorsal edge of the thoracic disc (Fig. 5B). Expansion of the *wg* expression domain in this mutant disc was also confirmed by using *wg-lacZ* reporter (data not shown). High levels of *lacZ* protein were observed in dorsocentral SMCs in wild-type discs (Fig. 5A) but not in *punt-ts* discs (Fig. 5B). This result indicates that both dorsocentral proneural cluster induction and repression of *wg* expression are promoted by endogenous Dpp signalling.

To our surprise, in thoracic discs from *punt-ts* mutants shifted to milder conditions (25°C) at the same stage (we will refer to this mutant as 'mild *punt-ts*') one or a few extra SMCs are formed (Fig. 5C). It is also worth noting that extra SMCs appear to be formed in a more posterior region compared to the endogenous dorsocentral SMCs (Fig. 5C compare with Fig. 5A). In the mild *punt-ts* disc, the *wg* expression domain is slightly expanded dorsally and posteriorly (Fig. 5C). One possible explanation for this phenotype is that the region receiving sufficient levels of both Dpp and Wg signals to induce dorsocentral proneural cluster has expanded in mild *punt-ts* mutants. We will discuss more about this controversial issue later.

wg activity is necessary for induction of ectopic dorsocentral bristle formation by ectopic Dpp signalling

Finally, we examined whether *wg* activity is required for *tkv** induced ectopic dorsocentral bristle formation. Fig. 6A shows a bristle pattern of the allelic combination of the *wg* mutants (wg^{IL114}/wg^{Sp1}). aDC is constantly missing in flies of this genotype. Ectopic *tkv** expression by *tsh-GAL4* fails to induce any ectopic dorsocentral bristles (Fig. 6B and compare to Fig. 1C). In contrast to the dorsocentral bristles, *wg* independent macrochaetes, such as aPA and pSA, are ectopically induced by *tkv** even in the *wg* mutant background (Fig. 6B). These results confirm that Wg signalling is absolutely required for ectopic dorsocentral bristle formation.

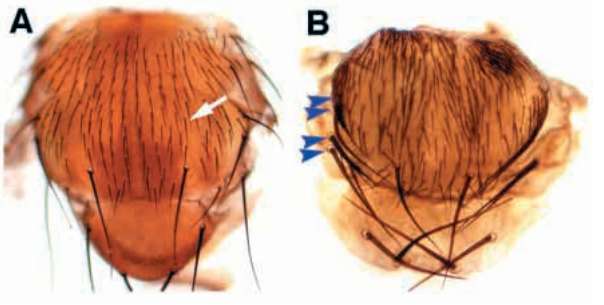

Fig. 6. Effect of *tkv** overexpression on bristle development in a *wg* mutant background. (A) Bristle pattern of the wg^{IL114} *tsh-GAL4*/wg^{Sp1} notum. aDC is constantly missing in the flies of this genotype (position where it should be formed is indicated by an arrow). (B) Pharate adult notum of wg^{IL114} *tsh-GAL4*/wg^{Sp1}; *UAS-tkv*/+*. Ectopic *tkv** expression by *tsh-GAL4* in this *wg* mutant no longer induces ectopic bristles in the dorsocentral region of the notum. Duplication of the *wg*-independent bristles (aPA and pSA) is frequently observed (indicated by blue arrowheads).

DISCUSSION

Dpp signalling participates in dorsocentral bristle development

We have shown that ectopic Dpp signalling induces additional dorsocentral proneural clusters and SMCs all along the dorsal side of the *wg* expression domain in the thoracic discs. Mosaic expression of the *tkv** indicated that Dpp signalling is required cell autonomously to induce ectopic proneural clusters. Loss-of-function experiments using *punt-ts* flies also indicated that endogenous Dpp signalling is necessary for the formation of the dorsocentral SMCs. Moreover, in the *wg* mutant flies (wg^{Sp1}/wg^{IL114}), ectopic Dpp signalling did not induce any additional dorsocentral bristles. These results indicate that the dorsocentral proneural cluster is formed through the activities of both Dpp and Wg signalling.

There are many genes known to be regulated by both Dpp and Wg signalling. For instance, a midgut enhancer of the *Ultrabithorax* gene has been shown to be regulated directly by both Wg and Dpp signal transducers (Eresh et al., 1997; Riese et al., 1997). *vestigial (vg)* quadrant enhancer has been shown to be activated by Dpp signalling (Kim et al., 1996, 1997). *vg* expression is also regulated by Wg signalling in the wing pouch (Kim et al., 1996, 1997). The regulatory mechanism for the *cis*-element(s) of the DC-enhancer is totally unknown. Cell autonomous effects of both Dpp and Wg signals (Fig. 1D, and R. G. Phillips et al., personal communications) suggest the possibility that a Dpp and Wg signal transducer directly effects the DC-enhancer to induce proneural genes. However, we cannot rule out another possibility that Dpp and/or Wg signalling control the expression (or activity) of other prepattern genes that directly activate the DC-enhancer. Analysis of the DC-enhancer element is necessary to address how Dpp and Wg signals cooperate in the induction of the proneural genes at the dorsocentral region.

Our data from both gain-of-function and loss-of-function experiments suggest that Dpp signalling also has an important role in the specification of the *wg* expression domain in the thoracic disc. A mutual inhibitory interaction between *dpp* and *wg* in the leg disc has been extensively analyzed (Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffmann, 1996; Theisen et al., 1996). Wg is expressed ventrally and Dpp is expressed at high levels dorsally along the A/P boundary in the leg discs and both expressions are controlled by Hh (Basler and Struhl, 1994). By contrast, in the wing disc, Hh controls expression of *dpp* but not *wg*. The involvement of inhibitory interactions between *wg* and *dpp* in transcriptional regulation in the wing disc has been controversial. Penton and Hoffmann (1996) reported that the *punt* mutant clone, $punt^{P62}$, ectopically expressed *wg* only in a restricted portion of the wing pouch. However, Theisen et al. (1996) reported that the pattern of *wg* expression in the wing disc of the *punt-ts* mutants is normal even if the animals are upshifted to the nonpermissive temperature, 25°C, for 70 hours BPF, while the maximal ectopic *wg* expression in the leg disc is seen after 40 hours at the restrictive temperature. We have shown that *wg* expression is expanded dorsally in the thoracic discs of $punt^{P1}/punt^{135}$, the same allelic combination as Theisen et al. used, however, we set much more severe conditions, with a temperature shift from 18 to 29°C. We suggest that transducing activity associated with Dpp signalling is reduced in the *punt-ts* mutant at 25°C, but it still retains partial activity to restrict the *wg* expression domain in the thoracic disc (Fig. 5C). At 29°C, Dpp signalling activity is reduced below a threshold level and the *wg* expression domain expands (Fig. 5B). *dpp* expression in the wing disc seems not to be regulated by Wg signalling, since the *dpp* expression pattern is normal in the *wg-ts* mutants under nonpermissive conditions (Theisen et al., 1996; Y. Tomoyasu et al., unpublished data).

Interestingly, even when high levels of *tkv** were expressed in the whole thoracic disc, complete repression of *wg* expression was not observed. A narrow band of *wg* expression always remained, presumably corresponding to the lateral side of the endogenous *wg* expression domain (Fig. 2B). It is also worth noting that *wg* expression never expanded toward the lateral side in the *punt-ts* mutant discs (Fig. 5B and C). These results suggest that the mechanism which establishes the lateral side of the *wg* expression boundary may be different from that which defines the dorsal side of its expression domain. The lateral side of the *wg* expression domain may be regulated by a Dpp-independent mechanism.

Models for formation of the dorsocentral bristles

It appears that different thresholds of Dpp signalling are required for proneural gene induction and repression of *wg* expression. Dpp is secreted from the A/P border and generates a concentration gradient along the A/P axis within the thoracic disc. The schematic model shown in Fig. 7A illustrates a possible relationship between Dpp signal activity and position in the thoracic disc, along the A/P axis. This model assumes that Dpp molecules simply diffuse and generate a gradient of Dpp signal activity along the A/P axis. Presumably, low levels of Dpp still reach the most anterior region of the thoracic disc and this level of Dpp signal activity is sufficient for *wg* repression (Fig. 7C). The threshold levels required for proneural gene induction, on the other hand, appear to be higher. Only those cells which are located in the vicinity of both *dpp* and *wg* expression domains receive sufficient levels of both signals for proneural induction (Fig. 7C).

It is possible that alterations in Dpp signal activity cause a shift in the activities slope, with an upshift in gain of function mutants and a downshift in loss of function mutants (Fig. 7A). This model is consistent with our experimental results except for the results that were observed in the mild *punt-ts* mutants. According to this model, weak reduction of Dpp signal transduction activity should result in a downshift of the slope and this change should have reduced both the area of *wg* repression and the area of proneural induction. This was not the case. The *wg* expression area was slightly reduced, as would be expected in response to the weak reduction of Dpp signalling, however, the proneural induction area was not reduced. To explain this phenotype, it is necessary to consider the effect of Emc, a negative regulator of proneural gene products. Asymmetric distribution of Emc protein affects the threshold level for proneural induction. Emc is thought to play an inhibitory role after proneural gene expression. We have shown that a high level of Dpp signalling can overcome the inhibitory effect of Emc (Fig. 4B). This indicates that cells within the Emc expression domain require a higher threshold level of Dpp signalling activity for proneural gene induction than those cells outside of this domain (Fig. 7A). In this case, a slight downshift in the Dpp signal activity slope would result

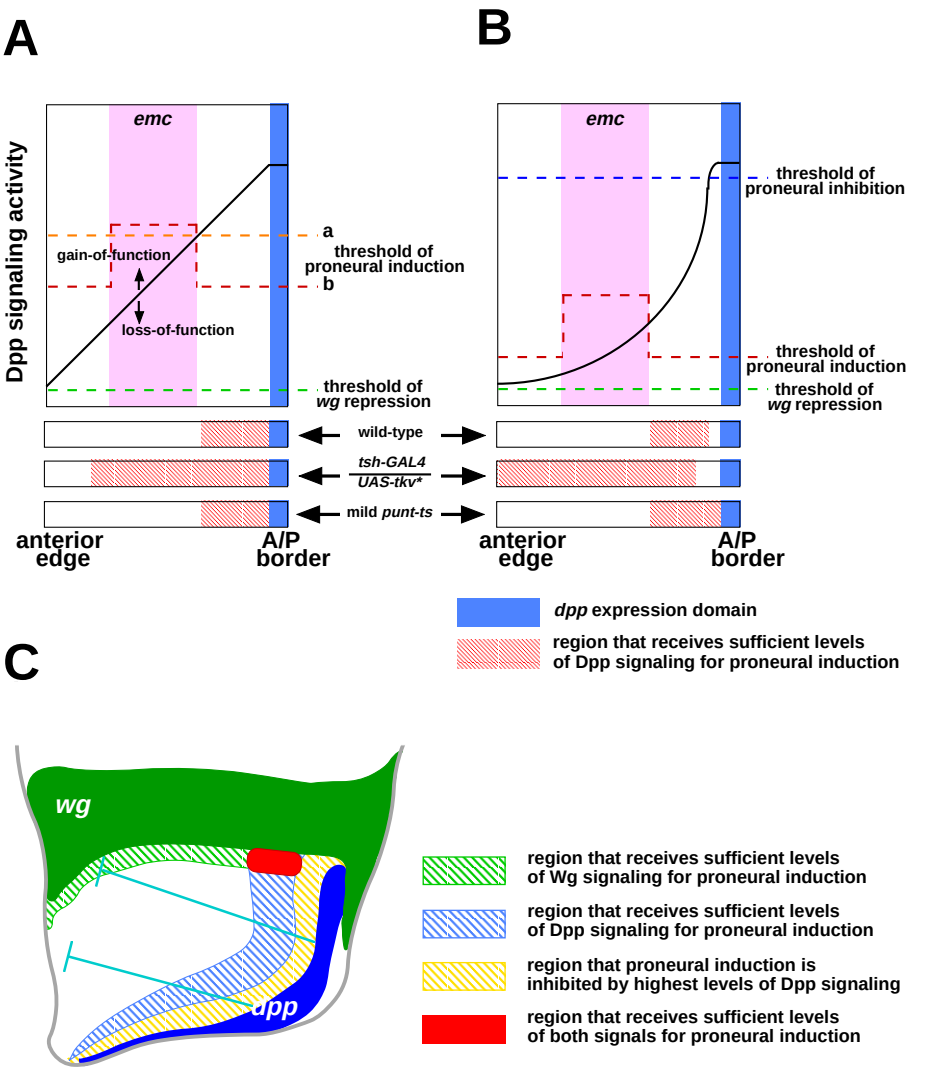

Fig. 7. Models for dorsocentral proneural cluster formation. Two hypothetical models for Dpp signalling activity along the A/P axis within the thoracic discs are illustrated in A and B. *dpp* is expressed in A cells which are located near the A/P compartment boundary (drawn with blue). After secretion, Dpp proteins are expected to diffuse anteriorly and posteriorly and generate a concentration gradient which is drawn as a linear slope in A and nonlinear slope in B. Cells respond differently depending on the activity of Dpp signalling. The thresholds of Dpp signalling activity for proneural induction and *wg* repression are different. A low level of Dpp signalling activity, as would exist at the most anterior region of the thoracic disc, appears to be sufficient for the repression of *wg* expression. High levels of Dpp signalling seem to be necessary for proneural induction. The threshold of Dpp signal activity for proneural induction is likely to be modulated by a proneural inhibitor protein, *Emc*. The region in which *emc* is expressed is shown in pink (A,B). Taking into consideration this inhibitory activity, the threshold level for proneural induction should not be drawn as a straight line (A-a, orange broken line) but as a line, with a higher threshold level for proneural induction in the *emc* expression area and a lower threshold outside of this (A-b, red broken line). Model B also adopts a hypothetical activity of Dpp signalling for the inhibition of proneural induction, in which the highest level of Dpp signalling is necessary for this inhibitory action (B). It can be thought that the Dpp signal activity slope is shifted up in the gain-of-function mutants and down in the loss-of-function mutants. The regions that are estimated to be a proneural cluster according to the models in the wild type, gain-of-function mutant (*UAS-tkv*: tsh-GAL4*) and partial loss-of-function mutant (mild *punt-ts*) are indicated (drawn in red with horizontal bars at the bottom of A and B). Model B precisely reflects our results. A thoracic disc is schematically illustrated in C. *wg*-expressing cells are shown in green and *dpp*-expressing cells are shown in blue. Dpp signalling (indicated with light blue lines) is sufficient to suppress *wg* expression even at the most anterior region. The hypothetical regions that have acquired sufficient levels of Dpp and Wg signalling for proneural induction are shaded in blue and white or green and white, respectively. The dorsocentral proneural cluster is formed where these regions overlap (shown in red). Proneural clusters are not formed in the region that receives highest levels of Dpp signalling.

in weak reduction of the *wg* repression area, while the proneural induction area would not be reduced. Thus, the region receiving sufficient levels of both Dpp and Wg signalling to induce proneural genes seems to have expanded, resulting in the formation of additional SMCs.

There is a substantial distance between dorsocentral SMCs and the *dpp* expression domain in wild-type discs (Fig. 1B). One explanation for the existence of this gap is that the highest level of Dpp signalling inhibits the formation of proneural clusters. This hypothetical Dpp signal activity is useful to explain the observation that additional dorsocentral SMCs were formed more posteriorly in the mild *punt-ts* discs (Fig. 5C). A down shift of the Dpp activities slope would release the area in which proneural induction is inhibited by the highest levels of Dpp signalling from such inhibition. However, there is one more paradox to the adoption of the inhibitory action of Dpp signalling. Considering this inhibitory effect in terms of the model shown in Fig. 7A, ectopic activation of Dpp signalling should have expanded the area in which proneural induction is inhibited. This was not the case for *UAS-tkv*: tsh-GAL4* discs (Fig. 2B). One solution to this paradox is to alter the linear activity slope, as illustrated in Fig. 7A, to the nonlinear slope, as illustrated in Fig. 7B. The alternative model is able to simulate both phenotypes of *UAS-tkv*: tsh-GAL4* and mild *punt-ts* without contradiction. However, it is clear that this model still includes several assumptions which may be addressed by future experiments. For instance, a concentration gradient of Dpp protein within the thoracic disc should be directly visualized. The mechanism by which the highest levels of

Dpp signalling inhibits proneural induction is unclear and should be studied at the molecular level.

It is worth noting that the effective range of *wg* from its source for proneural cluster induction seem to be different to that of *dpp*. The dorsocentral proneural cluster is formed within approximately five cell diameters from the *wg* expression domain, whereas it can be formed more than ten cell diameters from the *dpp* source (Fig. 3A). This difference must contribute to the oval shape of the proneural cluster, which is longest along the A/P axis. *wg* expression is not uniform in the notal stripe, it is lower at the A/P compartment border (Fig. 1B). It is possible that the difference in *wg* expression levels along the A/P axis also affects the precise positioning of the dorsocentral proneural cluster.

Interaction to other genes in the macrochaete prepatterning on the notum

Recently, it has been reported that *pnr*, which encodes a GATA family transcription factor, and *ush*, which encodes a novel zinc finger protein, have a regulatory role in dorsocentral proneural cluster formation, presumably at the level of *ASC* gene expression (Cubadda et al., 1997; Haenlin et al., 1997). It has shown that Pnr transactivates the α-globin promoter in a cultured cell system and that Ush negatively regulates the activity of Pnr (Cubadda et al., 1997). They have not described whether or not Pnr proteins act as a transcriptional activator against DC-enhancer of *ASC* genes. The relation between Dpp signalling and these transcriptional regulators is largely unknown. Dpp signalling may regulate the expression or activity of these proteins. Some interesting results are also reported by Calleja et al. (1996), who have shown that *wg* expression is affected in *pnr* mutants. In pnr^{V1}/pnr^{VX1} discs, both are loss-of-function alleles of the *pnr* gene, resulting in no *wg-lacZ* expression in the thoracic disc. On the other hand, *wg* expression extends dorsally in the disc of another heteroallelic combination, pnr^{D1}/pnr^{md237} (pnr^{D1} seems to be a gain-of-function allele). These results suggest that *pnr* and *ush* may regulate *wg* expression in the thoracic disc.

Ectopic Dpp signalling also induces *wg* independent proneural cluster formation (Fig. 3B). It has been shown that formation of these proneural clusters depends on the activity of the *iro* locus. This suggests that Dpp signalling positively interacts with the products of the *iro* locus (Dambly-Chaudiere and Leyns, 1992; Gomez-Skarmeta et al., 1996; Gomez-Skarmeta and Modolell, 1996; Kehl et al., 1998; Leyns et al., 1996). Whether an epistatic relationship exists between *dpp* and *iro* in *wg* independent proneural cluster is unknown. Further studies are necessary to determine how *dpp* and other prepattern genes cooperate in the regulation of *ac* and *sc* in the thoracic disc.

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