PATTERN FORMATION OF DROSOPHILA DEVELOPING NOTUM

YOSHINORI TOMOYASU

DEPARTMENT OF MOLECULAR BIOMECHANICS
SCHOOL OF LIFE SCIENCE
THE GRADUATE UNIVERSITY FOR ADVANCED STUDIES

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ABBREVIATIONS

A/P:	anterior / posterior
BMP:	Bone Morphogenetic Protein
BPF:	before puparium formation
D/V:	dorsal / ventral
DC:	dorsocentral
FRT:	FLP recombinase recognition terget
GFP:	green fluorescence protein
HLH:	helix-loop-helix
JNK:	c-Jun amino-terminal kinase
PBS:	phosphate-buffered saline
PA:	postalar
SA:	supraalar
SMC:	sensory mother cell
UAS:	upstream activation sequence

GENE SYMBOLS AND THEIR FULL NAMES

ac:	*achaete*
ACS:	*achaete-scute complex*
ara:	*araucan*
caup:	*cauporican*
dad:	*Daughters against dpp*
d-axin:	*d-axin*
Dl:	*Delta*
dpp:	*decapentaplegic*
dsh:	*dishevelled*
emc:	*extra macrochaetae*
hh:	*hedgehog*
iro:	*iroquois*
mirr:	*mirror*
N:	*Notch*
neur:	*neuralized*
pnr:	*pannier*
put:	*punt*
sax:	*saxophone*
sc:	*scute*
tsh:	*teashirt*
tkv:	*thick veins*
Ubx:	*Ultrabithorax*
ush:	*u-shaped*
vg:	*vestigial*
wg:	*wingless*

CHAPTER 1.

GENERAL INTRODUCTION

Background and purpose of this study

Almost all animals develop from a single cell, fertilized egg, with multiple and progressive processes. In these processes, that we call DEVELOPMENT, fertilized egg is divided, proliferates, and differentiates into specific tissues or organs to form precise adult body. A major challenge in developmental biology is the elucidation of how a pattern is formed.

One general mode of pattern formation of the living things is based on the concept of "Morphogen" and "Positional information". This concept can be well illustrated by considering the patterning of "French Flag" (Fig. 1-1) (Wolpert, 1969; Wolpert, 1971; Wolpert, 1998). To establish the pattern that similar to the French Flag in developing field, it is necessary that cell acquires an identity (or positional value) that is related to their positions in whole field. This means, cells should acquire the positional information. The simplest mechanism to provide the positional information to cells is based on a gradient of some substances. If gradient exists from one end to the other, this gradient can efficiently specify the position of cells depending on its concentration (Fig. 1-1A) (Wolpert, 1969; Wolpert, 1971; Wolpert, 1998). A chemical that is expressed by some cells and diffuses through surrounding tissues providing other cells with information about their relative position is called a morphogen. Recently, it was reported that some secreted growth factors act as morphogen in *Drosophila* imaginal discs (Lecuit et al., 1996; Nellen et al., 1996; Neumann and Cohen, 1997; Zecca et al., 1996). Decapentaplegic (Dpp), a BMP family member protein, is expressed just anterior to the boundary of anterior and posterior compartment of the wing imaginal disc (Fig. 1-1B) (Basler and Struhl, 1994; Tabata and Kornberg, 1994). Dpp protein emanating from *dpp*-expressing cells accumulates

as a concentration gradient and acts directly on responding cells (Nellen et al., 1996). Cells seem to respond to some threshold concentrations of Dpp to express several target genes, such as *optomotor-blind* (*omb*) or *spalt* (*sal*) (Lecuit et al., 1996; Nellen et al., 1996). Hence, Dpp gradient organizes the spatial patterns of several target gene expressions by eliciting their transcription at different distances from *dpp*-expressing cells. In other words, Dpp acts as morphogen in the pouch region of the wing imaginal disc.

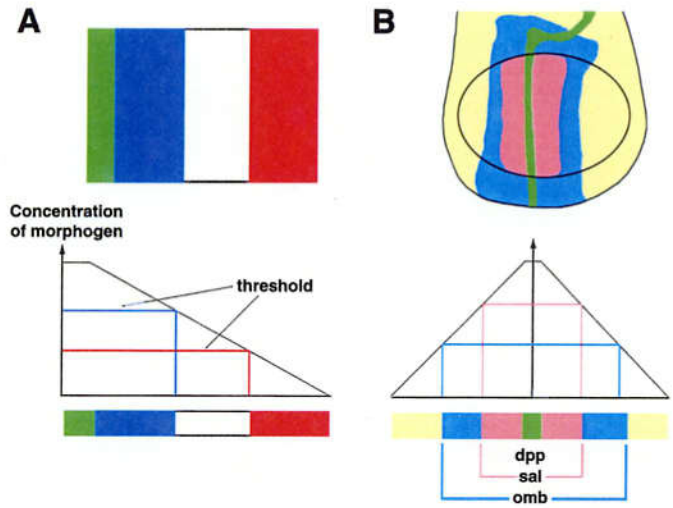

Fig. 1-1. The French flag model (A) and pattern formation of Drosophila wing imaginal disc (B).

Another general mode of pattern formation is "Prepattern" proposed by Stern in 1967. This concept is based on that in order to make a pattern it is necessary to generate a spatial variation in something which resembles in some way the pattern. Namely, the appearance of overt pattern depends on the expression of underlying prepattern. This mode of pattern formation is useful for explain a precise and complex pattern, such as bristle pattern of *Drosophila*. The difference between mechanisms of pattern formation based on morphogen and prepattern is shown in Fig. 1-2.(Wolpert, 1971).

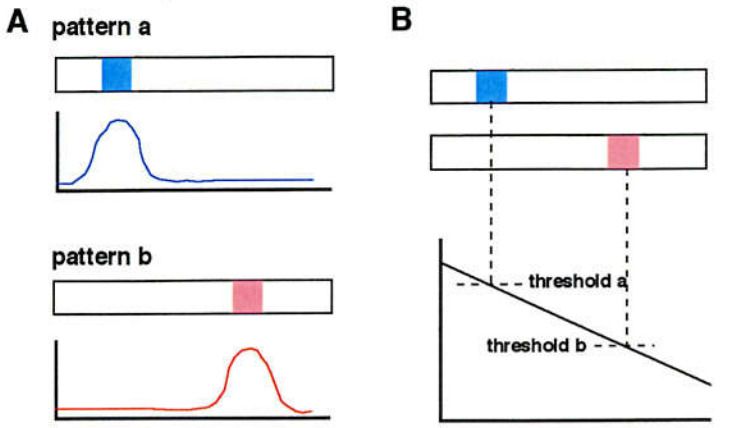

Fig. 1-2. The difference between mechanisms of pattern formation based on prepattern (A) and positional information (B). In **pattern a**, one cell in a line undergoes a specific cytodifferentiation, and in prepattern terms this requiers a singularity in some property at that region (A, above). If, as **pattern b** the site of cytodifferentiation changes, then the singularity also changes (A, below). In terms of positional information, there are no spatial singularity (B) and the pattern arise from interpretation of the cells. For **pattern a** the the cells respond to **threshold a** whereas **threshold b** for **pattern b** (B).

On the dorsal part of the mesothorax (called notum) of *Drosophila*, large bristles (macrochaetes) develop in fixed numbers at constant positions (Fig. 1-4) (Hartenstein and Posakony, 1989; Huang et al., 1991). Pattern formation of macrochaetes on the notum has provided an ideal model system for studying two-dimensional patterning. Stern, C. et al. proposed that this patterning of macrochaetes on the notum is carried out based on the prepattern concept (Stern and Tokunaga, 1968). Recently, several genes which contribute the establishment of prepattern (we call these genes "prepattern genes") on the notum were characterized. For instance, three genes residing at the *iroquois* (*iro*) locus, *araucan* (*ara*), *caupolican* (*caup*) and *mirror (mirr)*, which encode a novel family of homeoproteins (Dambly-Chaudiere and Leyns, 1992; Gomez-Skarmeta et al., 1996; Gomez-Skarmeta and Modolell, 1996; Kehl et al., 1998; Leyns et al., 1996), *pannier* (*pnr*) which encodes a GATA family transcription factor (Cubadda et al., 1997; Heitzler et al., 1996; Ramain et al., 1993), *extramacrochaetae* (*emc*) which encodes a helix-loop-helix protein devoid of a basic

domain(Botas et al., 1982; Ellis et al., 1990; Van Doren et al., 1991), were identified as prepattern genes. These genes are expressed in some specific regions of the notum, and seem to establish a prepattern on the notum field. Local specific expression of prepattern genes could explain the pattern formation of macrochaetes on the notum. However, one problem of prepatterning event on the notum (indeed this is a problem of prepattern concept itself) is how local maxima (or minima) of prepattern gene expression (or activation/ repression of the prepattern gene product) are generated. One possible explanation about this problem is that some morphogen gradients could contribute the regulation of prepattern genes expression on the notum, suggesting that these two modes of pattern formation are inseparable events in same developmental process.

In this study, I attempted to reveal how morphogen gradient contributes to establishment of prepattern, using the notum region of *Drosophila* as a model system. In chapter 2, I have focused on whether Dpp, as a morphogen gradient, plays a part in the prepatterning of the macrochaetes on the notum. In chapter 3, I tried to reveal a regulatory network of *pannier* (*pnr*) and *u-shaped* (*ush*), which seem to be prepattern genes, and Dpp signaling in the pattern formation of the notum, especially focusing on the regulation of the *wg* expression. In the last chapter, I summarised the presented results and discuss a general mode of pattern formation.

Development of adult structure of *Drosophila melanogaster*

There are three major patterns of insect development, ametabolous, hemimetabolous and holometabolous metamorphosis. Dipterian insects, including *Drosophila melanogaster* are holometabolous insect, and there is a dramatic transformation between the larval and adult stages, called pupal stage. In the pupal stage, old body of the larva is destroyed and the adult structures are formed from undifferentiated clusters of cells, imaginal discs and histoblasts (Girbert, 1994).

In *Drosophila*, there are fifteen major imaginal discs, one pair of antenna, eye, wing and haltere disc, three pairs of leg disc, and a genital disc, which reconstruct the entire adult structures except for the abdomen. The abdominal epidermis forms from a small group of imaginal cells, histoblasts (Fig. 1-3)(Girbert, 1994).

The half of dorsal part of adult mesothorax (heminotum), which is mainly analysed in this study, develops from a part of wing imaginal disc. Wing imaginal disc is composed of three major part, wing pouch region, hinge region, and presumptive notum region (Fig. 1-4) (Bate and Arias, 1993). Presumptive notum region of wing imaginal disc will be referred as "thoracic disc". To elucidate the mechanisms of pattern formation of adult structure, not only adult phenotypes but also imaginal disc

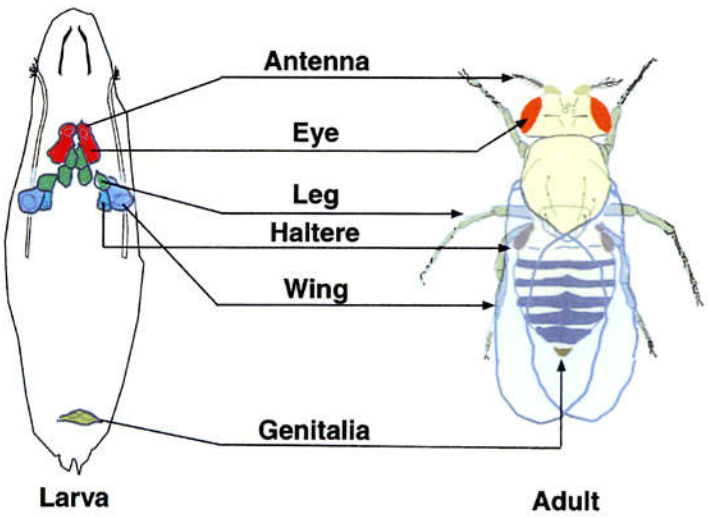

Fig. 1-3. The location and developmental fates of the imaginal discs in *Drosophila melanogaster*(ref).

phenotypes should be analysed, because rough patterning of adult form is carried out until the end of imaginal disc stage. Hence, I first analysed the adult phenotypes in the first half of chapter 2, then analysed imaginal disc phenotypes in the latter half of chapter 2 and in chapter 3.

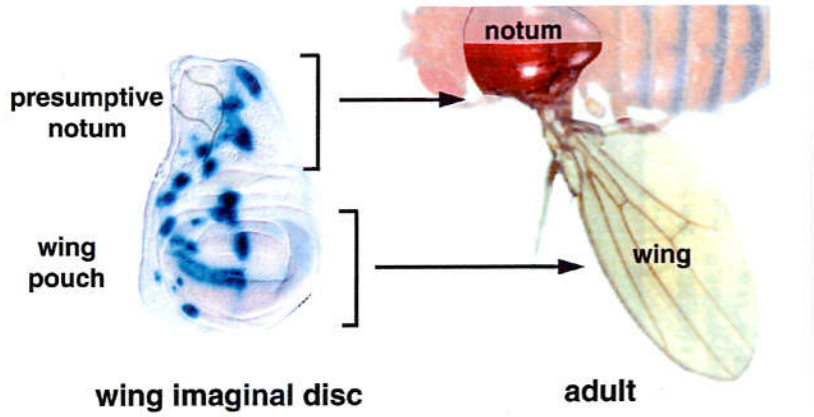

Fig. 1-4. Developmental fate of wing imaginal disc

Techniques of *Drosophila* developmental genetics

Because of the facility of genetics, fruit fly *Drosophila melanogaster* is one of the good model animals to study developmental biology. Furthermore, it makes this model animal more attractive that the methods for making transgenic flies with the use of transposable element (P-element) vectors was well established (Rubin and Spradling, 1982). The availability of stable, single copy, integrative transgenesis enabled a range of powerful techniques to be developed in *Drosophila*. Insertional mutagenesis with engineered transposable elements enabled us to complete large-scale mutant screen, and to easily clone some genes affected by insertion of transposon (Cooley et al., 1988). In addition, some useful techniques that combined genetic and molecular approaches are also based on this transgenic technique. Following three techniques are indispensable to recent studies of *Drosophila* developmental biology, and also to this study.

1. Enhancer trap technique

The random generation of operon fusions in prokaryotes enables the isolation and characterization of genes simply by knowing or postulating their expression patterns (O'Kane and Gehring, 1987). Enhancer trap technique in *Drosophila* is analogous to this method of bacterial genetics (O'Kane and Gehring, 1987). Random integration of reporter gene that is fused to a weak promoter (heat shock protein 70 promoter) by P-element transformation into *Drosophila* genome leads to an increase in expression of the reporter gene reflecting the activity of nearby enhancer-like elements in the genome. Usually, β–glactosidase which is encoded by *lacZ* gene or Green fluorescence protein (GFP) are used as reporter. This technique is useful not only for screening some genes based on their pattern of expression, but also for visualizing known gene's expression easily by staining with anti β–glactosidase antibody or detecting the activity of β–glactosidase. In this study, a variety of reporter strains such as *neur-lacZ*, *wg-lacZ* and *emc-lacZ* were used to visualize the expression patterns of some genes.

2. Gal4-UAS system

The ability to express genes in a directed fashion is a useful means of analysing its role in development. Gal4-UAS system is one of powerful techniques for controlling ectopic gene expression in *Drosophila* (Brand and Perrimon, 1993). This system consists of two components, Gal4 driver and UAS-gene X. Gal4 drivers are fly strains that expresses yeast transcriptional activator Gal4 in numerous cell- and tissue-specific patterns. Gal4 drivers are generated by enhancer trap technique, or transgenesis of *gal4* gene fused with transcriptional regulatory sequences from a defined promoter. A Gal4-dependent target gene can be constructed by subcloning any sequence (gene X) behind Gal4 binding site, upstream activation sequence (UAS). This fly strain is called as "UAS-gene X". The target gene is silent in the absence of Gal4, however, when Gal4 driver and UAS-gene X are crossed, the progeny that has both Gal4 and UAS-gene X are express gene X only where Gal4 is expressed. In this study, *tsh-Gal4*, *hs-Gal4*, and *pnr-Gal4*, were used for ectopic gene expression.

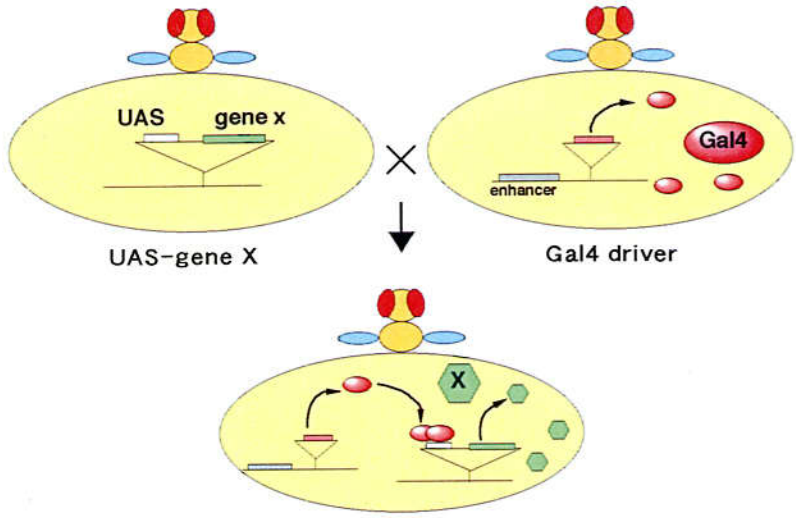

Fig. 1-5. Directed gene expression by Gal4-UAS system

3. Mosaic analysis by FLP-FRT system

Mosaic means individual whose cells are not all of the same genotype. In *Drosophila*, it has been possible to introduce cells of one genotype within an organism that geneticcally distinguishable. These cells proliferate and form a group of cells called a clone. By marking these clones, it has been possible to answer many developmental questions, for instance the construction of developmental fate maps. Clones of mutant tissue are usually generated by mitotic recombination. The use of the FLP recombinase and its recombination targets (FLP-FRT system) greatly facilitates to induce mitotic recombination in *Drosophila* somatic cells (Golic and Lindquist, 1989; Xu and Rubin, 1993). Method for producing and marking clones using FLP-FRT system are illustrated in Fig. 1-6. This technique is useful for the study of genes that are essential for the viability of the whole organism, because usually, specific removal of their functions in particular tissue does not affect the viability of animals severely.

In this study, clones of *pnr, d-axin*, and *tkv* mutant were generated using this technique.

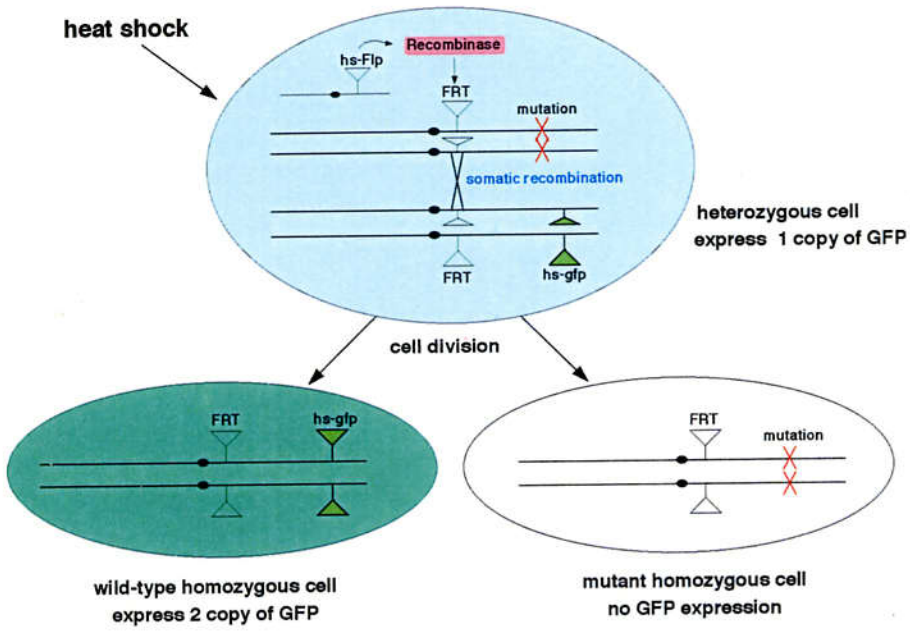

Fig. 1-6. Generation of mitotic clones using FLP-FRT system. A strain containing a chromosome carrying geneX mutant and a centroere-proximal FRT element on the same arm is crossed to another carrying the same FRT element as well as a hs-Flp on a separate chromosome. Clones of cells homozygous for the geneX mutation can be produced by inducing mitotic recombination between FRT site after heat-shock induction of the FLP recombinase. For marking clones, the distal part of the FRT-carrying arm which dose not carry the mutation geneX also carries some marker genes, such as *gfp* gene or *lacZ* gene. The cells homozygous for geneX mutant have no marker gene while cells homozygous for geneX wild-type have two marker genes. Hence, mutant clones are marked by the absence of marker gene expression.

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CHAPTER 2.

ROLL OF DECAPENTAPLEGIC SIGNALLING IN PREPATTERN FORMATION OF DORSOCENTRAL MECHANOSENSORY ORGAN IN *DROSOPHILA MELANOGASTER*

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 signaling itself is insufficient to establish the dorsocentral proneural cluster. Some factor(s) from posterior side must participate in this action in cooperation with Wg signaling. I have examined the role of Dpp signaling in dorsocentral bristle formation by either ectopically activating or conditionally reducing Dpp signaling. Ubiquitous activation of Dpp signaling 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 signaling during disc development resulted in the inhibition of dorsocentral proneural cluster formation and expansion of the *wg* expression domain. These results suggest that Dpp signaling has two indispensable roles in dorsocentral bristle formation, induction of the dorsocentral proneural cluster in cooperation with Wg signaling and restriction of the *wg* expression domain in the notum region of the wing imaginal disc.

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* (Fig.2-1). Prepattern formation of macrochaetes on the notum has provided an ideal model system for studying two-dimensional patterning.

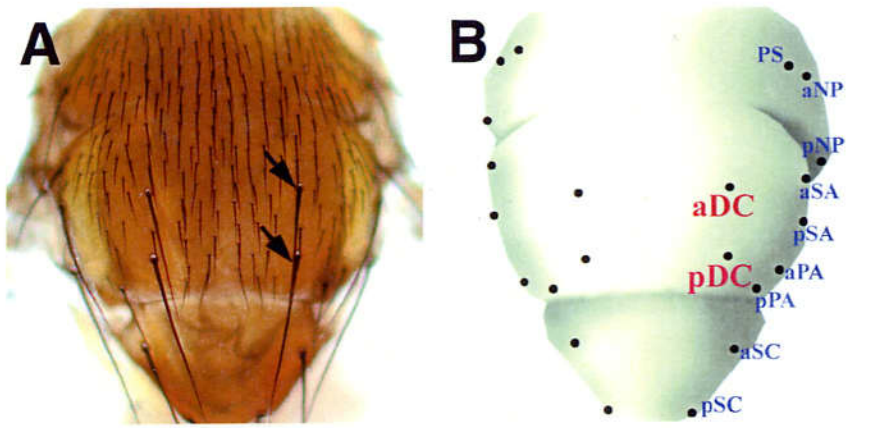

Fig. 2-1. wild-type macrochaete pattern on the notum (A), and, locations and names of each bristles (B). Two dorsocentral bristles (DC), an anterior DC (aDC) and a posterior DC (pDC) are indicated by arrows.

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 (thoracic disc) (Hartenstein and Posakony, 1989; Huang et al., 1991). 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).

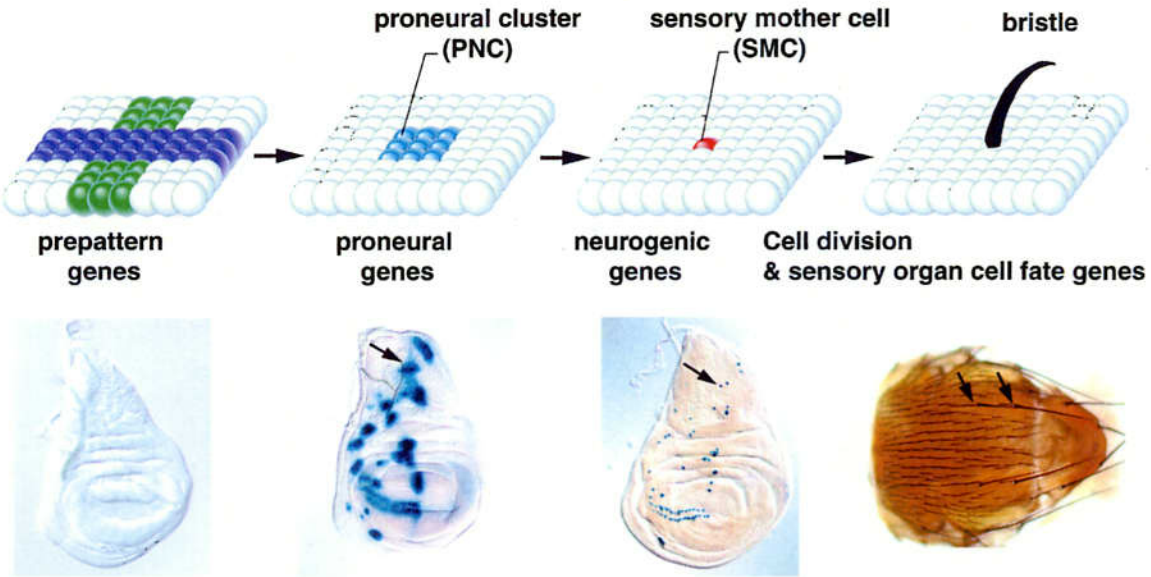

Fig. 2-2. Model for sensory organ formation.

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 (Stern and Tokunaga, 1968). 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 (Jan and Jan, 1990; Simpson, 1996). The existence of 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).

Several candidates of prepattern 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 ventral 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 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 (Ramain et al., 1993). M. 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. Recently, Garcia-Garcia et al. reported that Pnr directly regulates the dorsocentral enhancer element (Garcia-Garcia et al., 1999).

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 heterodimer with the HLH proteins encoded by the *ASC* and/or *daughterless*, thereby altering or interfering with their activity.

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 signaling 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.

In this chapter, I 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 signaling participates in this process in two major ways. One is induction of the proneural cluster in cooperation with Wg signaling 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.

Table 1 Drosophila culture medium

Cornmeal	50g
Dry yeast	40g
Glucose	100g
Wheat with embryo buds	20g
Agar	8g
Propionic acid	8ml
p-Hydroxybenzoic acid	10ml

Mess up to 1l with H_2O

The mutants and transgenic flies used in this work are as follows.

Gal4 driver lines.

hs-GAL4 and *tsh-GAL4* driver (Calleja et al., 1996; Shiga et al., 1996).

LacZ lines for reporting the expression of several genes.

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 and *punt* mutants.

wg^{Sp1} (Neumann and Cohen, 1996), and *wg*^{IL114} (Nusslein-Volhard and Wieschaus, 1980). *punt*^{P1} and *punt*^{135} (Letsou et al., 1995; Simin et al., 1998; Theisen et al., 1996). *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 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, which 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 in a PCR technique 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 NotI-XhoI 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

To mosaic expression of *tkv**, I used AyGal4 system that is combined Gal4-UAS and Flp-FRT systems (Fig. 2-3) (Ito et al., 1997). In the AyGAL4 construct, a flip-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

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 with fluorescence of GFP.

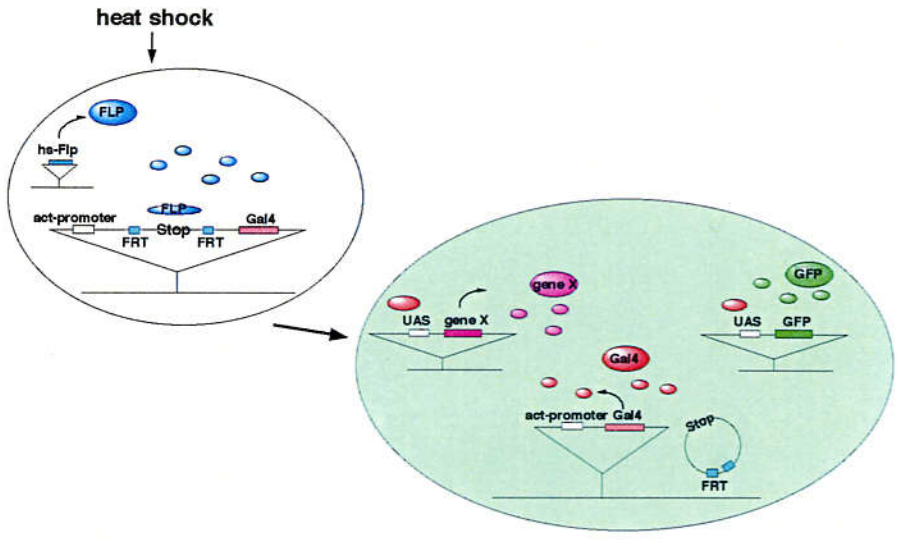

Fig. 2-3. AyGal4 system

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 as hours "Before Puparium Formation" (BPF). Each dot in Fig. 2-6 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 over night. 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™ (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-ß-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 signaling induces extra macrochaetes formation on the notum

Fig. 2-1 shows the wild-type macrochaete pattern of the notum. Anterior-dorsocentral bristle (aDC) and 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, dorsocentral proneural cluster is 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. 2-4). This suggests that Wg signaling 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 proneural cluster (Fig. 2-4B). This expression pattern and the property of Dpp as a morphogen suggest that Dpp signaling may also participate in prepattern formation of the macrochaetes on the notum.

First, I attempted to ectopically activate Dpp signaling in the thoracic disc during larval development using the GAL4-UAS system (Brand and Perrimon. 1993. See also Fig. 1-5). 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 signaling in a ligand independent manner (Hoodless et al., 1996; Nellen et al., 1996). I have tested several GAL4 drivers that promote GAL4 expression in the thoracic disc. Overexpression of either *tkv** or *punt* (data not shown) using *tsh-GAL4* driver (Shiga et al., 1996, and the GAL4 expression pattern in the wing disc is also shown in Fig. 2-5E) alters the macrochaete pattern on the notum (Fig. 2-5A). More than seven macrochaetes (per heminotum) are

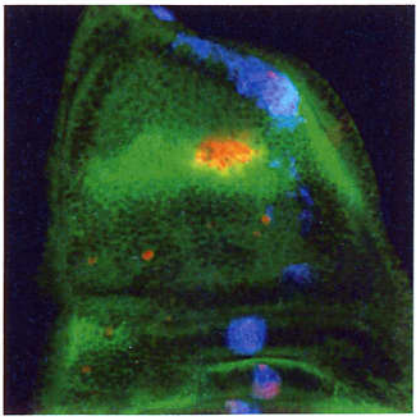

Fig. 2-4. Positions of dorsocentral proneural cluster, *wg* expression and *dpp* expression domain on the thoracic disc. dorsocentral proneural cluster (red) is located adjacent to the dorsal posterior side of the *wg* expression domain (green). dorsocentral proneural cluster is also close to the *dpp* expression domain (blue). Positions of dorsocentral proneural cluster is visualized with *neur-lacZ* expression (red), the *wg* expression with anti-Wg antibody (green) and the *dpp* expression with GFP expression by dpp^{blk}-*GAL4* at 18 °C (blue). Disc is shown with anterior left and dorsal up.

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. 2-5B). To look for a correlation between the timing of ectopic Dpp signaling 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. 2-5C) or *punt* (Fig. 2-5D) induces extra macrochaetes without significant notum morphology change. Fig. 2-6 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 signaling seems to be near the beginning of endogenous proneural gene expression in the thoracic disc

(Cubas et al., 1991). These results suggest that Dpp signaling participates in the prepatterning of the macrochaetes, presumably in the transcriptional activation of proneural genes in the thoracic disc.

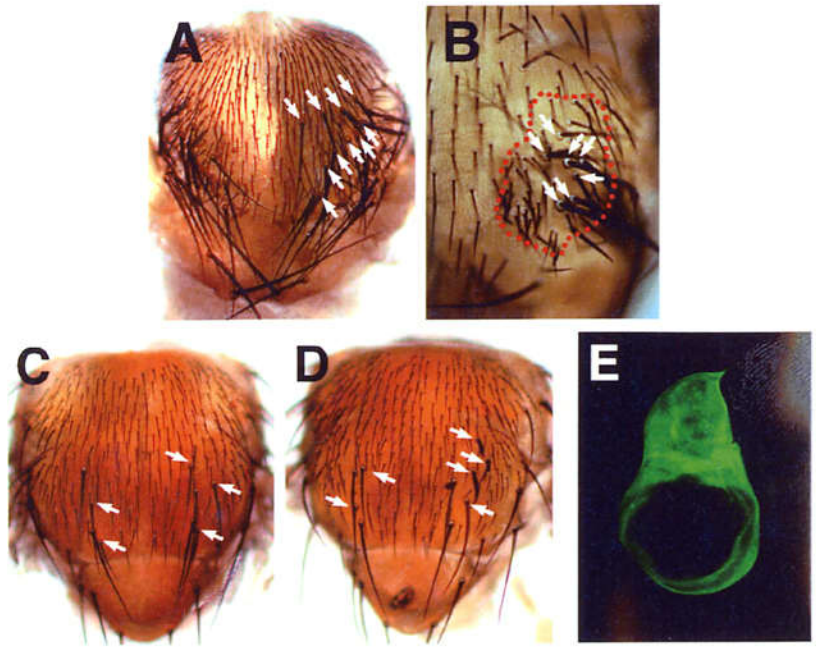

Fig. 2-5. Notal macrochaete phenotypes seen after ectopic Dpp signal activation. (A) pharate adult *UAS-tkv*: tsh-GAL4* notum. Overexpression of the constitutively active form of the type-I Dpp receptor (Tkv*) in the notal region causes a drastic change of the macrochaete pattern. Some of the ectopically induced macrochaetes in this region are indicated by arrows. (B) Clone of ectopic *tkv** expression induced by Ay-GAL4 system (Ito et al., 1997). Boundaries of the mosaic are outlined with a red dotted line. Additional macrochaetes (indicated with arrows) are induced only within the clone. (C,D) Mild activation of Dpp signaling by overexpression of either *tkv** (C) or Dpp type-II receptor *punt* (D) during early third larval stage (40 h BPF) using *hs-GAL4* driver induces ectopic macrochaetes. (E) *tsh-GAL4* expression pattern in the wing disc visualized by GFP expression of *UAS-gfp: tsh-GAL4*.

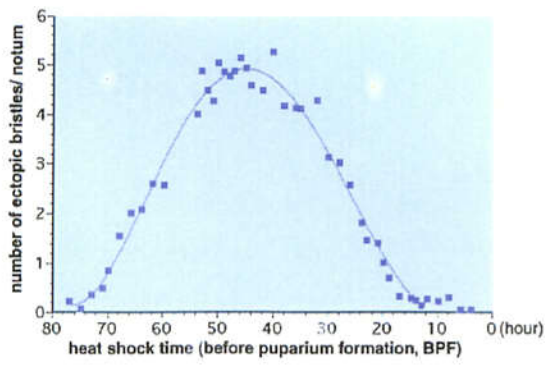

Fig. 2-6. Average number of the ectopically induced macrochaetes per notum of *UAS-tkv*: hs-GAL4* flies is plotted against heat shock timing (BPF).

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

To investigate more precisely the positioning of ectopically formed macrochaetes, I observed the locations of SMCs in the thoracic discs. Ubiquitous *tkv** expression in the thoracic disc using *tsh-GAL4* induces numerous ectopic SMCs (Fig. 2-7B). Ten to fifteen ectopic SMCs formed along the dorsal side of the *wg* expression domain and also several SMCs formed ventral to the *wg* expression domain. This asymmetric induction suggests that Wg signaling is necessary for the induction of extra SMCs by ectopic Dpp signaling. Interestingly, ectopic activation of Dpp signaling also alters the *wg* expression. In the wild-type thoracic disc, *wg* is expressed in a stripe of cells with a smooth boundary (Fig. 2-7A). In the *UAS-tkv*: tsh-GAL4* disc, *wg*-expressing cells exist within a narrow stripe and occasionally appear as small patches (Fig. 2-7B). Weak expression of *tkv** using *hs-GAL4* driver also induces additional SMCs and repression of the *wg* expression (Fig. 2-7C). This level of ectopic Dpp signaling induces additional SMCs only on the posterior side of the anterior compartment near the endogenous Dpp source (Fig. 2-7C). Relatively higher levels of *tkv** expression, in the 2 x *UAS-tkv*: hs-GAL4* disc, induces ectopic SMCs more anteriorly (Fig. 2-7D). These results indicate that high levels of Dpp signaling activity are necessary to induce SMCs. Low levels of ectopic Dpp signaling could recruit cells, which have already received sub-threshold levels of endogenous Dpp signaling, to form additional SMCs. On the other hand, even in the 1 x *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. 2-7C). Thus, low levels of Dpp signaling appear to be sufficient to repress *wg* expression.

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

threshold level of Dpp signaling required for SMC induction and that required for repression of *wg* expression.

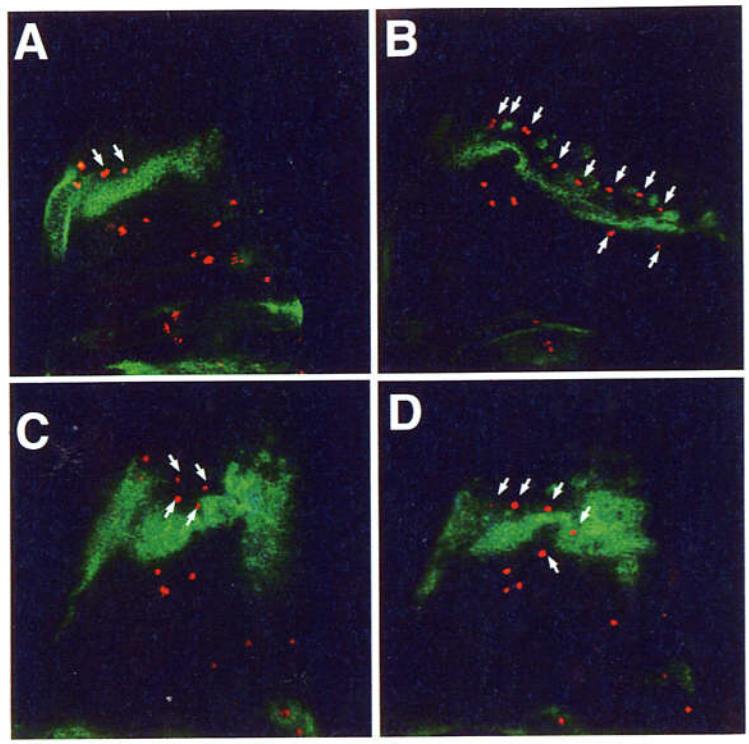

Fig. 2-7. Ectopic Dpp signaling affects the pattern of SMCs and *wg*-expression. Thoracic discs from late third instar larvae of wild type (A), *UAS-tkv*: tsh-GAL4* (B), *UAS-tkv*: hs-GAL4* (C) and *2 x UAS-tkv*: hs-GAL4* (2 copies of transgenes) (D) with heat shock at 40 hours BPF are labeled with *neur-lacZ* 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 DC SMCs are indicated by arrows. (B-D) Ectopic Dpp signaling induces additional SMCs, indicated by arrows, and also affect *wg* expression pattern.

Ectopic Dpp signaling 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 the dorsocentral proneural cluster appears only adjacent to the dorsal posterior side of the *wg*-expressing cells (Fig. 2-8A). Ectopic Dpp signaling using *tsh-GAL4* driver abnormally extends *ac* expression to the anterior end of the thoracic disc (Fig. 2-8B). Dorsocentral specific proneural gene expression was also monitored using *DC-lacZ* reporter (Gomez-Skarmeta et al., 1995). This reporter line selectively expresses *ß-galactosidase* in the dorsocentral proneural cluster in the thoracic disc (Fig. 2-8C and Gomez-Skarmeta et al., 1995). As this reporter contains an SMC enhancer, it also expresses *ß-galactosidase* in all of the SMCs in the wing disc (Culi and Modolell, 1998). *ß-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. 2-8D). 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. 2-8D). These results seem to indicate that Wg signaling 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 ventral 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

signaling appears to cooperate with other factor(s) to induce several *wg* independent proneural clusters.

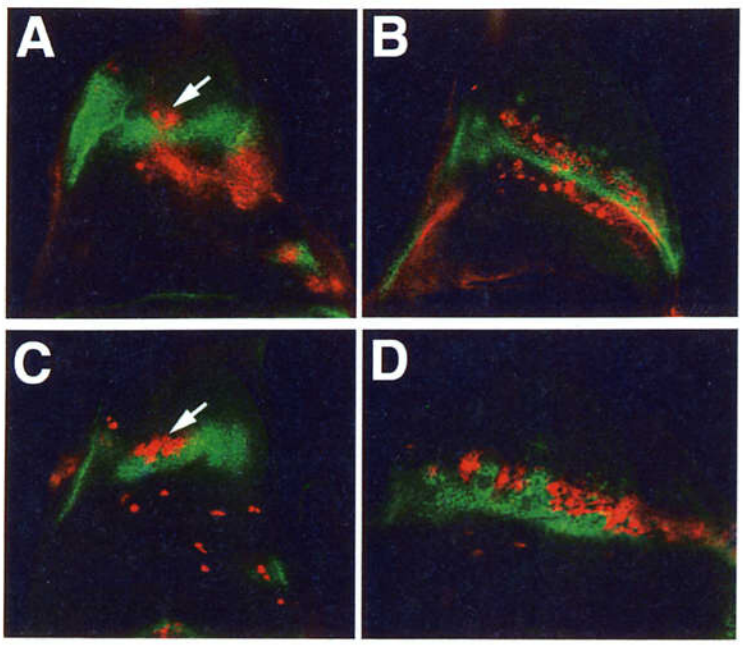

Fig. 2-8. Induction of the DC proneural cluster by ectopic Dpp signaling.
Late third instar thoracic discs of wild-type (A, C) and *UAS-tkv*: tsh-GAL4* (B, D). *wg* expression (green) and proneural clusters (red) are monitored with anti-Wg antibody and either *ac-lacZ* reporter (A, B) or *DC-lacZ* reporter (C, D). *ac-lacZ* expression localizes in the proneural clusters and high levels of accumulation are observed in some SMCs in this stage. The DC 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). DC proneural cluster is also monitored using *DC-lacZ* reporter whose expression is selectively localized in the DC proneural cluster (indicated by an arrow in C). This reporter also contains the SMC enhancer and expresses *β-galactosidase* in all of 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. All discs are shown with anterior left and dorsal down.

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

Anterior expansion of the dorsocentral proneural cluster in the presence of ectopic Dpp signaling could result from alteration of the *ASC* modulator(s) (Fig. 2-9). 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. 2-5C. I 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 signaling (Fig. 2-10B). This result indicates that *emc* expression is independent of Dpp signaling (Fig. 2-9B). Importantly, ectopic proneural clusters and SMCs are induced even in the *emc* expressing region (Fig. 2-10B, compare with Fig. 2-7B and 2-8B). Therefore it is possible that ectopically activated Dpp signaling causes the induction of proneural genes at high levels and that these activities could overcome the inhibitory effects of the Emc protein.

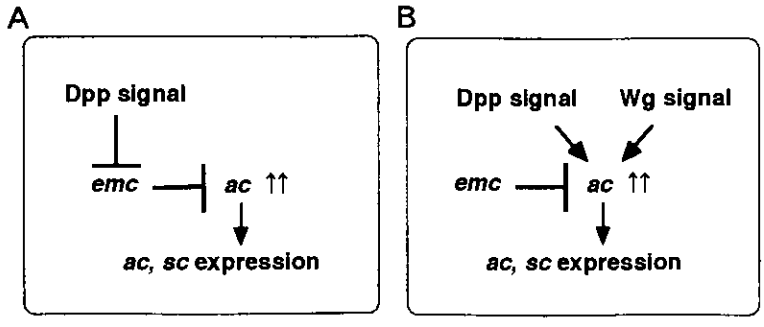

Fig. 2-9. Two possible relationships between Dpp signaling and Emc in the regulation of proneural gene expression.

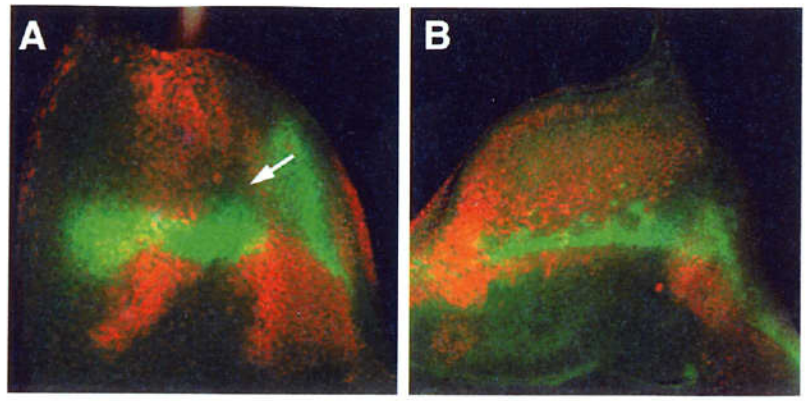

Fig. 2-10. Expression of *emc* in the wild-type (A), and *UAS-tkv*: tsh-GAL4* thoracic disc (B). Expression of *wg* (green) and *emc-lacZ* (red) are shown. The *emc* expression pattern is rather complex. Endogenous DC proneural cluster is formed in the region where no or little *emc* expression is observed (arrow in A). High levels of *emc-lacZ* expression are observed even in a *UAS-tkv*: tsh-GAL4* disc (B). Ectopic dorsocentral proneural clusters are formed in the region where *emc* is highly expressed (B, compare with Fig. 2-8D). 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 arrow heads in B). All discs are shown with anterior left and dorsal down.

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

Dpp signaling 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), I used conditional loss-of-function Dpp signaling mutants. Some allelic combinations of the *punt* mutations exhibit temperature sensitivity for Dpp signaling (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 stage. I 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. 2-11B). 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. 2-11A) but not in *punt-ts* discs (Fig. 2-11B). This result indicates that both dorsocentral proneural cluster induction and repression of *wg* expression are promoted by endogenous Dpp signaling.

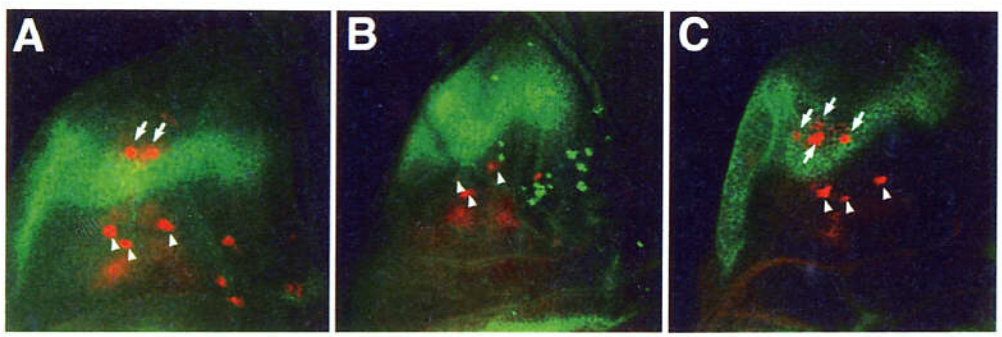

Fig. 2-11. Reduction of Dpp signaling activity leads to ectopic *wg* expression and inhibition of dorsocentral SMC formation. Thoracic discs in the late third larval stage labeled with anti-Wg antibody (green) and 3.7 *sc-lacZ* expression (red) are shown (A-C). Positions of the DC SMCs are indicated with arrows. (A) wild-type thoracic disc. (B) *punt-ts* thoracic disc from larvae which were transferred from 18°C to 29°C about 48 hours BPF, I referred this mutant as 'severe *punt-ts*'. In the severe *punt-ts* disc, *wg* expression expands to the dorsal edge of the disc. dorsocentral SMCs are no longer observed within the expanded *wg* expression domain. SMCs ventral to the *wg* expression domain still exist (indicated with arrow heads). (C) A thoracic disc of *punt-ts* which was shifted to mild heat condition (at 25°C), I referred this as 'mild *punt-ts*'. In the mild *punt-ts* disc, the *wg* expression domain is slightly expanded toward dorsal and additional SMCs are formed (arrows in C). All discs are shown with anterior left and dorsal down.

To our surprise, in thoracic discs from *punt-ts* mutants shifted to milder condition (25°C) at same stage, I will refer this mutant as 'mild *punt-ts* ', one or a few extra SMCs are formed (Fig. 2-11C). It is also worth noting that extra SMCs appear to be formed in a more posterior region compared to the endogenous dorsocentral SMCs (Fig. 2-11C compare with Fig. 2-7A). In the mild *punt-ts* disc, the *wg* expression domain is slightly expanded dorsally and posteriorly (Fig. 2-11C). One possible explanation of 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. I will discuss more about this controversial issue later.

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

Finally, I examined whether *wg* activity is required for *tkv** induced ectopic dorsocentral bristle formation. Fig. 2-12A 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. 2-12B and compare to Fig. 2-5A). 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. 2-12B). These results confirm that Wg signaling is absolutely required for ectopic dorsocentral bristle formation by Dpp signaling.

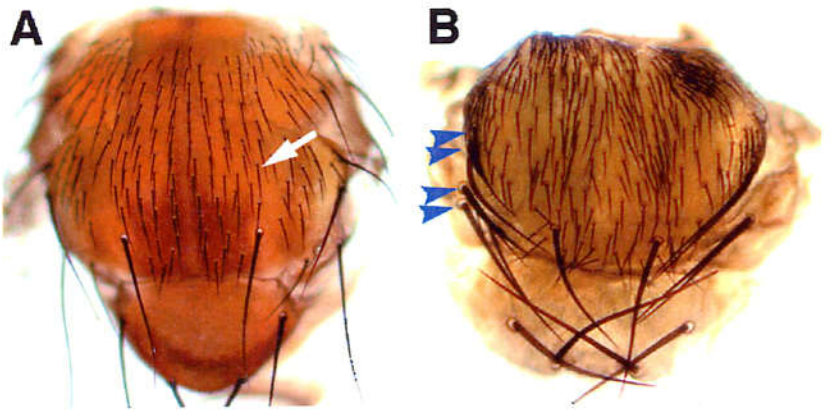

Fig. 2-12. Effect of *tkv** overexpression on bristle development in *wg* mutant background. Bristle pattern of the wg^{IL114} *tsh-GAL4*/wg^{Sp1} notum (A), and pharate adult notum of wg^{IL114} *tsh-GAL4*/wg^{Sp1}; *UAS-tkv**/+ (B). Ectopic *tkv** expression by *tsh-GAL4* in this *wg* mutant no longer induces ectopic bristles in the dorsocentral region of the notum (B). Duplication of the *wg*-independent bristles (aPA and pSA) is frequently observed (indicated by blue arrow heads).

Discussion

Dpp signaling participates in dorsocentral bristles development

In this chapter, I have shown that ectopic Dpp signaling 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 signaling is required cell autonomously to induce ectopic proneural clusters. Loss-of-function experiments using *punt-ts* flies also indicated that endogenous Dpp signaling is necessary for the formation of the dorsocentral SMCs. Moreover, in the *wg* mutant flies (wg^{Sp1}/wg^{IL114}), ectopic Dpp signaling 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 signaling.

There are many genes known to be regulated by both Dpp and Wg signaling. 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 signaling (Kim et al., 1997; Kim et al., 1996). *vg* expression is also regulated by Wg signaling in the wing pouch (Neumann and Cohen, 1997; Zecca et al., 1996). Regulatory mechanism of the *cis*-element(s) of the DC-enhancer is totally unknown. Cell autonomous effects of both Dpp and Wg signals (Fig. 2-5D and Phillips et al. personal communications) suggest the possibility that Dpp and Wg signal transducer directly effects the DC-enhancer to induce proneural genes. However, I can not rule out another possibility that Dpp and/or Wg signaling control the expression (or activity) of other prepattern genes that directly activate the DC-enhancer. Analysis of DC-enhancer element is necessary to address how Dpp and Wg signals cooperate in the induction of the proneural genes at the dorsocentral region.

My data from both gain-of-function and loss-of-function experiments suggest that Dpp signaling also has an important role in 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. I 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, I set much more severe conditions, with a temperature shift from 18 to 29°C. I suggest that transducing activity associated with Dpp signaling 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. 2-11C). At 29°C, Dpp signaling activity is reduced below a threshold level and the *wg* expression domain expands (Fig. 2-11B). *dpp* expression in the wing disc seems not to be regulated by Wg signaling, since *dpp* expression pattern is normal in the *wg-ts* mutants under nonpermissive conditions (Theisen et al., 1996; Tomoyasu et al., unpublished data).

Models for formation of the dorsocentral bristles

It appears that different thresholds of Dpp signaling 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 2-13A 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. 2-13C). 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. 2-13C).

It is possible that alterations in Dpp signal activity cause a shift in the activities slope, with an up-shift in gain-of-function mutants and a down-shift in loss-of-function mutants (Fig. 2-13A). This model is consistent with my experimental results except for the results that were observed in the mild *punt-ts* mutants. According to this model, weak reduction of Dpp signaling activity should result in a down-shift 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 signaling, 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. I have shown that a high level of Dpp signaling can overcome the inhibitory effect of Emc (Fig. 2-10B). This indicates that cells within the Emc

expression domain require a higher threshold level of Dpp signaling activity for proneural gene induction than those cells outside of this domain (Fig 2-13A). In this case, a slight downshift in the Dpp signal activity slope would result 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 signaling 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. 2-4). One explanation for the existence of this gap is that the highest level of Dpp signaling 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. 2-11C). A down shift of the Dpp activities slope would release the area in which proneural induction is inhibited by the highest levels of Dpp signaling from such inhibition. However, there is one more paradox to the adoption of the inhibitory action of Dpp signaling. Considering this inhibitory effect in term of the model shown in Fig. 2-13A, ectopic activation of Dpp signaling should have expanded the area in which proneural induction is inhibited. This was not the case for *UAS-tkv*: tsh-GAL4* discs (Fig 2-7B). One of solution to this paradox is altering the linear activity slope, as illustrated in Fig. 2-13A, to the nonlinear slope, as illustrated in Fig. 2-13B. 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 that may be addressed by future experiments. For instance, a concentration gradient of Dpp protein within the thoracic disc should be directly visualized. Recently, distribution of Dpp proteins in the pouch region of the wing disc was directly visualized using Dpp-GFP fusion proteins (Entchev et al., 2000; Teleman and Cohen, 2000). Same approach could be available for visualizing the Dpp protein distribution in the thoracic disc. The mechanism by which the highest levels of Dpp

signaling inhibit proneural induction is also unclear and should be studied at molecular level.

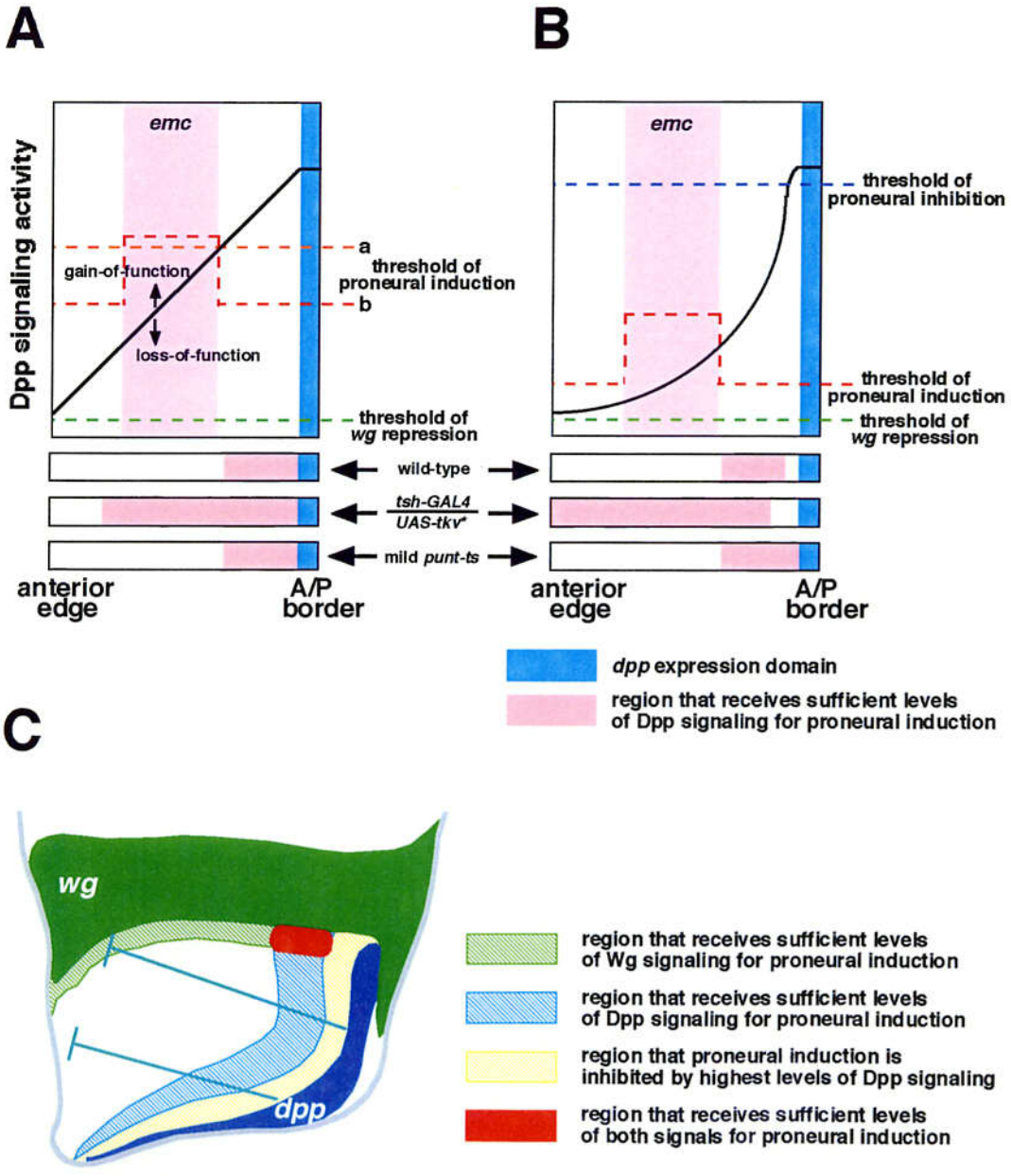

Fig. 2-13. Models for DC proneural cluster formation. Two hypothetical models for Dpp signaling activity along the A/P axis within the thoracic discs are illustrated in (A) and (B). *dpp* is expressed in A cells which are located near the A/P compartment boundary (drawn with blue). After secretion, Dpp 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 signaling. The thresholds of Dpp signaling activity for proneural induction and *wg* repression are different. A low level of Dpp signaling 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 signaling seems 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 broken 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 signaling for the inhibition of proneural induction, in which the highest level of Dpp signaling 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 is 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 of 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 signaling (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 signaling 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 signaling.

Interaction to other genes in the macrochaete prepatterning on the notum

It has previously 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* genes expression (Cubadda et al., 1997; Garcia-Garcia et al., 1999; 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). Garcia-Garcia et al have described that Pnr proteins act as a transcriptional activator against DC-enhancer of *ASC* genes. The relationship between Dpp signaling and these transcriptional regulators is largely unknown. Some interesting results are also reported by Calleja et al. (1996), who have shown that *wg* expression is affected in *pnr* mutants. In pnr^{VI}/pnr^{VX1} discs, both seems to be loss-of-function alleles of *pnr* gene, results in no *wg-lacZ* expression in the thoracic disc. On the other hand, *wg* expression extends dorsally in the disc of the 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.

In next chapter, I attempt to elucidate the relationship between Dpp signaling and Pnr/Ush, mainly focused on the regulation of wg expression in the thoracic disc.

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CHAPTER 3.

THE DECAPENTAPLEGIC MORPHOGEN GRADIENT REGULATES THE NOTAL WINGLESS EXPRESSION THROUGH INDUCTION OF PANNIER AND U-SHAPED IN *DROSOPHILA*

Summary

In previous chapter, it is reported that Dpp morphogen gradient contributes the positioning of dorsocentral proneural cluster in the thoracic disc in two major ways, induction of proneural gene and restriction of *wingless* expression. It has previously been reported that two prepattern genes, *pannier* and *u-shaped*, regulate the positioning of the dorsocentral proneural cluster along the dorsoventral axis, by activating the dorsocentral enhancer element of *achaete-scute* complex, and also regulate the *wingless* expression in the thoracic disc. However, the relationship between Dpp signaling and these transcriptional regulators is not well understood. Here, I show that expression of *pannier* and *u-shaped* is regulated by Dpp signaling in a concentration-dependent manner, low threshold for inducing *pannier* and high threshold for *u-shaped*. Furthermore, the mechanisms for establishing the notal *wingless* expression were also examined. Analysis of *wingless* expression in wild-type and several allelic combinations of *pannier* mutants revealed that Pannier has two opposing roles, induction and repression, in the regulation of the *wingless* expression. I also revealed that notal *wingless* expression is affected by Wingless signaling itself. These finding reveal a detail relationship between Dpp morphogen gradient and prepattern genes in the thoracic disc.

Introduction

The positioning of dorsocentral (DC) proneural cluster seems to be regulated by several independent mechanisms. In chapter 2, it is reported that Decepentaplegic (Dpp) morphogen gradient contributes the positioning of DC proneural cluster in the presumptive notum region of the wing imaginal disc (thoracic disc) in two major ways, induction of proneural gene and restriction of *wingless* (*wg*) expression. Sato et al. also reported the involvement of Dpp in the regulation of notal *wg* expression (Sato et al., 1999).

In contrast, It has previously been reported that two transcriptional regulaters, Pannier (Pnr) and U-shaped (Ush), regulate the positioning of the dorsocentral proneural cluster along the dorsoventral (D/V) axis, and regulate the *wg* expression in the thoracic disc. *pnr*, which encodes a GATA family transcription factor, is expressed in the dorsal half of the thoracic disc, and is necessary for the formation of dorsocentral mechanosensory bristles which are formed in the *pnr* expression domain (Garcia-Garcia et al., 1999; Haenlin et al., 1997). Recently, Garcia-Garcia et al. reported that Pnr directly binds to DC enhancer element and regulates the proneural gene expression in the thoracic disc (Garcia-Garcia et al., 1999). *ush* encodes a protein belonging to the friend of GATA-1 (FOG-1) family protein, which modulates the transcriptional activity of GATA-1 (Fox et al., 1999). *ush* is expressed in the hinge region and in dorsal-most region of the thoracic disc largely overlapping the area of expression of *pnr* (Cubadda et al., 1997). It has previously been reported that Ush directly binds to Pnr in vitro and in yeast. Also Ush inhibits the transactivation of α-globin promoter sequences by Pnr in cultured cells (Haenlin et al., 1997). Loss-of-function of *ush* results in the opposite phenotype observed in the loss-of-function *pnr* mutants (Cubadda et al., 1997). Thus, Ush seems to directly inhibit the Pnr function in sensory organ development. The mechanism for the positioning of DC proneural cluster by Pnr and Ush is summarised in Fig. 3-1.

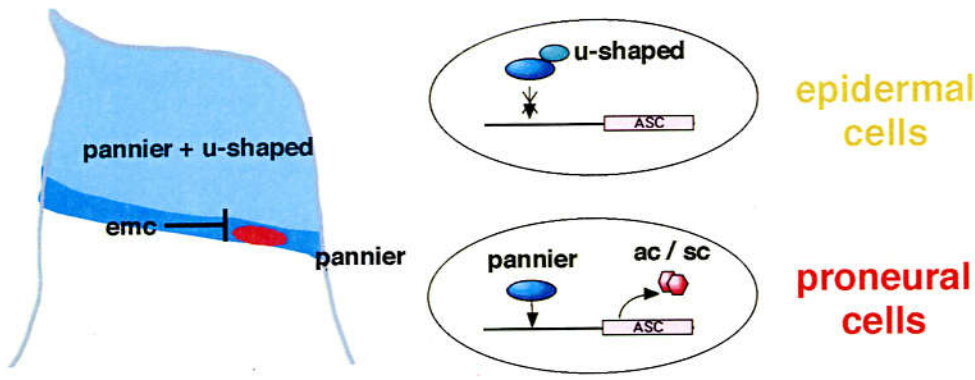

Fig. 3-1. Mechanism for the positioning the DC proneural cluster by Pnr/ Ush.

Similar to the Dpp signaling, Pnr and Ush seem to be also involved in the regulation of notal *wg* expression. Calleja et al. (1996), reported that *wg* expression is affected in *pnr* mutants. In pnr^{V1}/pnr^{VX1} discs, both seems to be loss-of-function alleles of *pnr* gene, results in no *wg-lacZ* expression in the thoracic disc. On the other hand, *wg* expression extends dorsally in the disc of the another heteroallelic combination, pnr^{D1}/pnr^{md237} (pnr^{D1} seems to be a gain-of-function allele). These findings indicate the possibility that Pnr (and presumably Ush) also modulates the regulation of notal *wg* expression. Recently, Garcia-Garcia et al. reported that Pnr and Ush regulate the *wg* expression in the thoracic disc (Garcia-Garcia et al., 1999).

These similarities between the effects of Pnr/Ush and Dpp morphogen gradient suggest that these two systems are closely related. In this chapter, I tried to reveal a relationship between Pnr/Ush and Dpp morphogen gradient in the prepatterning of thracic disc. Experiments which examined the *pnr* and *ush* expression

in loss-of and gain-of-function mutants for Dpp signaling revealed that both *pnr* and *ush* expression is positively regulated by Dpp in a concentration-dependent manner. Detailed mechanism for the regulation of notal *wg* expression by Pnr/Ush was also analysed. Analysis of *wg* expression in wild-type and several allelic combinations of *pnr* mutants revealed that Pnr has two opposing roles, induction and repression, in the regulation of the *wg* expression. I also revealed that notal *wg* expression is affected by Wg signaling itself. These results indicate that Dpp signaling establishes the notal *wg* stripe through the induction of *pnr* and *ush*.

These findings revealed a detail relationship between Dpp morphogen gradient and prepattern genes. I will also discuss the mechanisms for establishing the two-dimensional coordinates by Wg and Dpp in the thoracic disc. Recently, a paper describing the regulation of notal wg expression by Dpp through the induction of *pnr* and *ush* was published (Sato and Saigo, 2000). This study complements and extends their findings.

Materials and Methods

Fly stocks and crosses

Flies were raised at 25°C except for the put^{135}/put^{P1} and wg^{l-12} homozygous flies. The mutants and transgenic flies used in this work are as follows.

Mutants.

pnr^{VX1}, pnr^{VX6}, pnr^{D1}, *Df(3L)sbd45*, pnr^{md237} as *pnr* mutants (Ramain et al., 1993). $d\text{-}axin^{S044230}$, dsh^{va153}, and wg^{l-12} as Wg signaling mutants (Hamada et al., 1999; Theisen et al., 1994; van den Heuvel et al., 1993). put^{135}, put^{P1}, tkv^{a12}, as Dpp signaling mutants (Letsou et al., 1995; Nellen et al., 1994; Penton et al., 1994; Theisen et al., 1996).

Gal4 driver lines.

pnr^{md237} flies which is a allele of *pnr* were also used as *pnr-Gal4* driver (Calleja et al., 1996). *tsh-Gal4* as a Gal4 driver in *tsh* expression domain (Shiga et al., 1996).

UAS lines for ectopic gene expression

$UAS\text{-}gfp^{T10}$, *UAS-tkv**, *UAS-ush*, *UAS-dad* for overexpression of these genes (Cubadda et al., 1997; Tomoyasu et al., 1998; Tsuneizumi et al., 1997), and *Ubx >> lacZ >> tkv** for mosaic expression (Lecuit et al., 1996).

Lines for reporting the expression of several genes.

Wg^{17en40} as a reporter of *wg* expression, and dpp^{P10638} as a reporter of *dpp* expression (Twombly et al., 1996). *Act5c>stop>lacZ* and *UAS-Flp* for the lineage tagging method (Weigmann and Cohen, 1999).

dsh^{va153}/*FM7i act-gfp* flies were crossed with *FM7i act-gfp/Y;* pnr^{md237} $UAS\text{-}gfp^{T10}$/ +, and selected dsh^{va153}/*Y;* pnr^{md237} $UAS\text{-}gfp^{T10}$/ + larvae with absence of ubiquitous GFP expression. $put^{P1}/st\ put^{135}\ e$ flies, designated as put-ts are permissive at 18°C and non-permissive at 29°C (Letsou et al., 1995; Theisen et al., 1996; Tomoyasu et al., 1998). wg^{l-12} homozygous flies are permissive at 17°C and non-

permissive at 25°C (van den Heuvel et al., 1993). For lineage tracing, *Act5c>stop>lacZ; UAS-Flp* flies were crossed with *pnr ^{md237} UAS-gfp^{T10}/ TM6B.*

Clonal analysis

Mutant clones were induced by Flp/FRT mediated mitotic recombination (see chapter. 1) in the larvae of the following genotypes.

yw hsFlp; FRT82 pnr ^{VX6}/ FRT82 hs-Myc-gfp

yw hsFlp; FRT82 pnr ^{D1}/ FRT82 hs-Myc-gfp

yw hsFlp; FRT82 pnr^{V1}/ FRT82 hs-Myc-gfp

yw hsFlp; FRT82 d-axin^{S044230} / FRT82 hs-Myc-gfp

yw hsFlp; FRT82 d-axin^{S044230} pnr ^{VX6}/ FRT82 hs-Myc-gfp

yw hsFlp; FRT40 tkv ^{a12}/ FRT40 arm-lacZ; pnr ^{md237} UAS-GFP^{T10}/ +

Larvae of these genotypes were heat shocked at 35°C for 1 hour to induce mitotic recombination. Two hours before fixation, the resulting late third instar larvae were subjected to a second heat shock (1hour at 37°C) to induce GFP expression.

Clones of genetically marked cells expressing Tkv* were induced in the larvae of the genotype of

yw hsFlp; Ubx >> lacZ >> tkv/+, pnr ^{md237} UAS-gfp^{T10}/ +* .(>> means a FRT site)

Larvae of this genotype were heat shocked at 35°C for 1 hour to induce flip-out of FRT cassette containing the *LacZ* gene flanked by two FRT sites.

Imaginal disc staining

In situ hybridization, X-gal staining, and antibody staining was performed using standard procedures. Antibodies used in this chapter and their dilutions are as follows. anti-ß-galactosidase rabbit polyclonal antibody (1:1000; Cappel); anti-rabbit IgG LRSC-conjugated (1:200; Jackson); anti-mouse IgG FITC-conjugated (1: 200; Jackson); anti-mouse IgG Cy5-conjugated (1: 100; Jackson); anti-Wg antibodies

(4D4) (Brook and Cohen, 1996) (1:50). The anti-Wg antibody developed by S. Cohen was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa.

Lineage tracing experiment

One problem to analyze the expression pattern of several gene is whether the cell expressing some gene at some stage (e.g. late third larval stage) is same cell at earlier stage (e.g. second larval stege) or not. To visualize the region of cells that have expressed some gene at earlier stage, I used lineage tracing technique developed by Weigmann K. et al in 1999 (Weigmann and Cohen, 1999). The scheme outlining lineage tracing is shown in Fig. 3-2.

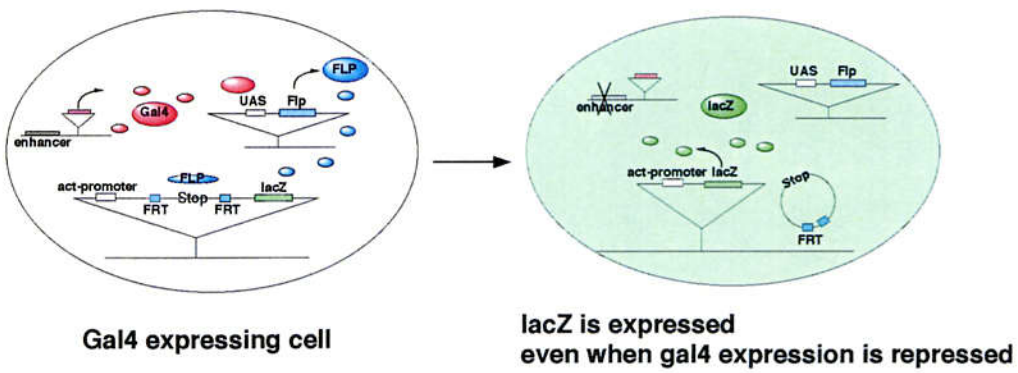

Fig. 3-2. Lineage tracing method

Results

ush expression is positively regulated by Dpp signaling

In previous chapter, I showed that Dpp signaling has two indispensable roles in the positioning of DC proneural cluster, induction of proneural gene expression at the dorsocentral area and restriction of *wg* expression, in the thoracic disc. It has previously reported that Pnr and Ush, the products of prepattern genes, also regulate the position of DC proneural cluster and regulation of *wg* expression domain in the thoracic disc. One possible explanation is that Dpp signaling affects the prepattern through the induction of these genes.

To confirm this possibility, I first examined *ush* expression in temperature sensitive allelic combination of *punt* (*put*), which encodes a type-II Dpp receptor (Letsou et al., 1995; Ruberte et al., 1995). Under non-permissive conditions, *ush* expression is strongly reduced in *put-ts* discs (Fig. 3-3A, B). Overexpression of Daughter against dpp (Dad) (Tsuneizumi et al., 1997), a negative regulator of Dpp signaling, also represses *ush* expression (Fig. 3-3C). Thus, *ush* expression is positively regulated by Dpp signaling in the thoracic disc.

pnr expression is also regulated by Dpp signaling

In contrast to *ush*, *pnr* expression is still observed even in the *put-ts* discs (Fig. 3-4A, B). However, in tkv^{a12} clones, *pnr* expression is significantly reduced in a cell autonomous manner (Fig. 3-4D, F). Reduction of *pnr* expression is more severe in tkv^{a12} clones located far from the Dpp source (Fig. 3-4D, F indicated with arrows) than in the clones located near the Dpp source (Fig. 3-4D, F indicated with an arrowhead). It would be possible that the tkv^{a12} cells respond to Dpp signaling by

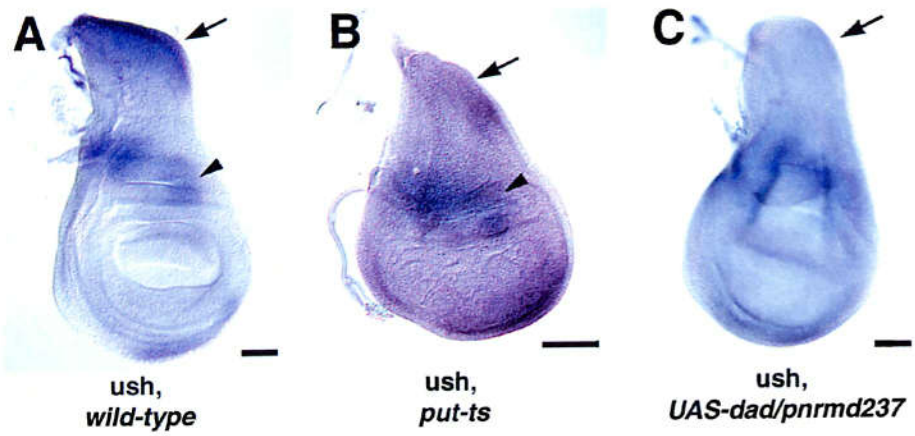

Fig. 3-3. *ush* is induced by Dpp signaling. *ush* expression in the wild-type (A), *put-ts* (shifted to the non-permissive condition at 72hr BPF) (B) and *UAS-dad/ tsh-GAL4* (a ubiquitous GAL4 driver in the notum) (C). *ush* expression in the dorsal-most region is strongly reduced in both discs (indicated by arrows). *ush* expression in the hinge region is not affected (A, B, indicated by arrowheads). All discs are shown with anterior left and dorsal up. mRNA expression is detected with a DIG-labeled antisense RNA probe. Scale bars represent 50 μm.

means of the existence of another Dpp type-I receptor, Saxophone (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994). Therefore, tkv^{a12} mutant clones located near the Dpp source can still receive some levels of Dpp signals, and cause weak expression of *pnr*. These findings indicate that Dpp signaling controls *pnr* expression in the thoracic disc.

The results from *put-ts* showing *pnr* expression was not altered seems to contradict the results with tkv^{a12} clones, however, this could be explained by the difference in remaining Dpp signal level between tkv^{a12} homozygous cells and *put-ts* cells. tkv^{a12} cells located in the wing pouch region where the Dpp signal is necessary for cell proliferation do not proliferate (Burke and Basler, 1996). In contrast, *put-ts* cells in the pouch region proliferate and form some wing blade tissues in the pharate adult stage (Fig. 3-4B and unpublished data). Thus, Dpp signal levels in *put-ts* cells should be higher than those in tkv^{a12} homozygous cells. The level of Dpp signal in the *put-ts*

disc seems to sufficient to induce *pnr* but insufficient to induce *ush*. In contrast, the Dpp signal level in *tkv* ^{a12} cells may be insufficient to induce both *pnr* and *ush*. Taken together, these results indicate that the expression of *pnr* is also regulated by Dpp signaling, and that different thresholds are set for the induction of *pnr* and for *ush* : high levels of Dpp signaling are necessary for inducing *ush* but low levels are sufficient to induce *pnr*.

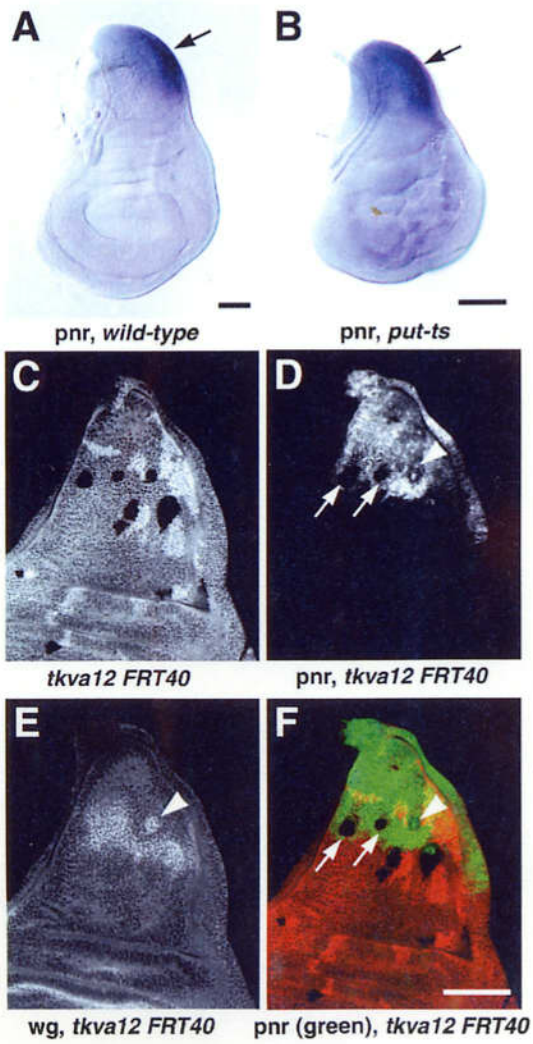

Fig. 3-4. Alteration of *pnr* expression in Dpp signaling mutants. *pnr* expression in the wild-type (A), *put-ts* (B), *tkv* ^{a12} mutant clones (C-F). The *put-ts* disc retains *pnr* expression similar to that of the wild-type (B). *pnr* expression (D, green in F) is completely abolished in a cell autonomous manner in *tkv* ^{a12} clones (C, absence of red in F) located far from the Dpp source (indicated by arrows). However, a clone located near the Dpp source (indicated by an arrowhead in D-F) still retains some levels of *pnr* expression. This result suggests that the *tkv* ^{a12} mutant clones still receive low levels Dpp signaling activity. This posterior clone ectopically expresses *wg* expression (E) suggesting that *ush* expression is lost in this clone. *pnr* expression is detected with a DIG-labeled antisense RNA probe (A, B), and by GFP expression of *UAS-gfp pnr* ^{md237} (D, F). *wg* expression is monitored with an anti-Wg antibody. *tkv* ^{a12} clones were induced at 50 hours BPF. All discs are shown with anterior left and dorsal up. Scale bars represent 50 μm.

Ectopic induction of *pnr* and *ush* by Tkv*

The analysis of *pnr* and *ush* expression in the loss-of-function mutants for Dpp signaling indicates that Dpp signaling is a required factor for *pnr* and *ush* expression in the thoracic disc. However, this relationship is somewhat paradoxical, because the Dpp that provides the positional information along the A/P axis regulates the *pnr*, *ush*, and *wg* that provide the D/V information in the thoracic disc. There are two possibilities for the role of Dpp signaling in *pnr* and *ush* induction. The first one is that Dpp is a main upstream regulator for *pnr* and *ush*, and Dpp signaling activity in the thoracic disc reflects the expression domains of these genes. Alternatively, other factor(s) (independent of Dpp signaling) may specify the *pnr* and *ush* expression domains and Dpp participates only in a permissive role in the expression of these genes. To investigate these possibilities, I examined whether ectopic Dpp signaling can ectopically induce *pnr* and *ush* in the thoracic disc. Overexpression of Tkv* by *tsh-Gal4* driver can ectopically induce *pnr* and *ush* in the ventral region of the thoracic disc (Fig. 3-5A, B). Mosaic expression of Tkv* also ectopically induced *pnr*, in a cell autonomous manner (Fig. 3-5C). These results support the first possibility, that Dpp plays an essential role in determining the expression domain of *pnr* and *ush* in the thoracic disc. However, cells in the central region of the thoracic disc do not seem to have the competence to express *pnr* and *ush* in response to ectopic Dpp signals (Fig. 3-5A-C indicated by arrowheads). Hence, other factor(s) also partially define the *pnr* and *ush* expression domain, independently of Dpp signaling.

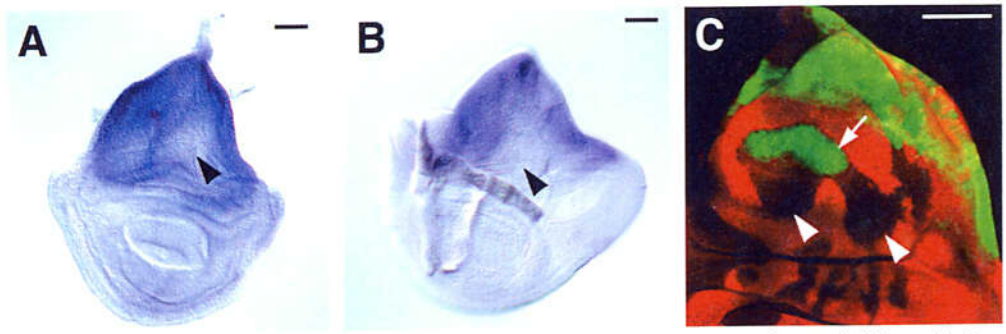

Fig. 3-5. Ectopic induction of *pnr* and *ush* by ectopic Dpp signaling. *ush* expression (A) and *pnr* expression (B) in *UAS-tkv*/ tsh-GAL4* discs. *pnr* and *ush* expression is ectopically induced by Tkv*. Mosaic expression of Tkv* by the Ubx promoter (indicated by the absence of red in C) also ectopically induces *pnr*. However, in the central region of the notum, Dpp signaling can not induce *ush* or *pnr* (indicated by arrowheads in A-C). *ush* expression is detected with a DIG-labeled antisense RNA probe. Pnr expression is monitored with a DIG-labeled antisense RNA probe (B), and by GFP expression of *UAS-gfp pnr* ^{md237} (C). All discs are shown with anterior left and dorsal up. Scale bars represent 50 μm.

dpp expression is restricted dorsally in the thoracic disc

Experiments of overexpression of Tkv* revealed that Dpp partially defines the expression domains of *pnr* and *ush* in the thoracic disc. However, in the late third larval stage, *dpp* expression seems to be orthogonal to the *pnr*, *ush*, and *wg* expression domains. One possible explanation is that the *dpp* expression domain is dramatically changed through wing disc development. To investigate this possibility, I analyzed the *dpp* expression monitored by *dpp* ^{P10638} throughout wing disc development. *dpp* expression in the thoracic disc is slightly changed, however, no dramatic change was observed throughout disc development (Fig. 3-6). In the early stage, cells located at the A/P border of the dorsal half of the thoracic disc predominantly expressed *dpp*. *pnr* and *ush* appeared to be induced by the Dpp secreted from this early *dpp* expression domain (Fig. 3-6A).

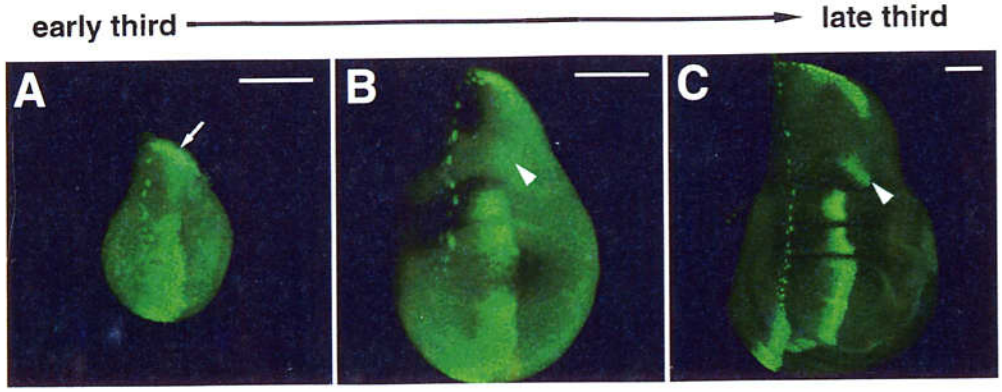

Fig. 3-6. *dpp* expression in the wing disc throughout development. *dpp* expression was monitored by an enhancer trap line, *dpp* ^{P10638}. Wing discs in the early (A), mid (B) and late (C) third larval stage were stained. *dpp* expression in the A/P border of the wing disc seems to be discontinuous throughout disc development. *dpp* expression in the notal region is predominantly observed in the dorsal half of the thoracic disc at the early third instar larval stage (A, indicated with an arrow). Ventral patch of the additional *dpp* expression domain is evident in mid to late third larval stage (B, C, indicated with arrowheads). All discs are shown with anterior left and dorsal up. Scale bars represent 50 μm.

The *wg*, *pnr* expression domain in the wild-type thoracic disc

The *pnr* and *ush* expression domains are determined by Dpp morphogen gradient. The next question is: how Pnr and Ush regulates the *wg* expression in the thoracic disc? In the adult notum, *pnr* and *wg* are expressed in stripes in an almost complementary manner (Simpson, 1996) (Fig. 3-7E, F). In order to know the precise positioning of the *wg* and *pnr* expression domains throughout wing disc development, I visualized the *wg* and *pnr* expression domain in the discs of late second to late third larval stages by anti-Wg antibody and GFP expression driven by *pnr* ^{md237}, respectively. GFP expression driven by *pnr* ^{md237} is first detected around the dorsal region of the thoracic disc in late second larval stage (Fig. 3-7A). Wg protein is not detected in the thoracic disc at this stage (Fig. 3-7A-ii). Wg is initially detected in the *pnr* domain at the early third larval stage (Fig. 3-7B-ii). The *pnr* domain expands anteriorally (Fig.

3-7C) and occupies the entire dorsal thoracic disc (Fig. 3-7D). In the mid to late third larval stage, some of the *wg* region expand ventrally from the *pnr* domain (Fig. 3-7D).

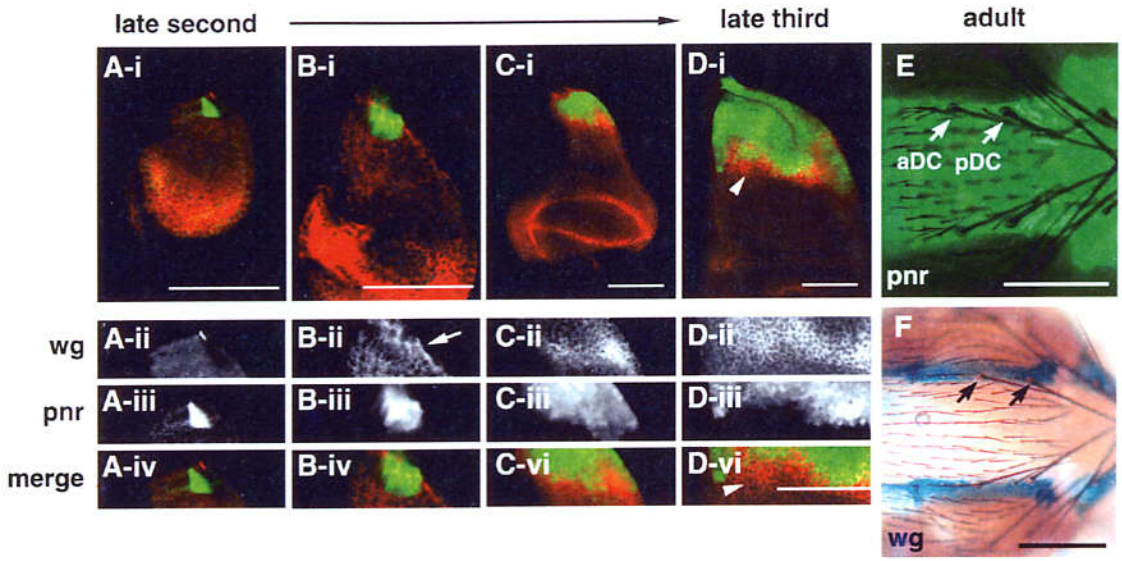

Fig. 3-7. The temporal and spatial relationship between *wg* and *pnr* in the wild-type thoracic disc. *wg* and *pnr* expression domains are monitored by anti-Wg antibody (A-D) or lacZ expression of *wg* ^{17en40} (F) and GFP expression driven by *pnr* ^{md237}, respectively. *wg* (red in A-C-i and separated images in A-C-ii) and *pnr* (green and A-C-iii) domains at the late second (A), early third (B), mid third (C) and late third (D) larval stages. *wg* (red) is initially induced only within the *pnr* expression domain in the early third larval stage (B, indicated by an arrow in B-ii). *wg* expression separated from the *pnr* domain in the mid to late third larval stage (C, D, separated *wg* expression is indicated by an arrowhead in D). It is also worth noting that *pnr* induction seems to initiate from the posterior side of the thoracic disc and expands anteriorally in the later stages (A-D). *pnr* (E) and *wg* (F) expression domains in the adult stage. Two large bristles (aDC; anterior dorsocentral bristles and pDC; posterior dorsocentral bristles) are marked with arrows. *pnr* and *wg* are expressed in a nearly complementary pattern. All discs are shown with anterior left and dorsal up. Scale bars represent 50 μm (A-D) and 250 μm (E, F).

Pnr has two opposing roles in the regulation of *wg* expression

To investigate the Pnr function in the regulation of notal *wg* expression, I analysed *wg* expression in some allelic combinations of *pnr* alleles and in homozygous mutant clones of several *pnr* mutants. One of the major problems in analysing Pnr function is the complexity of *pnr* alleles. To avoid this problem, I first focused only on loss-of-function alleles, *pnr* ^{md237}, *pnr* ^{VX6} and a deficiency line of the *pnr* locus, *Df(3R)sbd45*. The *pnr* ^{md237} is a P-element insertion line presumably resulting in reduced *pnr* gene expression (Calleja et al., 1996). The *pnr* ^{VX6} that encodes only nine amino acids of the N terminal side of wild-type Pnr protein (designated Pnr+) is an amorphic allele (Fig. 3-9) (Ramain et al., 1993). Hence, the amount of Pnr+ protein seems to be greatly reduced in both *pnr* ^{md237}/ *Df(3R)sbd45* and *pnr* ^{md237}/ *pnr* ^{VX6} discs. In *pnr* ^{md237} homozygous discs, notal *wg* stripe seems to shift dorsally (Fig. 3-8). In both *pnr* ^{md237}/ *Df(3R)sbd45* and *pnr* ^{md237}/ *pnr* ^{VX6} discs, *wg* is ectopically expressed on the dorsal side of the thoracic disc (Fig. 3-8B, C). These results suggest that Pnr represses *wg* expression on the dorsal side of the thoracic disc. I also monitored the *pnr* expression domain by GFP expression of *pnr* ^{md237} *UAS-gfp* in the same discs. Interestingly, the *wg* expression domain in both *pnr* ^{md237}/ *Df(3R)sbd45* and *pnr* ^{md237}/ *pnr* ^{VX6} discs is restricted to the more dorsal side than that in wild-type, and is completely abolished around the ventral edge of the *pnr* expression domain where *wg* is expressed in wild-type (Fig. 3-8B, C compare with Fig. 3-8D). This suggests that Pnr induces *wg* expression around the ventral edge of the *pnr* expression domain in the wild-type thoracic disc. In both *pnr* ^{VX6}/*pnr* ^{md237} and *Df(3R)sbd45/pnr* ^{md237} discs, there still might be some contribution of the Pnr+ protein from the *pnr* ^{md237} chromosome. However, mosaic analysis of *pnr* ^{VX6} clones revealed that these phenotypes are almost identical to the *pnr* complete loss-of-function phenotypes. In the *pnr* ^{VX6} clone located on the dorsal side of the wild-type *wg* expression domain, *wg* is cell autonomously induced (Fig. 3-8D). In contrast, *wg* expression is repressed

in the *pnr* ^{VX6} clones located in the wild-type *wg* expression domain (Fig. 3-8D). These results suggest that Pnr has two opposing roles in notal *wg* expression. One is to repress *wg* expression on the dorsal side of the thoracic disc (repressor function), and the other is to induce *wg* expression around the ventral edge of the *pnr* expression domain (activator function). In addition, in the *pnr* ^{md237} homozygous disc, the *wg* expression domain appears simply to be shifted dorsally (Fig. 3-8A). However, this dorsal shift could be explained by the lower level of Pnr activator function and partial loss-of-repressor-function in the regulation of notal *wg* expression.

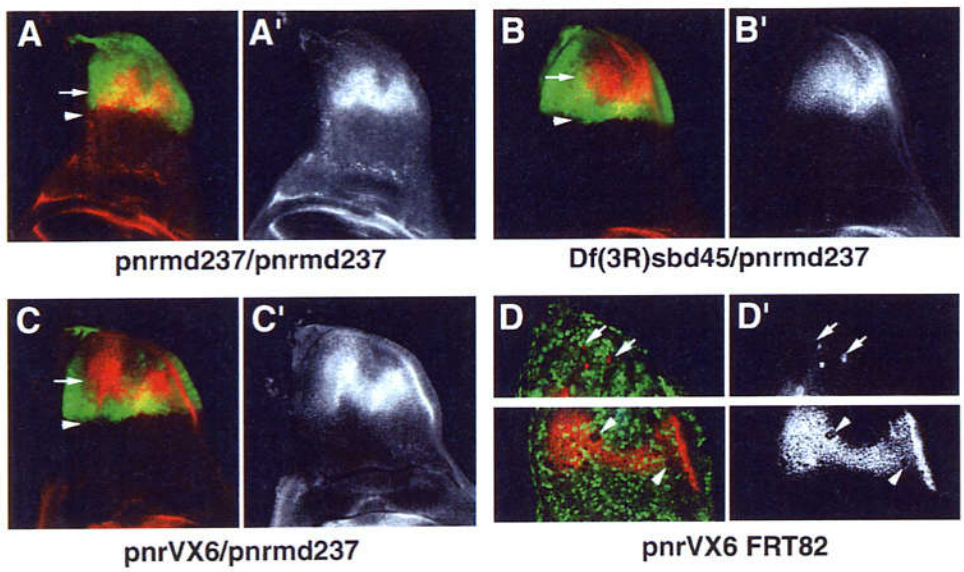

Fig. 3-8. Alteration of notal *wg* expression in *pnr* mutants. *wg* and *pnr* domains are monitored by anti-Wg antibody and GFP expression driven by *pnr* ^{md237}, respectively. Both merged image (A-D) and Wg single staining image (A'-D') are shown. *wg* (red in A-C, and A'-C') and *pnr* (green) expression in *pnr* ^{md237} homozygous (A), *Df(3R)sbd45/ pnr* ^{md237} (B) and *pnr* ^{VX6}*/pnr* ^{md237} (C) discs. *wg* is ectopically expressed in the dorsal side of the thoracic disc (A-C, indicated by arrows), and is lost around the ventral edge of the *pnr* expression domain (A-C, indicated by arrowheads. Compare with Fig. 3-7D). *wg* expression in *pnr* ^{VX6} clones (D). Endogenous *wg* (red) is reduced in *pnr* ^{VX6} clones located in the *wg* expression domain (absence of green, indicated by an arrowhead), however, *wg* is ectopically expressed in *pnr* ^{VX6} clones located in the dorsal side of the thoracic disc (indicated by an arrow). Note that not all clones located in the dorsal side of the thoracic disc express *wg*, suggesting the existence of local specific effects. All discs are shown with anterior left and dorsal up. Mutant clones were induced at 50 hours BPF (D). Scale bars represent 50 μm.

Allele specific effects of two antimorphic alleles, *pnr* ^{D} and *pnr* $^{VX1/4}$, in the regulation of notal *wg* expression.

I also analysed *wg* expression in other classes of *pnr* alleles that were previously categorised as class A (including *pnr* ^{D1}, *pnr* ^{D2}, *pnr* ^{D3}, *and pnr* ^{D4}; designated *pnrD*) and class B (including *pnr* ^{VX1} and *pnr* ^{VX4}; designated *pnr* $^{VX1/4}$) mutants (Haenlin et al., 1997; Ramain et al., 1993).

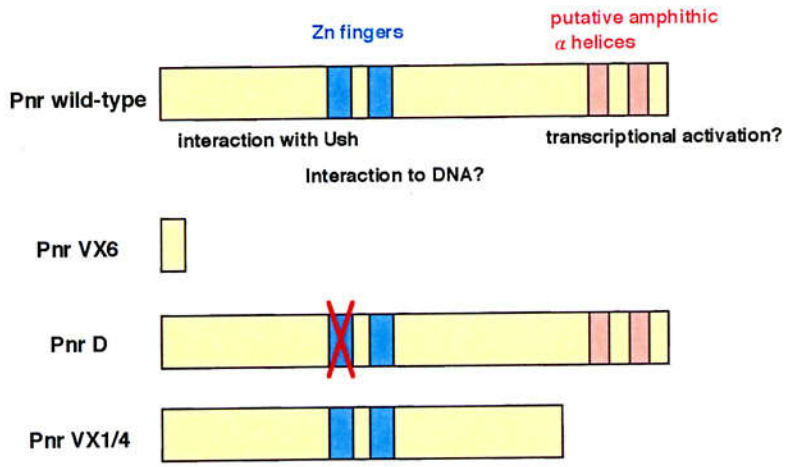

Fig. 3-9. Structure of wild-type and mutant pnr proteins

pnr ^{VX1} encodes truncated type of Pnr proteins that lack the C terminal activation domain, and the *Pnr* ^{VX1} protein seems to have a dominant negative effect on Pnr+ in bristle formation (Fig. 3-9) (Ramain et al., 1993). In *pnr* ^{VX1}/*pnr* ^{md237} discs, *wg* expression is reduced or sometimes completely abolished (Fig. 3-10A). In *pnr* ^{VX1} homozygous clones located in the wild-type *wg* expression domain, *wg* is not expressed in the same way as in *pnr* ^{VX6} clones (Fig. 3-10B). However, in contrast to the *pnr* ^{VX6} clone, *wg* is never ectopically expressed in the *pnr* ^{VX1} located on the dorsal side of the thoracic disc (Fig. 3-10B). Reduction of notal *wg* expression in *pnr* ^{md237}/*pnr* ^{VX1} discs or *pnr* ^{VX1} clones indicates that Pnr ^{VX1} protein has lost the activator function in the regulation of *wg* expression. However, Pnr ^{VX1} protein still

seems to possess the repressor function, because *wg* expression is still repressed on the dorsal side of the thoracic disc.

The *pnr* ^{D1} is associated with a point mutation resulting in protein with single amino acid change in the N terminal zinc finger, that interacts with Ush (Fig. 3-9) (Haenlin et al., 1997). A previous report showed that Ush can not interact with Pnr ^{D1} in vitro and in yeast, and fail to repress the transactivation of the α-globin promoter by Pnr ^{D1} in CEF cells (Haenlin et al., 1997). The *pnr* / ^{D1}*pnr* ^{md237} discs or *pnr* ^{D1} homozygous clones display an opposing phenotype to that of *pnr*^{VX1} / *pnr* ^{md237} or *pnr* ^{VX1} clones. The *pnr* ^{D1} mutation has no effect on *wg* expression in the normal *wg* expression domain and ectopically induces *wg* on the dorsal side of the thoracic disc (Fig. 3-10C, D). Thus, Pnr ^{D1} protein seems to lack the repressor function but still maintains the activator function in the regulation of notal *wg* expression.

Taking these results and Galcia-Galcia's previous report together, both *pnr*^{VX1} and *pnr* ^{D1} lose one of two Pnr+ functions. So, *pnr*^{VX1} is a loss-of-activator-function allele, while *pnr* ^{D1} is a loss-of-repressor-function allele.

Pnr-Ush complex represses *wg* expression on the dorsal side of the thoracic disc.

The lack of the repressor function of Pnr ^{D1} protein suggests that the interaction between Pnr and Ush is necessary for repressing *wg* expression on the dorsal side of the thoracic disc. *ush* mRNA is expressed in the dorsal-most region of the thoracic disc, and does not overlap with the *wg* domain (Fig. 3-11A). In contrast, *pnr* mRNA is expressed on the dorsal side of the thoracic disc, slightly overlapping the *wg* domain (Fig. 3-11B). Hence, *wg* expression seems to be repressed by the Pnr-Ush complex in the region where both *pnr* and *ush* are expressed, and to be induced by Pnr in the region where only *pnr* is expressed. This hypothesis is confirmed by the ectopic *ush* expression. When exogenous *ush* is induced in the *pnr*

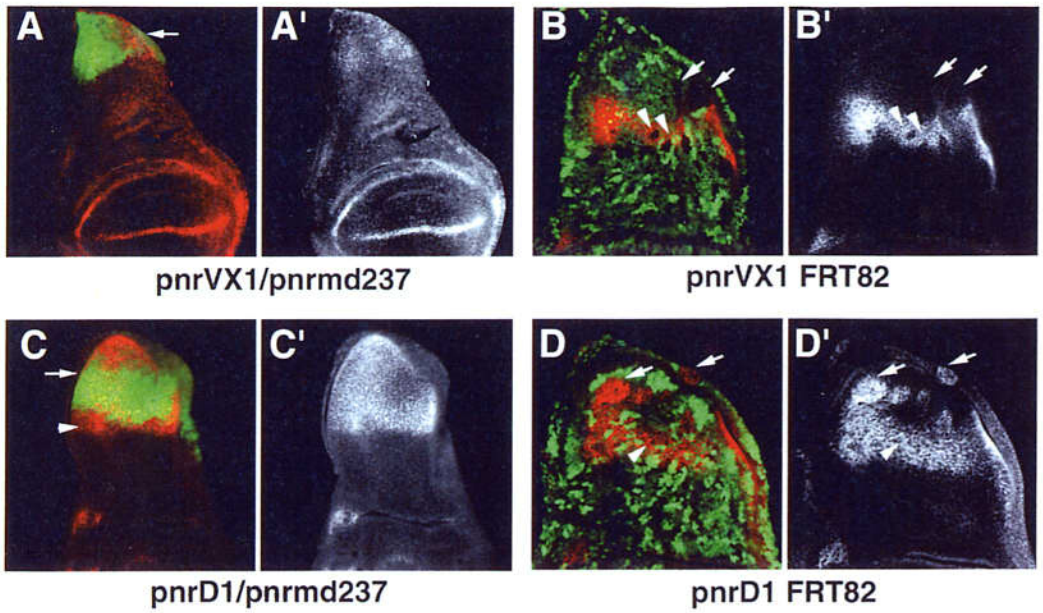

Fig. 3-10. *wg* expression in antimorphic allele of *pnr*. *wg* and *pnr* expression domains are monitored by anti-Wg antibody and GFP expression driven by *pnr* ^{md237}, respectively. Both merged image (A-D) and Wg single staining image (A'-D') are shown. *wg* (red) and *pnr* (green) expression in *pnr* ^{VX1}/ *pnr* ^{md237} disc (A). Almost all notal *wg* expression is abolished. Weak *wg* expression is observed in the dorsal side of the thoracic disc (E, indicated by an arrow). (B) *wg* expression is lost in *pnr* ^{VX1} clones located in the endogenous *wg* domain (absence of green, indicated by an arrowhead in B). *wg* is not ectopically expressed in *pnr* ^{VX1} clones located in the dorsal side of the thoracic disc (indicated by an arrow in B). (C) *wg* (red) and *pnr* (green) expression in *pnr* ^{D1}/ *pnr* ^{md237} disc. *wg* expression domain expands dorsally (indicated by an arrow). Endogenous *wg* (red) is still expressed in *pnr* ^{D1} clones located in the *wg* expression domain (absence of green, indicated by an arrowhead in D), and is ectopically expressed in *pnr* ^{D1} clones located on the dorsal side of the thoracic disc (indicated by an arrow in D). All discs are shown with anterior left and dorsal up. Mutant clones were induced at 50 hours BPF (B, D). Scale bars represent 50 μm.

expressing region, notal *wg* expression is completely repressed (Fig. 3-11C). This result and previous reports indicate that Ush is also involved in the regulation of notal *wg* expression, and the binding of Ush to Pnr could switch the activator function of Pnr to a repressor function. In addition, the ability of Pnr ^{VX1} protein to repress *wg* expression indicates that the C terminal activator domain is not necessary for the repressor function of the Pnr-Ush complex.

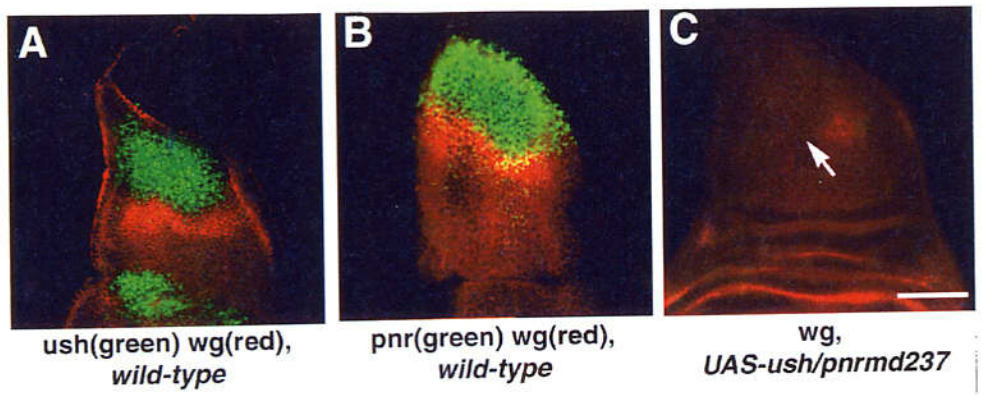

Fig. 3-11. Spatial relationship between *wg* and *ush/ pnr* expression domains in the wild-type thoracic disc (A, B). *ush* is expressed in the dorsal-most region (green in A). *wg* expression domain (red) does not overlap with the *ush* expression domain. The *pnr* expression domain (green in B) slightly overlaps the *wg* expression domain (red in B). Overexpression of Ush by a pnr-GAL4 driver (*pnr* ^{md237}) represses *wg* expression (C). *wg* expression is monitored by anti-Wg antibody. *pnr* and *ush* mRNA are detected by DIG labeled antisense RNA probes. All discs are shown with anterior left and dorsal up. Scale bars represent 50 μm.

Wg signaling also participates in the establishment of notal *wg* expression

Because some of the *wg* expressing region expand ventrally from the *pnr* expression domain at the late third larval stage, other factor(s) positively regulating notal *wg* expression should exist. I found that ventral expansion of the *wg* expression from the *pnr* expression domain does not occur in one of the Wg signaling mutants, disheveled (dsh). In *dsh* ^{va153} (a strong hypomorphic allele (Theisen et al., 1994)) hemizygous discs, *wg* is expressed only in a narrow stripe located in or just adjacent to the ventral side in the *pnr* expression region (Fig. 3-12A). This phenotype is also observed in a *wg* temperature sensitive mutant (in *wg* $^{l-12}$) (van den Heuvel et al., 1993). The notal *wg* expression domain in *wg* $^{l-12}$ homozygous flies shifted to non-permissive temperature at 50 hours before puparium formation (BPF) became narrower than that

of the wild-type (Fig. 3-12B, C). These results indicate that Wg signaling participates in the ventral expnasion of the *wg* expression from the *pnr* expression domain at the late third larval stage.

There are at least two possible explanations for the ventral expansion of *wg* expression from the *pnr* expression domain. The first is that *wg* expression expands ventrally by a self-inducing mechanism, and the second is that the *pnr* expressing region retreats dorsally in later stage and Wg signaling maintains *wg* expression itself in *pnr* non-expressing cells. To examine these possibilities, I used the lineage tagging method (Weigmann and Cohen, 1999) to monitor whether the cells at the ventral side of the *wg* expression domain, where *pnr* is not expressed in late third larval stage, have expressed *pnr* at an early stage. The region of cells that have expressed Gal4 derived from *pnr* ^{md237} is restricted more dorsally than the region of cells that express Gal4 in late third larval stage (Fig. 3-12D). This result indicates that the *pnr* expression domain does not retreat but appears to expand toward the ventral region during wing disc development, suggesting that the second explanation is not appropriate. Thus, notal *wg* expression is initiated on the ventral side in the *pnr* expression domain by a Wg signaling-independent mechanism and expands ventrally from the *pnr* expression domain by a Wg signaling-dependent mechanism.

I also monitored *wg* expression in *d-axin* mutant clones. *d-axin* encodes a negative regulator of Wg signaling and Wg signaling is constitutively activated in *d-axin* ^{S044230} homozygous cells (Hamada et al., 1999). In the *d-axin* ^{S044230} clones located in the ventral side to the normal *wg* stripe, *wg* is ectopically expressed (Fig. 3-12E). However, this ectopic expression of *wg* in *d-axin* mutant clones was suppressed by *pnr* ^{VX6}. In *d-axin* ^{S044230} *pnr* ^{VX6} double mutant clones, *wg* is never expressed (Fig. 3-12F). These results are contradicted with the result of lineage trace experiment. Because, the results from *d-axin* ^{S044230} *pnr* ^{VX6} double mutant clones favour the hypothesis that Pnr is necessary for *wg* expression on the ventral side to the *pnr* expression domain where *pnr* is not expressed in the late third larval stage. However, lineage trace experiments indicate that *pnr* does not appear to be expressed in this

region even at earlier stages. I suspect that *d-axin* ^{S044230} clones extopically express *pnr*, which in turn induce *wg*. These *d-axin* mutant clone analyses seem not to represent simple gain-of-function phenotypes for Wg signaling. However, the results of *wg* $^{l-12}$ and *dsh* ^{va153} strongly suggest that the activity of Wg signaling is required for the ventral expansion of the notal wg expression.

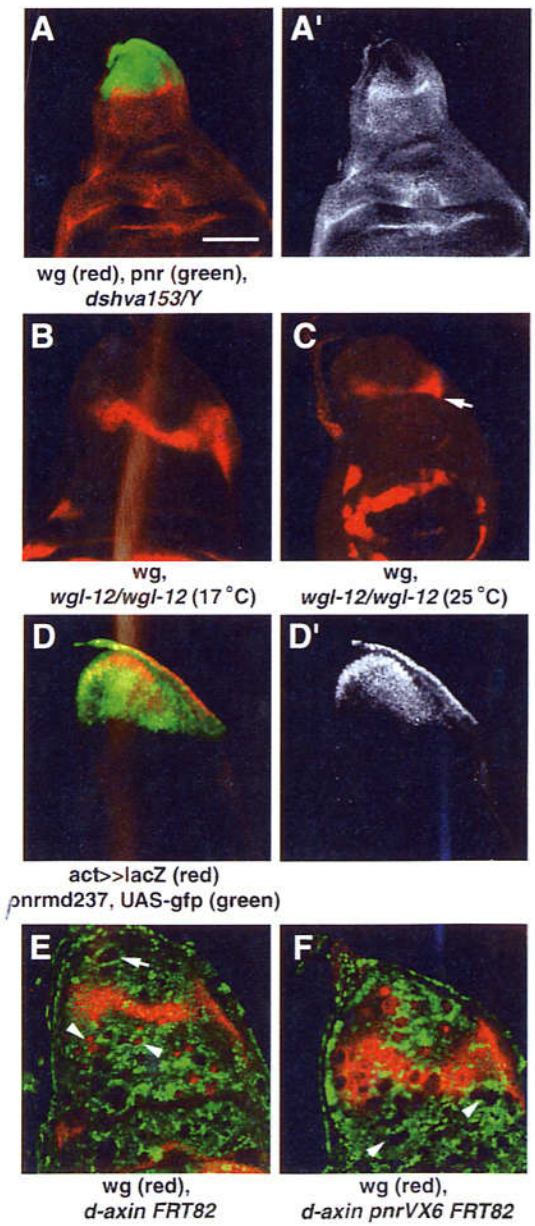

Fig. 3-12. Alteration of notal *wg* expression in Wg signaling. The *wg* (red) and *pnr* (green) expression domain in the *dsh*^{va153}/*Y* disc (A, Wg single staining image is also shown in A'). A narrow stripe of the *wg* expression domain is observed in or just adjacent to the ventral side of the *pnr* expression domain. *wg* expression in *wg* $^{l-12}$ homozygous disc (C, D). *wg* expression is not altered in in *wg* $^{l-12}$ homozygous disc at 17°C (B), however, it becomes narrower in the *wg* $^{l-12}$ homozygous disc shifted to non-permissive condition (25°C) at 50 hour BPF (C). (D) Lineage tracing in *act5c>stop>lacZ/+; UAS-flp/ pnr* ^{md237} *UAS-gfp* discs shows that the region of cells that have expressed Gal4 derived from *pnr* ^{md237} (red in D and D') is restricted more dorsally than the region of cells that express Gal4 in late third larval stage (Green in D). (E) *wg* (red) is ectopically expressed in the *d-axin* ^{S044230} clones (absence of green) located on the ventral side to the normal *wg* stripe (indicated by arrowheads in E) but not on the dorsal side (indicated by arrows in E). (F) *wg* (red) is not ectopically expressed in *pnr* ^{VX6} *axin* ^{S044230} clones located on the ventral side to the normal *wg* stripe (indicated by arrowheads in F). All discs are shown with anterior left and dorsal up. Mutant clones were induced at 50 hours BPF (E, F). The *wg* and *pnr* expression domains are monitored by anti-Wg antibody and GFP expression driven by *pnr* ^{md237}, respectively. Scale bars represent 50 μm.

Discussion

Dpp morphogen gradient is necessary for both A/P and D/V positioning of DC proneural cluster

The positioning of DC proneural cluster seems to be regulated by several independent mechanisms. In chapter 2, I showed that Dpp morphogen gradient is necessary for the A/P positioning of DC proneural cluster. It has previously been reported that two transcriptional regulaters, Pnr and Ush, regulate the positioning of the dorsocentral proneural cluster along the D/V axis, by activating the dorsocentral enhancer element of *ASC* genes, and also regulate the *wg* expression in the thoracic disc.

In this chapter, I showed that Dpp morphogen gradient regulates the *pnr* and *ush* in the thoracic disc. The mechanisms for regulating the *wg* expression by Pnr and Ush were also shown. A hierarchy of these genes during notum development is summarized in Fig.3-13. *dpp* is initially induced at the dorsal region of the A/P compartment boundary by Hh signaling (Basler and Struhl, 1994; Tabata and Kornberg, 1994) (Fig. 3-13A). Dpp signaling induces two target genes, *pnr* and *ush*. Analyses of *pnr* and *ush* expression in *put-ts* and *tkv* ^{a12} cells suggest that different thresholds are set for the induction of these genes, low levels for *pnr* and high levels for *ush* (Fig. 3-13B). *wg* is induced by Pnr where *ush* is not expressed (Fig. 3-13B). Simultaneously, the Pnr-Ush complex represses *wg* expression at the dorsal-most region of the thoracic disc (Fig. 3-13B). In the later stage, the *wg* expression domain expands ventrally from the *pnr* expressing region and *wg* starts to be expressed in the non-*pnr*-expressing cells. During this process, Wg signaling plays a crucial role and this separation did not occur in the Wg signaling mutants (Fig. 3-13A,C).

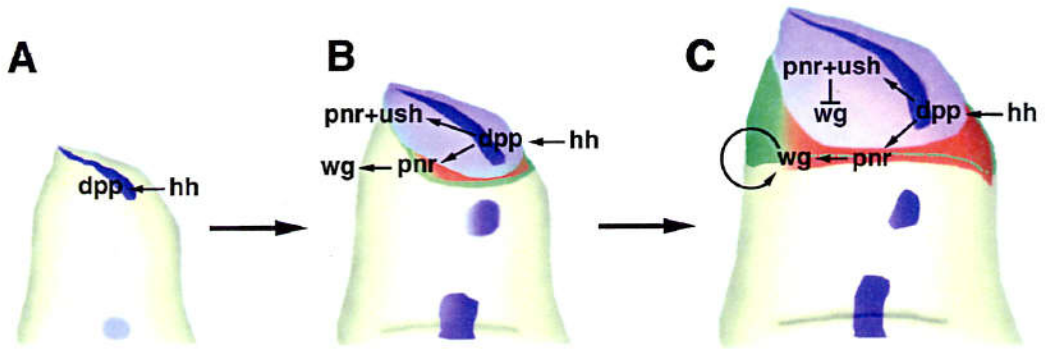

Fig. 3-13. Schematic models of the establishment of the *wg* stripe in the thoracic disc. *dpp*, *wg*, *pnr*, and *ush* expression domains are indicated in blue, red, green, and purple respectively. See text for details.

These findings proposed that Dpp morphogen gradient affects the prepattern on the notum through the induction of *pnr* and *ush*. Moreover, Dpp seems to contribute not only to the A/P positioning but also to the D/V positioning of DC proneural cluster through the induction of *pnr* and *ush* in the thoracic disc, because Pnr/ Ush presumably provides the positional information along the D/V axis. However, it does not seem that induction of *pnr* and *ush* is an only role of Dpp in the establishment of bristle prepattern in the thoracic disc. Because, over activation of Dpp signaling causes the expansion of DC proneural cluster towered the anterior, in contrast overexpression of *pnr* causes the expansion of DC proneural cluster only along the D/V axis. Thus, Dpp signaling seems to induce proneural gene expression independent of Pnr and Ush.

In contrast, Dpp seems to restrict the *wg* expression through the induction of *ush*, because repression of *wg* expression by ectopic Dpp signaling does not occur in pnr^{D1} mutant background (data not shown). pnr^{D1} encodes a protein that lacks the ability to interact with Ush, and Pnr^{D1} protein behaves as an activator in the regulation of notal *wg* expression even in the presence of Ush (Fig. 3-10C, D). Hence, pnr^{D1} seems to behave as the *ush* loss-of-function mutants. These data indicate that

the restriction of *wg* expression by Dpp signaling in the thoracic disc requires Ush. Furthermore, I reported only repressive effects of Dpp signaling on the *wg* expression in chapter 2, however, Dpp seems to also induce *wg* expression through the induction of *pnr* in the thoracic disc.

Both establishment of *pnr/ ush* expression domain and induction of proneural gene at dorsocentral area by Dpp protein are critical events for the precise positioning of DC proneural cluster.

Ush modulates the Pnr activity

As presented here and by Garcia-Garcia et al., the Pnr-Ush complex acts as a repressor for the induction of *wg* and DC enhancer-lacZ expression (DC enhancer is an enhancer of the *achaete/scute* proneural gene complex that activates gene expression in the dorsocentral area) (Garcia-Garcia et al., 1999). It is interesting that Ush does not simply inhibit Pnr function but switches the activator function of Pnr to a repressor function. Based on the result that the extra doses of Pnr can not revert the repressor activity of Pnr-Ush, Garcia-Garcia et al. proposed that the activator function of Pnr and the repressor function of the Pnr-Ush complex do not simply compete with each other on the notal *wg* enhancer element (Garcia-Garcia et al., 1999). However, it also seems to be possible that Pnr and the Pnr-Ush complex compete for the binding site at the notal *wg* enhancer, but the ability of Pnr-Ush complex to bind this site may be greater than that of Pnr. It is also worth noting that FOG-1, a mammalian homologue of Ush, represses the transactivation of α-globin and EKLF promoter by GATA-1, but enhances the transactivation of NF-E2 p45 promoter by GATA-1 in a culture cell system (Fox et al., 1999). It has been previously reported that dorsocentral (DC) bristles are ectopically formed but postvertical bristles on the head are missing in a loss-of-function allelic combination for *ush* or in pnr^{D1} heterozygous flies (Cubadda et al., 1997; Ramain et al., 1993). These observations suggest that the Pnr-Ush complex acts as a repressor for the DC enhancer, but acts as an activator for the

enhancer of postvertical bristles. Only a *cis*-regulatory element of the DC enhancer has been analyzed at the nucleotide level. Additional studies of the molecular analyses of the *cis*-regulatory elements of both *wg* and DC or other enhancers of the *achaete/scute* complex seem to be necessary in order to reveal the functions of Pnr and Ush.

Establishment of two-dimensional coordinates in the notum

Generally, at least two different coordinate axes are necessary for positional specification in a two-dimensional field. Morphogen gradients of Dpp and Wg provide this axial information during *Drosophila* imaginal disc development (Brook and Cohen, 1996; Jiang and Struhl, 1996; Lecuit et al., 1996; Lecuit and Cohen, 1997; Nellen et al., 1996; Neumann and Cohen, 1997; Penton and Hoffmann, 1996; Zecca et al., 1996). In both wing and leg discs, *dpp* is induced at the A/P compartment boundary by Hh signaling (Basler and Struhl, 1994; Tabata and Kornberg, 1994). In the leg disc, *wg* is also induced by Hh signaling. Mutual repression between Dpp and Wg signaling separates each expression territory, localizing *dpp* in the dorsal and *wg* in the ventral regions abutting the A/P border (a compartment-independent manner) (Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffmann, 1996). In contrast, *wg* is induced by Notch signaling only at the D/V compartment boundary in the wing pouch (a compartment-dependent manner) (Couso et al., 1995; de Celis et al., 1996; Diaz-Benjumea and Cohen, 1995; Rulifson and Blair, 1995). Then, secreted Dpp and Wg proteins provide positional information along the A/P and D/V axes, respectively, to establish Cartesian like coordinates in the pouch field. Relative positions of *dpp* and *wg* expression domains in the thoracic disc is more similar to that in the wing pouch (in both cases, their expression domains are orthogonal). However, a D/V compartment boundary does not exist in the notum (Garcia-Bellido, 1975; Garcia-Bellido et al., 1973). The results described here reveal that another compartment-independent mechanism acts to pattern the thoracic disc. Namely, the

D/V axis, provided by Pnr, Ush, and Wg, is initially established by the Dpp gradient, which mainly contributes the positional information along the A/P axis. One of the key issues of this patterning model is that Dpp signaling seems to act preferentially along the A/P axis of the thoracic disc. This is because two target genes, *pnr* and *ush,* are induced farther from the Dpp source along the A/P axis than along the D/V axis. One possible explanation for this phenomenon is that the diffusion of Dpp protein may be positively regulated along the A/P axis. However, such asymmetric induction is not observed on the dad induction (unpublished data), dad is one of the Dpp signaling targets in the wing disc (Tsuneizumi et al., 1997). This suggests that diffusion of Dpp protein is not directionally regulated in the notum region. An alternative explanation would be that an effective range of Dpp morphogen gradient is established in a relatively short range. Cells that respond to Dpp would proliferate or migrate preferentially along the A/P axis. *pnr* mRNA is detected mainly in posteriodorsal region of the thoracic disc (Fig. 3-4A). While, GFP expression of *UAS-gfp pnr* ^{md237} is seen along the entire dorsal side of the thoracic disc (Fig. 3-7D). This difference between the staining pattern of *pnr* mRNA and GFP expression of *UAS-gfp pnr* ^{md237} in the late third larval stage seems to be caused by a long half-life of *gal4* and/or *gfp* products, suggesting that cells that once have expressed *pnr* mRNA proliferate preferentially along the A/P axis. However, it seems to be difficult to explain the determination of *pnr* and *ush* expression domains only by the Dpp morphogen gradient. The existence of Tkv* insensitive cells for inducing *pnr* and *ush* (Fig. 3-5) indicates that some regional subdivision may occur independently of Dpp signaling. Discontinuous expression of *dpp* in the A/P border of the notum (Fig. 3-6) also suggests the existence of a Dpp-independent subdivision. D/V subdivision of the thoracic disc seems to be achieved by several parallel mechanisms, including Dpp signaling.

Similarity between formations of dorsal structure in embryogenesis and in metamorphosis

Because *Drosophila* is an holometabolous insect, it should destroy larval tissues and replace them with a different population of cells to form the adult structure during the pupal stage. Thus, formation of epidermal structure should occur reiteratively during embryogenesis and metamorphosis. Patterning of larval epidermal structure takes place during embryogenesis, however, patterning of adult structure is mainly performed in larval stage imaginal discs. In this work, I demonstrate that the Dpp morphogen gradient regulates *pnr* and *ush* expression to pattern the thoracic disc, which forms the dorsal structure of the adult, in the wing imaginal disc. It has previously been reported that *pnr* and *ush* are necessary for the formation of amnioserosa (Frank and Rushlow, 1996; Winick et al., 1993), the dorsal structure of the embryo, and that both *pnr* and *ush* expressions are also positively regulated by Dpp in a concentration-dependent manner during embryogenesis (Jazwinska et al., 1999). Furthermore, dorsal closure during embryogenesis and thorax closure in metamorphosis is also analogous, because both processes are regulated by the same signaling cascade, JNK signaling (Agnes et al., 1999; Zeitlinger and Bohmann, 1999). These similarities between embryogenesis and metamorphosis presumably reflect the evolutionary history of the development in holometabolous insect.

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CHAPTER 4.

CONCLUDING REMARKS

In 1971, Wolpert proposed in his review article that the pattern formation based on prepattern is the antithesis of that based on morphogen gradient and positional information.

In this study, I showed that Dpp morphogen gradient contributes the pattern formation by prepattern on the *Drosophila* developing notum in many ways. In chapter 2, I showed that Dpp signaling is necessary for inducing the dorsocentral proneural cluster in cooperation with Wg signaling, and restriction of *wg* expression domain in the thoracic disc. Furthermore, these effects seem to depend on the concentration of Dpp. These findings suggest that Dpp morphogen gradient contributes the prepattern of the macrochaetes on the notum, probably through the modulation of prepattern genes expression and/or activity. In chapter 3, a regulatory network of two prepattern genes, *pnr* and *ush*, and two secreted factors, Dpp and Wg, in the pattern formation of the notum, especially focusing on the regulation of the *wg* expression, was analyzed. Analysis of *wg* expression in wild-type and several allelic combinations of *pnr* mutants revealed that Pnr has two opposing roles, induction and repression, in the regulation of the *wg* expression. I also revealed that notal *wg* expression is affected by Wg signaling itself. Furthermore, I found that both *pnr* and *ush* expression is positively regulated by Dpp signaling in the presumptive notum region of the wing imaginal disc. A hierarchy of prepattern genes and morhogen during notum development is summarized in Fig. 5.

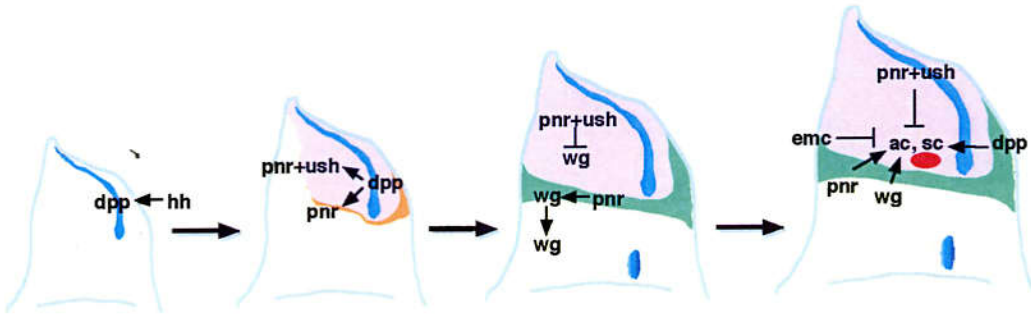

Fig. 5. Relationship between prepattern and morphogen gradient in the notum patterning.

These findings suggest that the pattern formation based on morphogen is not a antithesis of prepattern, but rather these two modes of pattern formation are parts of same developmental process in the patterning of the *Drosophila* developing notum.

In spite of these novel findings in this study, several problems remain to be addressed. For instance, Detail analysis of DC enhancer element of ASC is necessary for elucidating how Dpp signaling induces ac/ sc expression on the dorsocentral area (direct or indirect?). Furthmore, the relationship between Dpp signaling and other prepattern genes such as *iro*, *sal*, should also be addressed to the elucidation of contribution of Dpp morphogen gradient to the notum prepatterning. These studies will reveal the more precise relationship between prepattern and morphogen on the notum.

GLOSSARY

amorph a mutation that functionally inactivates a gene by producing either no product or a nonfunctional product (synonym: null allele)

antimorph a mutation that shows anomalous complementation such that the individuals are more severely affected when heterozygous with another mutation than when homozygous

balancer chromosome containing multiple inversions and markers that facilitate crossing schemes by their ease of detection and also by their suppression of recombination between homologs

deficiency rearrangement in which a piece of a chromosome is excised and the remaining large pieces reattached (synonym: deletion)

hemizygous chromosome or chromosomal region present in only one dose

hypomorph mutation in which the gene product is produced at lower levels than normal or is less active than normal

mosaic individual whose cells are not all of the same genotype

Reference

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