Available online at www.sciencedirect.com

SCIENCE DIRECT

Developmental Biology 289 (2006) 77 – 90

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

Regulation of expression of Vg and establishment of the dorsoventral compartment boundary in the wing imaginal disc by Suppressor of Hairless

Stefan Koelzer, Thomas Klein *

Institute for Genetics, University of Cologne, Zülpicherstr. 47, 50674 Cologne, Germany

Received for publication 24 May 2005, revised 26 September 2005, accepted 4 October 2005

This paper is dedicated to the late Prof. Jose Campos-Ortega.

Abstract

The transcription factor Suppressor of Hairless (Su(H)) belongs to the CSL transcription factor family, which are the main transcriptional effectors of the *Notch*-signaling pathway. Su(H) is the only family member in the *Drosophila* genome and should therefore be the main transcriptional effector of the *Notch* pathway in this species. Despite this fact, in many developmental situations, the phenotype caused by loss of function of *Su(H)* is too weak for a factor that is supposed to mediate most or all aspects of *Notch* signaling. One example is the *Su(H)* mutant phenotype during the development of the wing, which is weaker in comparison to other genes required for *Notch* signaling. Another example is the complete absence of a phenotype upon loss of *Su(H)* function during the formation of the dorsoventral (D/V) compartment boundary, although the *Notch* pathway is required for this process. Recent work has shown that Su(H)/CBF1 has a second function as a transcriptional repressor, in the absence of the activity of the *Notch* pathway. As a repressor, Su(H) acts in a complex together with Hairless (H), which acts as a bridge to recruit the co-repressors Groucho and CtBP, and acts in a *Notch*-independent manner to prevent the transcription of target genes. This raises the possibility that a de-repression of target genes can occur in the case of loss if function of *Su(H)*. Here, we show that the weak phenotype of *Su(H)* mutants during wing development and the absence of a phenotype during formation of the D/V compartment boundary are caused by the concomitant loss of the *Notch*-independent repressor function. This loss of the repressor function of *Su(H)* results in a de-repression of expression of target genes to a different degree in each process. Loss of *Su(H)* function during wing development results in a transient de-repression of expression of the selector gene *vestigial* (*vg*). We show that this residual expression of *vg* is responsible for the weaker mutant phenotype of *Su(H)* in the wing. During the formation of the D/V compartment boundary, de-repression of target genes seems to be sufficiently strong, to compensate the loss of *Su(H)* activity. Thus, de-repression of its target genes obscures the involvement of Su(H) in this process. Furthermore, we provide evidence that Dx does not signal in a *Su(H)*-independent manner as has been suggested previously.

Keywords: CSL transcription factors; CSL-independent *Notch* signaling; Compartment formation; *Drosophila* wing development; *Vestigial*; Deltex

Introduction

The *Notch*-signaling pathway plays important roles in specifying cell fates in many developmental and pathological processes in multi-cellular animals and humans (reviewed in Artavanis-Tsakonas et al., 1999). *Notch* proteins are type 1 trans-membrane receptors that are activated by ligands of the DSL protein family. In the genome of *Drosophila*, two DSL ligands are present, Serrate (Ser) and Delta (Dl). The binding of these ligands to Notch elicits a sequence of two proteolytic cleavages that release the intracellular domain of Notch (Nintra) into the cytoplasm, from where it travels to the nucleus (reviewed in Kopan, 2002). The two proteolytic cleavages are performed by membrane proteases of the ADAM and Presinillin families. The *Drosophila* ADAM family member Kuzbanian (Kuz) first cleaves Notch in the extra-cellular domain, close to the membrane (Klein, 2002; Lieber et al., 2002). This first cleavage is named S2, and it is the ligand-dependent step. It creates an intermediate that is called NEXT, which is immediately cleaved in the transmembrane domain by the γ-secretase complex that includes Presinillin (Psn) as well as Nicastrin (Nic) to release Nintra

* Corresponding author.
E-mail address: th.klein@uni-koeln.de (T. Klein).

0012-1606/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2005.10.008

(S3-cleavage). In the nucleus, Nintra acts together with the sequence specific DNA-binding protein Suppressor of Hairless (Su(H)) to activate the transcription of target genes. Besides these core elements, many additional proteins are involved in regulation of and signal transduction through the *Notch* pathway. One example is Deltex (Dx), which contains a Ring finger motif typical for E3 Ubiqutin ligases and binds to the intracellular domain of Notch (reviewed in Le Borgne et al., 2005). It is involved in signal transduction of the *Notch* signal in some developmental processes such as wing development, possibly in a *Su(H)*-independent pathway (Hori et al., 2004).

The *Notch*-signaling pathway plays a pivotal role during the establishment of the proximo-distal axis of the wing and the establishment of the dorsoventral compartment boundary (D/V boundary) (reviewed in Dahmann and Basler, 1999; Klein, 2001). It mediates the interactions between dorsal and ventral cells at the D/V boundary that lead to the expression of genes that are essential for establishment and patterning of the proximo-distal axis. The dorsal cell fate is defined by the activity of the Apterous (Ap) selector protein, which in addition controls the activity of the Notch pathway through the activation of expression of Ser and the Glycosyltransferase Fringe (Fng). Fng modifies the Notch receptor so that Ser can only signal to ventral and Dl to dorsal cells (Haines and Irvine, 2003). As a consequence, the activity of the pathway is restricted to a small stripe of cells along the D/V boundary. There, it induces transcription of genes essential for wing development and patterning of the proximo-distal axis (P/D axis), chief among them *vestigial* (*vg*) and *wingless* (*wg*) (reviewed in Klein, 2001). *vg* encodes a nuclear protein that forms a dimeric transcription factor with the TEA-domain DNA binding protein Scalloped (Sd) (Halder et al., 1998). Previous studies have revealed that the expression of target genes is activated by Su(H). Activation of *vg* has been studied in some details (Kim et al., 1997a,b). Its transcription is initiated through the activation of the *vestigial* boundary enhancer (*vg*BE). This enhancer contains a single Su(H) DNA binding site that is essential for its activity. Nevertheless, the mutant phenotype of *Su(H)* described in the literature is significantly weaker than that of *vg* null mutants and that of other genes required for the signal transduction in the *Notch* pathway. This discrepancy could argue for the existence of another, *Su(H)*-independent signaling mechanism. The existence of such a pathway has been suggested several times, although the evidence remains weak (reviewed in Mumm and Kopan, 2000).

However, the interpretation of the *Su(H)* mutant phenotype during wing development is hampered by the fact that the strength of the alleles of *Su(H)* analyzed in previous studies is not clear. Hence, it is possible that the weaker phenotype is caused by a residual activity of *Su(H)* (Gho et al., 1996).

The interactions between *ap*-expressing and non-expressing cells, mediated by the *Notch* pathway, are also required for the formation of the dorsoventral (D/V) compartment boundary (reviewed in Klein, 2001). This boundary prevents the mixing between dorsal and ventral cell populations. How the segregation of these two cell populations is achieved is not understood, but an attractive explanation is that both populations have differential adhesive properties. Because of these adhesive differences, the cells from each lineage try to minimize their contact with cells from the other lineage (reviewed in Dahmann and Basler, 1999). Although previous work showed that *Notch* signaling is required for the formation of this boundary, it also provided evidence that Su(H) is not (Miccheli and Blair, 1999). This has led to the conclusion that either a *Su(H)*-independent mechanism of signal transduction mediates the activity of the pathway or a transcriptional response to the Notch signal is not required.

Work on the function of the vertebrate homologue of Su(H), CBF-1, in cell culture and studies of the interaction of CBF-1 with the viral protein EBNA2, especially in the laboratory of D. Hayward, suggested that CBF-1 has a second function as a repressor of transcription in the absence of *Notch* signaling (reviewed in Lai, 2002). More recently, it has been shown that, in *Drosophila*, Su(H) interacts with Hairless (H) and the co-repressor proteins Groucho and dCtBP to repress transcription (Barolo et al., 2002). This raises the possibility of de-repression of expression of target genes in *Su(H)* mutants that could result in a weaker phenotype than observed for mutants of other genes required for Notch signal transduction (Koelzer and Klein, 2003; Morel and Schweisguth, 2000).

Here, we have analyzed the phenotype caused by homozygosity of a null allele of *Su(H)* (Morel and Schweisguth, 2000), during wing development. We confirmed that during pattern formation, the mutant phenotype is weaker than expected and found that this is caused by the loss of the repressor function of Su(H). The loss of *Su(H)* function results in a transient de-repression of expression of the selector gene *vestigial* (*vg*), mediated by a weak and transient activation of one of its enhancers, the *vestigial* boundary enhancer (*vg*BE). Furthermore, we show that Su(H) is involved in the formation of the D/V compartment boundary, despite previous reports on the contrary. This involvement is obscured by the de-repression of expression of the target genes that allow the process to occur in the absence of Su(H) function. In summary, the data reveal that the weaker phenotype of *Su(H)* mutants during wing development can be explained by the dual function of Su(H) and does not provide evidence for the existence of a *Su(H)*-independent signal transduction mechanism. Furthermore, we show that Dx does not signal in a *Su(H)*-independent manner during wing development as suggested previously.

Materials and methods

Fly strains

The following alleles were used in this work: $Su(H)^{\Delta 47}$ P(B)FRT40A (Morel and Schweisguth, 2000), Psn^{C1} (Struhl and Greenwald, 1999), Psn^{I2} (Ye et al., 1999), nic^{A7} (Hu et al., 2002), kuz^{1405}, kuz^{1403} (Sotillos et al., 1997), $Df(1)N^{81K}$ FRT101 (Brennan et al., 1997); ap^{UG035} and *ap-lacZ* (ap^{rK568}) (Cohen et al., 1992), $Su(H)^{Sf8}$ and H^{E31} (Lecourtois and Schweisguth, 1995); vg^{83b27R} and the *vg*BE (Williams et al., 1994).

UAS stocks: UAS*vg* (Kim et al., 1996); UAS GFP (Yeh et al., 1995). UAS *H* (Go et al., 1998), UAS *Su(H)* (Klein et al., 2000), UAS*dx* (Matsuno et al., 2002).

Gal4 drivers: *sd*Gal4 (Klein and Martinez-Arias, 1998); *ptc*Gal4 (Speicher et al., 1994); *dpp*Gal4 (Wilder and Perrimon, 1995).

The MARCM System is described in (Lee and Luo, 2001). To generate clones with the MARCM system that express UAS *dx*, females with of the genotype *y w* hsFlp 1.22 *tub*Gal4 UAS GFP; FRT 40A *tub*Gal 80 were crossed with *w*; FRT 40A; UAS*dx*/Sm6a-TM6b males. To generate cell clones that are mutant for $Su(H)^{d47}$ and express UAS*dx*, females of the *y w* hsFlp *tub*Gal4 UASGFP; FRT 40A Gal80 genotype were crossed against *w*; FRT40A $Su(H)^{d47}$; UAS*dx* males. The progenies of each cross were heat shock during the first larval instar (24–48 h after egg laying). The wing imaginal discs were prepared at the end of the third larval instar stage.

Histochemistry

Antibody staining were performed according to standard protocols. The anti Wg antibody was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. The anti-Dll antibody was a gift from G. Boekhoff-Falk (formerly G. Panganiban), anti-Dve antibody was a gift from F. Matzusaki (Nakagoshi et al., 1998). Fluorochrome conjugated antibodies were purchased from Molecular Probes.

Results

It has been known for some time that the wing phenotype of the so far available *Su(H)* alleles is weaker than expected for a gene that encodes the transcriptional mediator of the *Notch* pathway (Gho et al., 1996; Miccheli and Blair, 1999). This paradox was explained by the following possibilities: Since the *Su(H)* mutant phenotype resembles that of hypomorphic alleles of other genes required for *Notch* signaling (see below), it was assumed that the weaker phenotype might be caused by the existence of a residual activity of the *Notch* pathway in *Su(H)* mutants, either by a very long lasting maternal component of *Su(H)* or because the alleles used in most studies ($Su(H)^{SF8}$ and $Su(H)^{AR9}$) are strong but might not be null alleles (Gho et al., 1996; Morel et al., 2001). An alternative explanation is the existence of an alternative, *Su(H)*-independent signal transduction mechanism that weakly activates the expression of the *Notch*-target genes in the absence of *Su(H)* activity.

In order to discriminate between these possibilities, we first analyzed the wing phenotype caused by the homozygosity of a recently available null allele of *Su(H)*, $Su(H)^{d47}$ (Fig. 1, Morel and Schweisguth, 2000). We compared the phenotype of this allele with that caused by loss of function alleles of two components of the γ-secretase complex, encoded by *Presinillin* (*Psn*) and *nicastrin* (*nic*). We further included a loss of function allele of *apterous* (*ap*) in our analysis, which is the transcriptional regulator that controls the expression of the *Ser*-ligand and *fringe* (*fng*) on the dorsal side of the wing (Klein, 2001). We chose the expression pattern of Wg in the late third larval instar stage as a marker for this initial analysis. At this time, Wg is expressed in a stripe that straddles the D/V boundary and in two nested ring-like domains that highlight the anlagen of the proximal and medial regions of the proximal wing (PW) and encircle the more distally located elements such

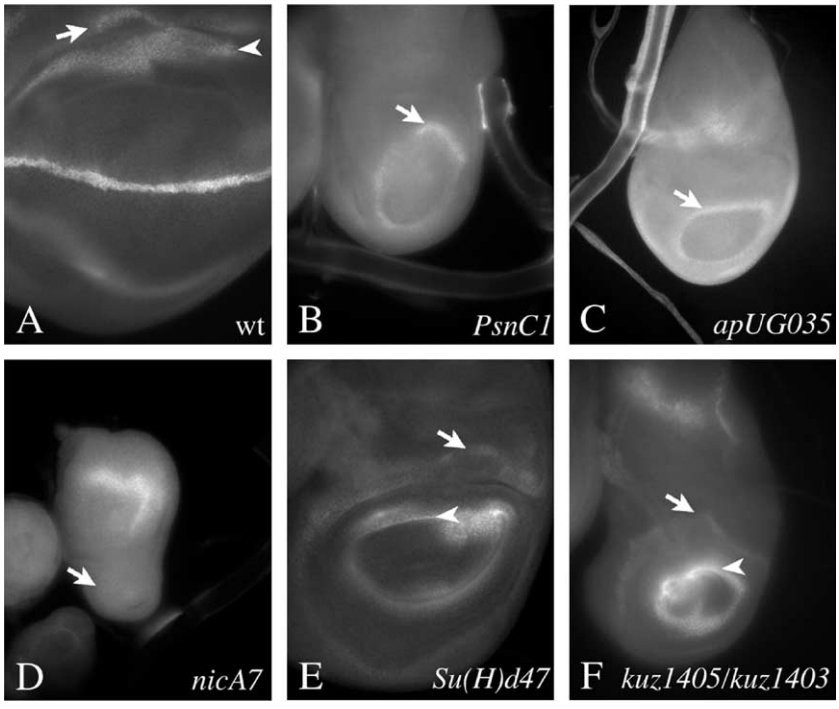


Fig. 1. Comparison of the phenotypes of wing imaginal discs, mutant for $Su(H)^{d47}$ with discs mutant for alleles of other genes involved in Notch signaling. (A) Expression of Wg in a wild-type wing imaginal disc at the end of the third larval instar stage. Wg is expressed in two ring-like domains in the anlage of the proximal wing (PW) (arrow and arrowhead) and along the D/V boundary. The inner ring-like domain (arrowhead) labels the medial area of the PW and encircles the more distally located anlagen such as the wing pouch. (B) Expression of Wg in a Psn^{C1} mutant wing imaginal disc. Only the outer ring-like domain of Wg is present, indicating that the more distal fates are lost. A similar phenotype is caused upon loss of *ap* and *nic* function (C, D). (E) In contrast, the inner ring-like domain of *wg* expression is present in $Su(H)^{d47}$ mutants (arrowhead in E). The phenotype resembles that of a hypomorphic allelic combination of *kuz*, kuz^{1403}/kuz^{1405}, which is depicted in panel F.

as the anlage of the wing blade (Fig. 1A; Koelzer et al., 2003; Neumann and Cohen, 1996). In *ap* mutants, only the outer ring-like expression domain of Wg is present (Fig. 1C). This domain marks the anlage of the medial region of the PW. The loss of the inner ring-like domain of expression of *wg* indicates that the anlagen of all regions located distally from the proximal region of the PW fail to develop. A similar defect was observed in discs mutant for *Psn* or *nic*: the inner ring-like domain was deleted or reduced to a spot of expression (Figs. 1B, D). In contrast to this, the inner ring-like domain of *wg* expression is present in wing imaginal discs that are homozygous for the null allele $Su(H)^{d47}$ (Fig. 1E). This observation suggests that in the absence of *Su(H)*-function, more distal structures form, than in mutants of other genes required for the activation of the *Notch* pathway.

This conclusion was confirmed by monitoring the expression of two genes whose expression marks the anlagen of more distally located wing structures, *defective proventriculus* (*dve*) and *Distal-less* (*Dll*) (Figs. 2A, D). Dve is expressed in a disc-like domain within the area framed by the inner ring-like domain of *wg* (Fig. 2D; Koelzer et al., 2003). Dll is expressed in a similar, albeit smaller domain that is restricted to the anlage of the wing blade (Fig. 2A; Neumann and Cohen, 1997). The expression of both genes was lost in the wing area of *Psn*, and *nic* mutant discs (Figs. 2C, F, G). The residual expression of Dve in *Psn* mutant wing discs is located in cells of the remaining proneural clusters (arrows in Fig. 2F). It appears that loss of *Psn* function leads to ectopic expression of Dve in proneural cluster cells of the wing.

In contrast, residual expression of both genes within the area of the wing can be found in *Su(H)* mutant and *kuz*-hypomorpic wing discs (Figs. 2B, E and H respectively). These observations confirmed that more distally located structures form in *Su(H)*-null mutant wings, and that the loss of *Su(H)* function therefore causes a milder defect than the loss of function of other genes involved in *Notch* signaling.

The weaker wing phenotype of Su(H)-null mutants is caused by the loss of the Notch-independent repressor function

The phenotype of $Su(H)^{d47}$ mutants resembles that caused by a combination of weak alleles of *kuz* (compare Figs. 1E, F and 2E, H). This similarity suggests that in *Su(H)* mutants, a residual activity of the *Notch* pathway might exist, possibly provided by the maternal component of *Su(H)*. In order to test this possibility, we generated double mutants of *Psn* and *Su(H)* null alleles. If the *Notch* pathway is weakly active in *Su(H)* mutants, this activity should be abolished if function of *Psn* is removed. Hence, the phenotype of the *Psn* mutant should be epistatic over that of *Su(H)*. As a test for the correctness of this prediction, we first generated wing imaginal discs double mutant for *Psn* and the hypomorphic allelic combination of *kuz*. As predicted, the *kuz*; *Psn*-double mutant wing discs looked like that of *Psn*-single mutants: the inner ring-like domain of *wg* expression as well as

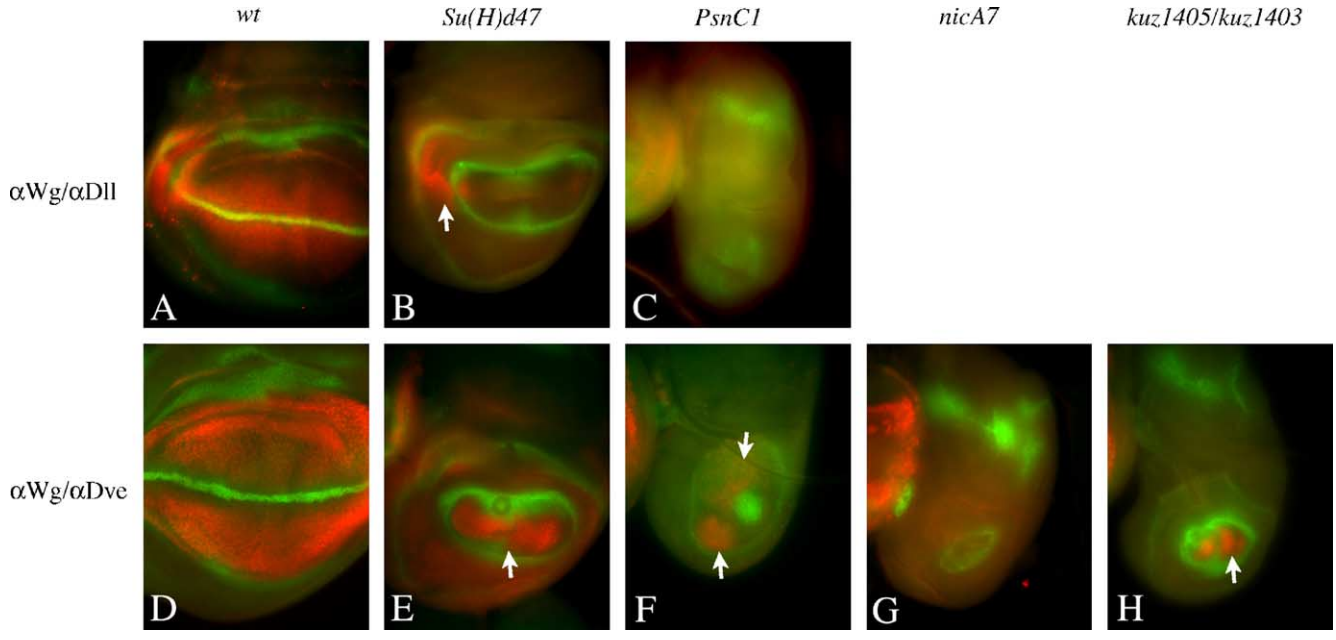


Fig. 2. Expression of Dll and Dve in mutants of genes that are involved in signal transduction through the *Notch* pathway. Expression of Wg, Dll and Dve is detected by antibody staining. Dll and Dve are depicted in red, Wg in green. Arrows highlight the expression of Dve and Dll in the mutant discs. (A, D) Expression of Dll (A) and Dve (D) in wild-type wing imaginal discs at the end of the third larval instar stage. Dll is expressed only in cells of the developing wing pouch, whereas the domain of Dve is slightly larger and reaches until the inner ring-like domain of Wg expression in the PW. Thus, the expression domain of Dve includes the wing pouch and the distal area of the PW. (B, E) Both genes are expressed in $Su(H)^{d47}$ mutant wing imaginal discs. The expression of Dll is restricted to the anterior region of the wing anlage (arrow in B), whereas expression of Dve is found in all cells in the area encircled by the inner ring-like domain of Wg (see arrow in E). (C, F) Expression of Dve and Dll is absent in Psn^{C1} mutants. The residual expression of Dve (red) that is highlighted in the disc depicted in panel F by the arrows is located in cells of the remaining proneural clusters. This expression is ectopically initiated after loss of *Psn* function. (G) Expression of Dve is also lost in nic^{A7} mutant wing discs. However, it can be detected in a *kuz*-hypomorphic (kuz^{1405}/kuz^{1403}) situation depicted in (arrow in H). Note that the phenotype of this hypomorphic combination looks very similar to that of the *Su(H)* loss of function mutant (compare E with H).

the expression domain of *dve* and *Dll* was lost, just as in the case of *Psn* mutants (Fig. 3A; data not shown). This indicates that a residual activity of the *Notch* pathway causes the "weak" phenotype of the *kuz*-hypomorphic allelic combination.

In contrast to this result, the *Su(H)*; *Psn*-double mutants looked like the weaker *Su(H)*-single mutant, and the expression of *Dll*, *dve* as well as the inner ring-like expression domain of *wg* was present (compare Figs. 3B, C with Figs. 2B, E). We could observe a similar weakening of the *nic* mutant phenotype, upon abolition of *Su(H)* function (Fig. 3D). Furthermore, the loss of *Su(H)* function led to a weakening of the *ap* mutant phenotype, indicated by the re-appearance of the inner ring-like domain of *wg* expression (Figs. 3G, H). Altogether, these results indicate that the weaker phenotype of *Su(H)* is not caused by the existence of a residual activity of the *Notch* pathway. Instead, it is caused by the loss of a function of *Su(H)* that is independent of the *Notch* pathway.

In the absence of *Notch* signaling, Su(H) acts as a repressor in a complex with H (Barolo et al., 2002; Furriols and Bray, 2001; Klein et al., 2000; Koelzer and Klein, 2003). To determine whether the loss of this repressor function is responsible for the weaker phenotype of *Su(H)* mutants, we tested if inactivation of the repressor complex through loss of *H* function leads to a similar weakening of the *Psn* mutant phenotype, as it is the case upon loss of *Su(H)* function. Indeed, we found that in *H Psn*-double mutants, the inner ring-like domain of *wg* as well as the expression of *Dll* and *dve* is present just as it is the case in the *Su(H)*; *Psn*-double mutants (Figs. 3E, F). These results show that the loss of the *Notch*-independent function of Su(H) as a repressor of gene expression is responsible for the "weaker" mutant phenotype. They further indicate that the loss of *Su(H)* function leads to a de-repression of one or more target genes of the *Notch* pathway.

De-repression of vestigial expression is responsible for the weaker phenotype of Su(H) mutants

The inner ring-like domain of *wg* expression as well as the expression of *dve* and *Dll* is established by Vg (Klein and Martinez-Arias, 1999; Koelzer et al., 2003; St. Pierre et al., 2002). Hence, it is possible that *vestigial* (*vg*) is one of the crucial genes that is de-repressed in *Su(H)* mutants. In order to test this assumption, we generated *vg Su(H)*-double mutant wing imaginal discs. Loss of *vg* function results in a wing phenotype that is very similar to that of *ap* or *psn* null mutants, causing a loss of expression of the inner ring-like domain of *wg* as well as that of *dve* and *Dll* (Fig. 4A, Kolzer and Klein, 2003). We found that the *vg Su(H)*-double mutant looked like the stronger *vg*-single mutant (Fig. 4B). This result suggests that *vg* function is important for the "weakness" of the *Su(H)* mutant phenotype. If this assumption is true, forced expression of *vg* in *Psn* mutants (where its expression is absent) should recover expression of Dll, Dve and the inner ring-like domain of Wg. Indeed, we

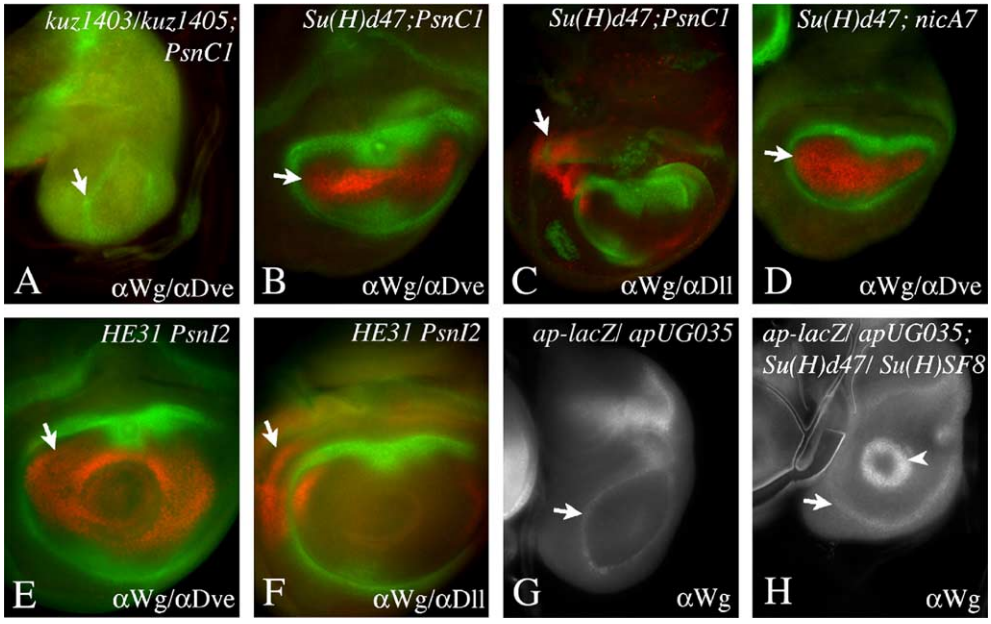


Fig. 3. The phenotype of *Su(H)* mutants is different from other mutants of other genes involved in the *Notch* pathway because of the loss of a second, *Notch*-independent function. Expression of Dll and Dve is depicted in red, that of Wg in green. (A) Expression of Dve and the inner ring-like domain of Wg are lost in a disc, double mutant for kuz^{1405}/kuz^{1403} and Psn^{C1}, indicating that residual activity of the *Notch* pathway was responsible for the expression of both genes in the hypomorphic *kuz*-mutant situation. (B, C) Expression of Wg/Dve (B) and of Wg/Dll (C) in $Su(H)^{d47}$; Psn^{C1}-double mutant wing imaginal discs. As in the *Su(H)* mutant discs and in contrast to *Psn* mutants, the inner ring-like domain of Wg expression and expression of Dll and Dve is detectable in the double mutant. Likewise, expression of the inner ring-like domain of Wg and that of Dve occurs in $Su(H)^{d47}$; nic^{A7}-double mutants (D). These observations suggest that the weaker phenotype of *Su(H)* mutants is not caused by a residual activity of the *Notch* pathway but because of the loss of a *Notch*-independent function of Su(H). (E, F) De-repression of expression of Dve and Dll also occurs if *H* function is abolished in *Psn* mutants. Thus, it appears that the loss of the repressor function of the Su(H)/H complex is responsible for the weaker mutant phenotype of the double mutants. (G, H) Loss of *Su(H)* function weakens the phenotype of *ap* mutant wing imaginal discs. (G) Expression of Wg in *ap* mutants. Only the outer ring-like domain of Wg-expression is observable (arrow). (H) The inner ring-like domain reappears in *Su(H) ap*-double mutants, indicating that loss of *Su(H)* function results in the establishment of distal fates that are normally absent in *ap* mutants.

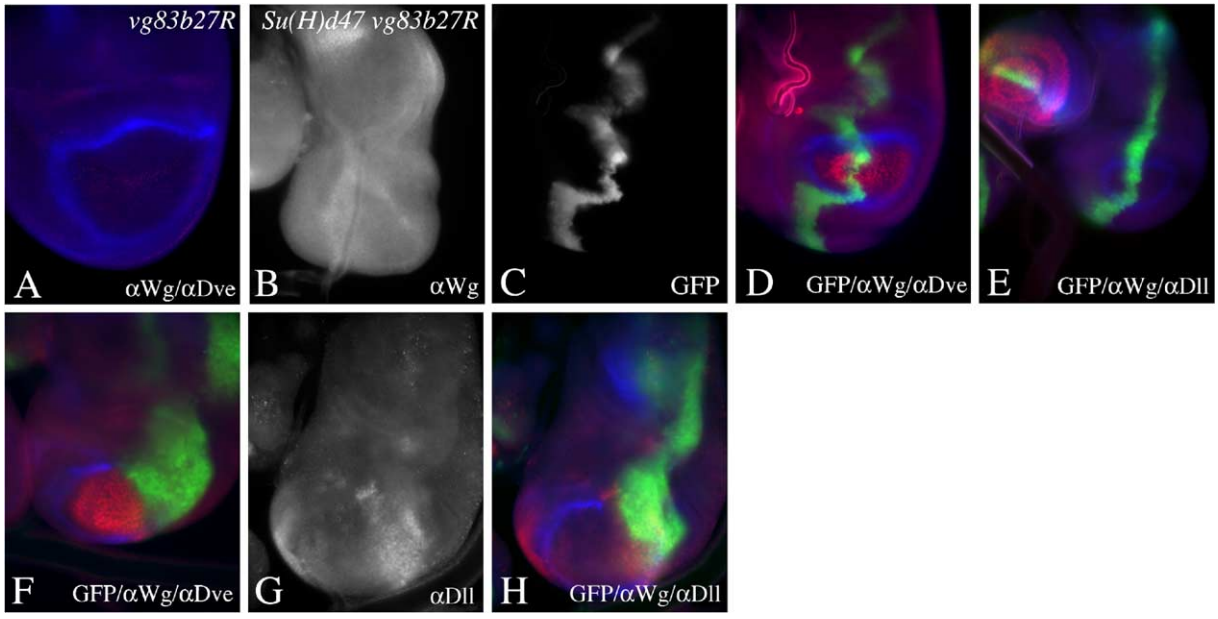


Fig. 4. De-repression of *vg* expression is responsible for the weaker phenotype of *Su(H)* mutants. (A) Expression of Wg (blue) and Dve (red) in a *vg*-single mutant disc. The inner ring-like domain of Wg as well as expression of Dve is lost. (B) Expression of Wg in a *vg Su(H)*-double mutant wing imaginal disc. The inner ring-like domain of Wg expression is lost, indicating that *vg* is epistatic over *Su(H)*. We further found that the expression of Dve is also lost. (C–H) Forced expression of *vg* in *Psn* mutant wing imaginal discs establishes the expression of the inner ring-like domain of Wg and that of Dve and Dll. Expression of Wg in (D–F, H) is shown in blue, that of Dve in (D, F) in red (D, F) and that of Dll in (E, H) in red. The expression of *vg* occurred with help of the *ptc*Gal4 line, which activates expression of UAS constructs in a stripe along the A/P boundary, depicted in panel C and in green in panels D–F, H. Two classes of phenotypes were observed if *vg* was expressed with *ptc*Gal4: one class expressed Dve and Wg, but not Dll (D, E). The second class expressed also Dll (F–H). Altogether, these results show that de-repression of expression of *vg* occurs in *Su(H)* mutants and that this de-repression is the major cause of the weaker phenotype.

found that this is the case: forced expression of UAS*vg* in *Psn* mutant discs with *ptc*Gal4 lead to two classes of phenotypes: In wing discs belonging to the first class, the expression of the inner ring-like domain *wg* as well as *dve*, but not of *Dll* (Figs. 4C–E) was recovered. In the second class, we also found expression of Dll in addition to that of *wg* and *dve* (Figs. 4F–H). In *Su(H)* mutants, Dll is expressed only in an anterior region of the wing (see Fig. 2B), indicating that additional factors are required that are present in this anterior region. *ptc*Gal4 is only very weakly expressed in the anterior region of the anlage of the wing. Thus, the existence of two classes of phenotypes can be explained by a weak expression of UAS*vg* in the anterior region that only occasionally reaches the threshold level required for induction of expression of Dll. Nevertheless, the experiment indicates that forced expression of *vg* can re-establish expression of *wg*, *dve* and *Dll* in *Psn* mutants, suggesting further that de-repression of expression of Vg causes the weak phenotype of *Su(H)* mutants.

If de-repression of *vg* occurs, we might be able to observe this directly. The *Notch* pathway regulates expression of *vg* through the activation of the *vg* boundary enhancer (*vg*BE) (Figs. 6A–D). A single Su(H)-binding site within this enhancer mediates the activation by the *Notch* pathway, and it has been shown that loss of this site or loss of *Su(H)* function leads to the loss of *vg* expression and activity of the *vg*BE in wing imaginal discs of the late third larval instar stage (Kim et al., 1996; Klein and Martinez-Arias, 1999). These published data do not support our suspicion that *vg* would be expressed in *Su(H)* mutant wing discs. However, since expression of the *vg*BE was analyzed only during the late third larval instar stage, we thought it is possible that de-repression occurs during earlier phases, and that this transient expression might be sufficient for establishment of the distal fates present in *Su(H)* mutants. We first monitored expression of the *vg*BE in *H*; *Psn*-double mutant discs and indeed found that the *vg*BE was weakly active in the double mutant, but not in *Psn*-single mutant (Figs. 5A–F). Thus, if the Su(H)-dependent repressor complex is destroyed in *Psn* mutants, Vg appears to be de-repressed.

We then monitored the expression of the *vg*BE in *Su(H)* mutant discs in earlier phases of the third larval instar stage and again found that it is transiently and weakly active until the middle of the third larval instar stage, but not in later stages (Figs. 6E–I). Likewise, a variant of the *vg*BE, whose expression cannot be activated by the *Notch* pathway, because its Su(H)-binding site is mutated (*vg*BE-Su(H); Kim et al., 1996), is weakly active in wild-type wing discs in earlier stages of the third larval instar (Figs. 6J, K).

We furthermore can detect a weak expression of Vg in a $Su(H)^{SF8}/Su(H)^{AR9}$ strong mutant allelic combination (data not shown). The phenotype is very similar to that caused by the null allele $Su(H)^{d47}$ (TK, own observation). We therefore believe that the residual expression of Vg is caused by the loss of *Su(H)* activity, rather than by a residual activity of it. Altogether, these results suggest that weak and transient expression of *vg* occurs in the absence of *Su(H)* activity. This transient expression appears to be sufficient to establish

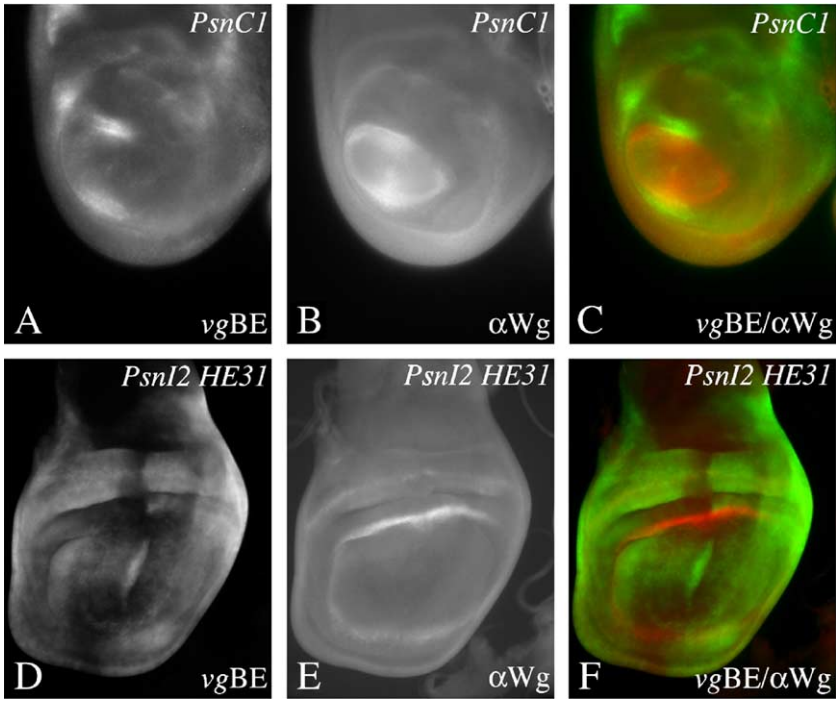


Fig. 5. Expression of the *vg*BE in *Psn*-single and *Psn H*-double mutants. Expression of Wg in red, activity of the *vg*BE in green (A–C). The *vg*BE is not active in *Psn*-single mutants in the region encircled by the ring-like domain of *wg* expression. (D–F) In contrast, activity of the enhancer can be detected inside this area in *Psn H*-double mutant discs.

the expression of Wg, Dll and Dve and causes the weaker phenotype.

Su(H) is involved in the formation of the dorsoventral compartment boundary

Notch-mediated interactions at the boundary of *ap*-expressing and non-expressing cells are also required to establish and maintain the D/V compartment boundary (Miccheli and Blair, 1999; Rauskolb and Irvine, 1999). However, Miccheli and Blair (1999) found that Su(H) is probably not involved in this process, suggesting that compartment boundary formation occurs via a *Su(H)*-independent *Notch* pathway. To rule out the possibility that the requirement of *Su (H)* was obscured by the fact that the *Su(H)* allele used in the study ($Su(H)^{SF8}$) might not be a complete loss of function allele, we have looked at the formation of the D/V compartment boundary in the null allele $Su(H)^{d47}$. As a marker for the D/V boundary, we used the smoothness of the boundary between *ap*-expressing and non-expressing cells (Fig. 7A). We found that this boundary was as smooth in $Su(H)^{d47}$ mutant wing imaginal discs as in the wild type, indicating that the compartment boundary has been established correctly in the absence of *Su(H)* function (Figs. 7E, F). In contrast, the boundary was irregular in *Psn* and *ap* mutant wing imaginal discs, indicating that formation of the D/V compartment boundary failed in these mutants (Figs. 7B–D). These results confirm the findings of Miccheli and Blair (1999) and suggest that the Notch pathway, but not Su(H), is required for the formation of the D/V compartment boundary.

However, knowing that loss of *Su(H)* function leads to a de-repression of *Notch* target gene expression, we speculated that Su(H) might be involved in compartment formation, but loss of its function results in a de-repression that is sufficiently strong to allow the formation of the compartment boundary to occur. An indication that this speculation might be correct was provided by the observation that in some of the $Su(H)^{d47}$ mutant wing imaginal discs, small violations of the compartment boundary were observed (data not shown). Thus, the process of boundary formation appears to be weakened.

If our assumption is correct, the boundary should be re-established in the *Psn* mutant if *Su(H)* or *H* activity is concomitantly removed. Indeed, we found that in contrast to *Psn*-single mutants, the boundary between *ap*-expressing and non-expressing cells is smooth in wing discs double mutant for *Su(H)*; *Psn* or *Psn H* (Figs. 7G, H, K, L). Thus, the compartment boundary formation is restored in the double mutants. In addition, over-expression of *H* throughout the wing results in an irregular boundary between *ap*-expressing and non-expressing cells (Figs. 7I, J), indicating that shifting the equilibrium towards the formation of the repressor complex prevents boundary formation. Altogether, these results have two important implications: Firstly, Su(H) is involved in formation of the D/V compartment boundary. Secondly, it is not necessary to postulate a *Su(H)*-independent mechanism of *Notch* signal transduction during this process of boundary formation. The requirement of Su(H) in this process is simply obscured by the de-repression of expression of its target genes.

Ap has two distinct roles during D/V compartment formation

The de-repression of expression of target genes required for boundary formation upon loss of *Su(H)* function should

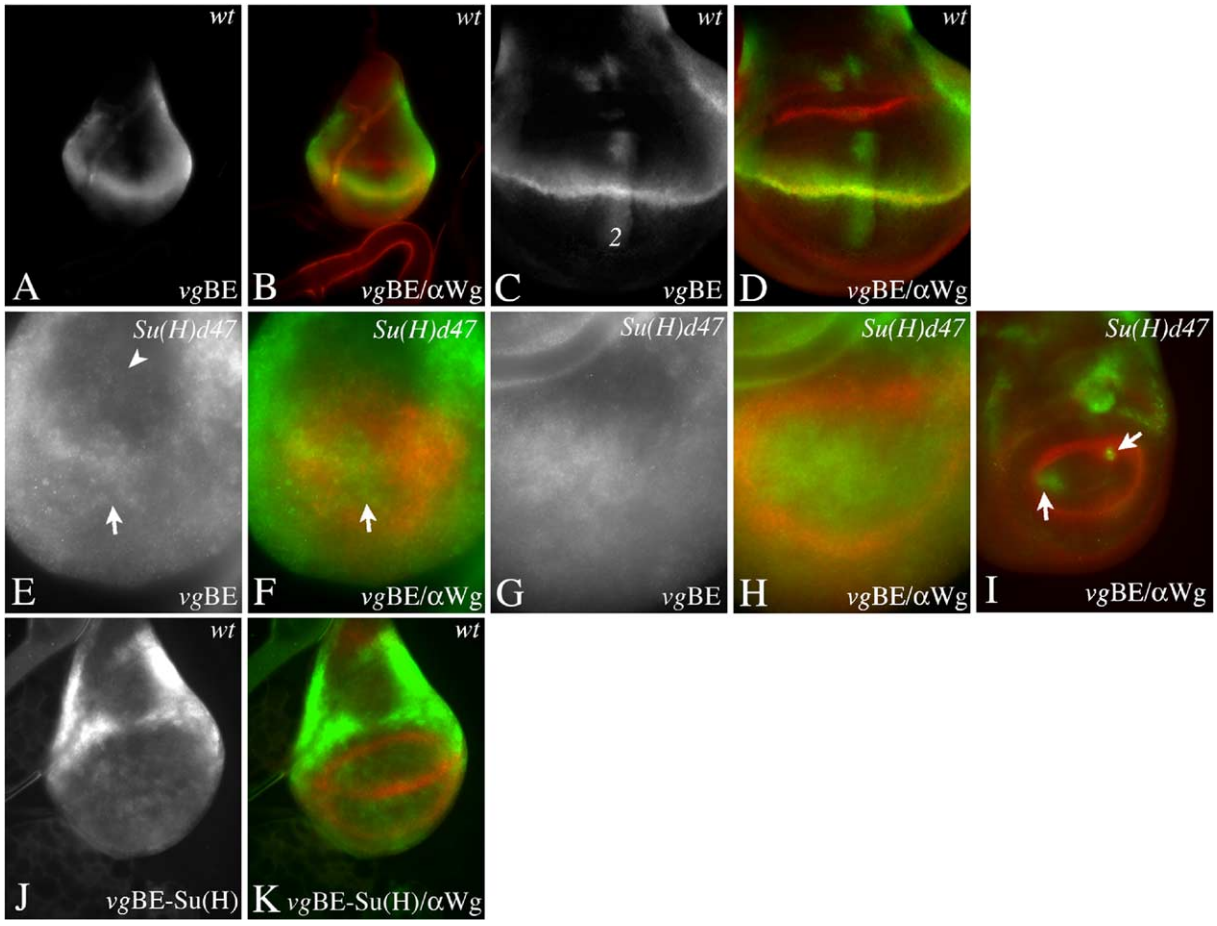


Fig. 6. Expression of the *vg*BE in *Su(H)*^{d47} mutant wing imaginal discs. (A, B, E, F) Wing imaginal discs at the beginning and (C, D, G, H, J, K) in the middle of the third larval instar stage. (I) Wing imaginal disc at the late third larval instar stage. Discs are stained with anti Wg and with anti β-Gal antibodies to reveal the activity of the *vg*BE. Wg is depicted in red and the *vg*BE in green in panels B, D, F, H, I, K. Discs in panels E–H are shown in twice the magnification as the rest. (A–D) Activity of the *vg*BE in wild-type wing imaginal discs. The enhancer is active in cells along the D/V boundary and from the middle of the third larval instar stage onwards in a second domain along the A/P boundary (domain 2, see C). (E–I) Activity of the *vg*BE in *Su(H)* mutant wing imaginal disc. The activation of the enhancer along the D/V boundary is lost and is replaced by a weak and diffuse activity throughout the region of the wing anlage (arrow in E, F). The arrow points to the region that is framed by the ring-like domain of Wg expression. A comparison of this region with that highlighted by the arrowhead indicates that the staining in the is stronger in the wing region. This suggests that the diffuse staining is above background staining. (I) The diffuse activity of the enhancer is lost in most regions during late phases of the third larval instar stage, especially in the region encircled by the inner ring-like domain of Wg expression. This observation further indicates that the diffuse staining observed in the wing area of discs in earlier phases is not background staining but specific expression of the *vg*BE. The residual expression within the wing area in panel I, highlighted by the arrows is in the remaining proneural cluster cells, where the *vg*BE is ectopically expressed if Su(H) function is lost. (J, K) Activity of a variant of the *vg*BE, *vg*BE-Su(H), that lacks a functional Su(H) DNA binding site in wild-type discs. Also this variant is weakly active throughout the anlage of the wing during early phases of the third larval instar stage.

occur in ventral as well as in dorsal cells. Thus, these genes, although required, are probably not providing an asymmetry in the cell properties that helps to separate dorsal from ventral cells. Therefore, this asymmetry has to be provided by Ap as an additional function besides the activation of the *Notch* pathway through the regulation of the expression of *Ser*. This assumption is substantiated by the observation that removing *ap* function in *H* or *Su(H)* mutants abolishes the formation of the boundary (Figs. 7M–O). We further confirm the finding of Milan and Cohen (1999) that expression of UAS *Ser* in a hypomorphic *apGal4*/*ap*^{UG035} mutant background cannot rescue boundary formation completely (data not shown). Hence, these results show that Ap provides an asymmetry that is required in addition to its function of activating *Notch* signaling during the formation of the compartment boundary.

The role of Vg during D/V compartment formation

As shown by this work, de-repression of *vg* expression is the major cause for the weaker pattern formation defect of *Su(H)* mutant wing discs. Using expression of *wg* as a marker for analysis, the loss of function phenotype of *vg* and *ap* mutants look very similar (compare Fig. 1C with Fig. 4A). Vg is expressed along the compartment boundary from early stages of wing development onwards, as a result of *Notch* signaling (see Figs. 6A–C; Williams et al., 1991). Thus, we wondered if Vg might also be involved in the formation of the D/V compartment boundary. Indeed, a recent report provided evidence for such an involvement by showing that the boundary between *ap*-expressing and non-expressing cells is slightly irregular in a loss of function allele of *vg*, named *vg*^{null} (Delanoue et al., 2002). However, when we generated a *vg* loss of function situation

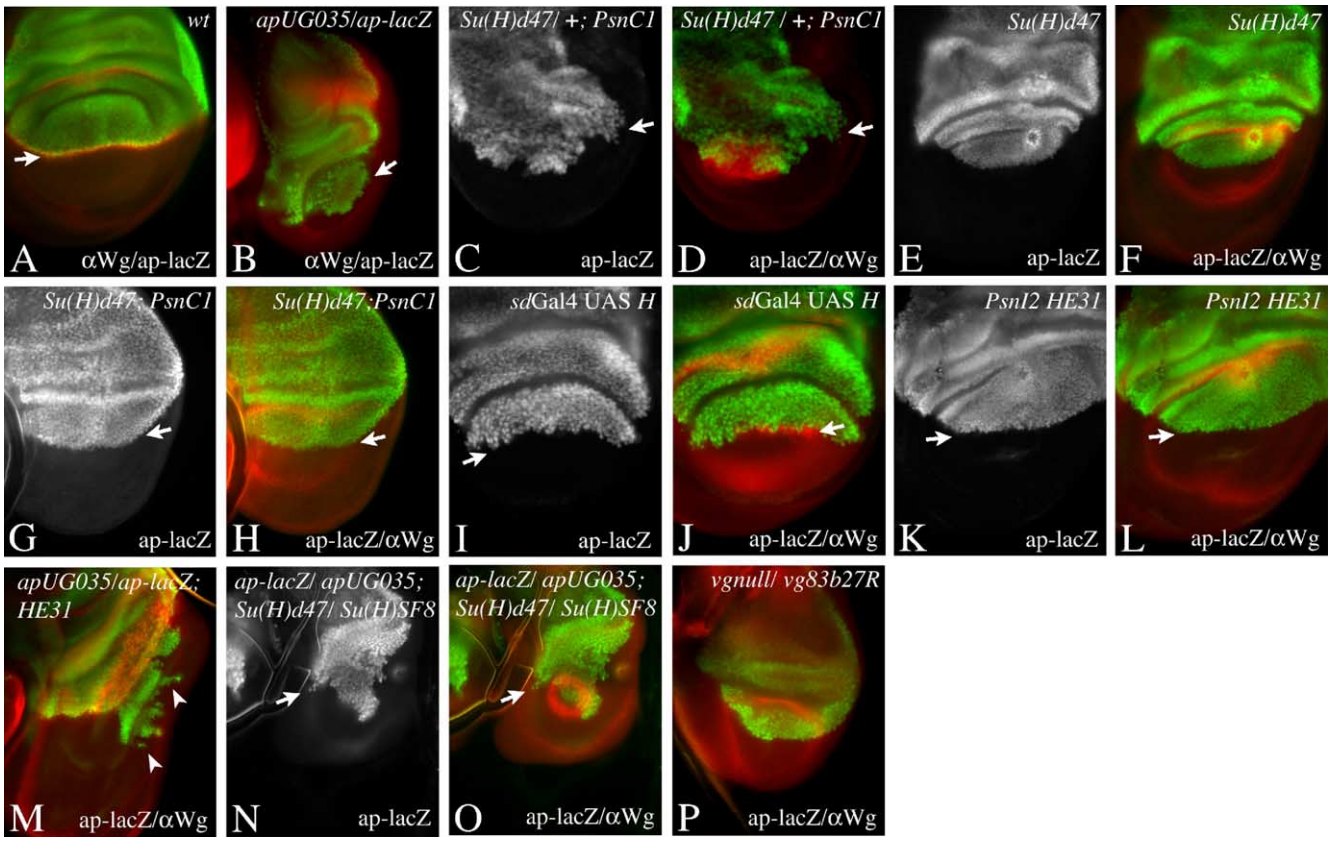


Fig. 7. Su(H) is involved in the formation of the D/V compartment boundary. Expression of Ap, detected by *ap*-lacZ, in green, expression of Wg in red. (A–F) Expression of *ap* in wild-type (A), *ap* mutant (B), *Psn* mutant (C, D) and *Su(H)* mutant (E, F) wing imaginal discs of the late third larval instar stage. In contrast to the wild-type disc, the boundary between *ap*-expressing and non-expressing cells is irregular in *ap* and *Psn* mutant discs, indicating that the compartment boundary has not been established. However, the boundary in *Su(H)* mutant discs is smooth just as that of a wild-type disc. Thus, the compartment boundary has been established in the absence of *Su(H)* function. (G–L) Su(H) is involved in the formation of the boundary. (G, H) In contrast to the *Psn* mutant discs, the boundary between *ap*-expressing and non-expressing cells is smooth in *Su(H)*; *Psn* (G, H)- and *Psn H* (K, L)-double mutant wing imaginal discs. Furthermore, over-expression of UAS *H* with *sd*Gal4 results in a failure of boundary formation (I, J). Altogether, these results show that removal of the Su(H)-dependent repressor complex can restore the compartment boundary in *Psn* mutants. (M–O) Ap has a second role during formation of the D/V compartment boundary, besides regulation the activity of the *Notch* pathway. (M) A disc double mutant for *ap* and *H*. The boundary between *ap*-expressing and non-expressing cells is irregular (arrowheads), indicating that establishment of the compartment boundary has failed. (N, O) Expression of *ap* in a *Su(H)d47/Su(H)SF8 ap-lacZ/apUG035*-double mutant. Also in this situation, the boundary is irregular and, thus, the compartment boundary failed to form. (P) Expression of *ap* in a *vg* null mutant wing disc. The boundary between *ap*-expressing and non-expressing cells is smooth, indicating that the D/V compartment boundary can form in the absence of *vg* function.

using vg^{null} in combination with another loss of function allele, vg^{83b27R}, we found that the compartment boundary in these wing imaginal discs is only slightly disturbed in comparison to *Psn* mutant discs (compare Figs. 7P and C). This result suggests that Vg probably does not play an important role in the formation of the D/V compartment boundary.

Deltex (Dx) signaling is dependent on the activity of Su(H)

Recent work suggests that the RING finger ubiquitin E3-ligase Deltex (Dx) mediates a *Su(H)*-independent *Notch* signal that activates the *vg*BE during wing development (Hori et al., 2004). This conclusion was based on the following experiment: If expressed by *dpp*Gal4, UAS*dx* can induce the expression of the *vg*BE in a stripe-like domain along the anterior of the A/P boundary. The authors showed by clonal analysis that the enhancer appears to be expressed in cells that lack a functional *Su(H)* gene. Thus, they concluded that Dx can activate the *vg*BE in a *Su(H)*-independent manner and is therefore a mediator of a novel *Notch*-signaling pathway. However, two alternative explanations can be brought forward: firstly, our data presented here indicate that the *vg*BE is transiently de-repressed in the absence of *Su(H)*. During the late third larval instar stage, the *vg*BE is expressed in an additional stripe-like domain (domain 2; see Figs. 6C and 8D) along the A/P boundary, which is included in the expression domain of the *dpp*Gal4 line. Domain 2 is probably not solely dependent on *Su(H)* function but also on a signal (or signals) emerging at the A/P boundary. We speculated that this signal might be sufficient to maintain or even enhance de-repression of the *vg*BE along the A/P boundary in the absence of *Su(H)* activity. Hence, the removal of *Su(H)* would not abolish expression of the *vg*BE within domain 2. A second possible explanation is the stability of the β-Galactosidase. It is possible that β-Galactosidase perdures in the mutant cells of the clones, especially since the clones generated by Hori et al. were small (induced during the second larval instar stage). The perdurance of β-Galactosidase would imply a *Su(H)*-independent induction of expression of the *vg*BE, which may not exist.

In order to evaluate these possible explanations, we performed two types of experiments. Similar to Hori et al. (2004), we expressed UAS*dx* with *dpp*Gal4 in the first experiment and induced *Su(H)* mutant cell clones. As a measure for the activity of the *Notch* pathway, we monitored the expression of the *vg*BE as well as Wg in the *Su(H)* mutant territories. In contrast to Hori et al. (2004), we found a clear reduction in or abolition of the expression of the *vg*BE in most *Su(H)* mutant cells (lower arrow and arrowhead in Figs. 8A–C). However, a weak background expression was also often observed (Fig. 8). Especially in domain 2, the expression of the *vg*BE was often only reduced in small mutant areas. Only in large clones, the expression was lost (arrowheads in Figs. 8A–C). Furthermore, the expression of Wg (detected by antibody stainings) was always abolished in the clones (Figs. 8B, C). Altogether, these data suggest that Dx is not able to activate expression of *Notch* target genes in the absence of *Su(H)* function.

In order to be able to monitor the ability of Dx to activate *Notch*-target genes at other positions in the wing pouch, especially the D/V boundary, we used the MARCM system (Figs. 8D–L). This system allows to generate heat-shock induced and positively (GFP-) labeled *Su(H)* mutant clones that concomitantly express UAS*dx*. We heat-shocked the larvae during the first larval instar stage (24–48 h after egg laying), to obtain large clones.

As expected, we found that expression of *vg*BE and to a lesser degree that of Wg were induced in control clones that only expressed UAS*dx* (Figs. 8D–F). Furthermore, we observed additional cell proliferation as it is typical for ectopic activation of the *Notch* pathway (Diaz-Benjumea and Cohen, 1995). All these effects were abolished, if the cells lacked the function of *Su(H)* (Figs. 8G–I). In the *Su(H)* mutant cell clones, expression of UAS*dx* was unable to induce cell proliferation or ectopic expression of *wg* and the *vg*BE. Furthermore, clones that crossed the D/V boundary interrupted the endogenous expression of these markers along the D/V boundary although the clone cells expressed *dx* (Figs. 8G–I). In contrast, in clones located in domain 2, we sometimes found only a reduction of the expression of the *vg*BE (arrowhead in Figs. 8G, I). Nevertheless, expression of *wg* was never observed (Figs. 8G, H). We found a similar behaviour in control clones that were mutant for *Su(H)* but did not express UAS*dx* (Figs. 8J–L). This indicates that the expression of the *vg*BE in domain 2 is not absolutely dependent on *Su(H)* function. Thus, the residual expression of the *vg*BE observed in *Su(H)* mutant cells in this domain is not caused by a *Su(H)*-independent activity of Dx. Since our analysis indicates that outside domain 2 the activity of the *vg*BE is clearly dependent on Su(H), irrespective of the presence or absence of over-expression of Dx, we conclude that Dx does not mediate a *Su(H)*-independent signal.

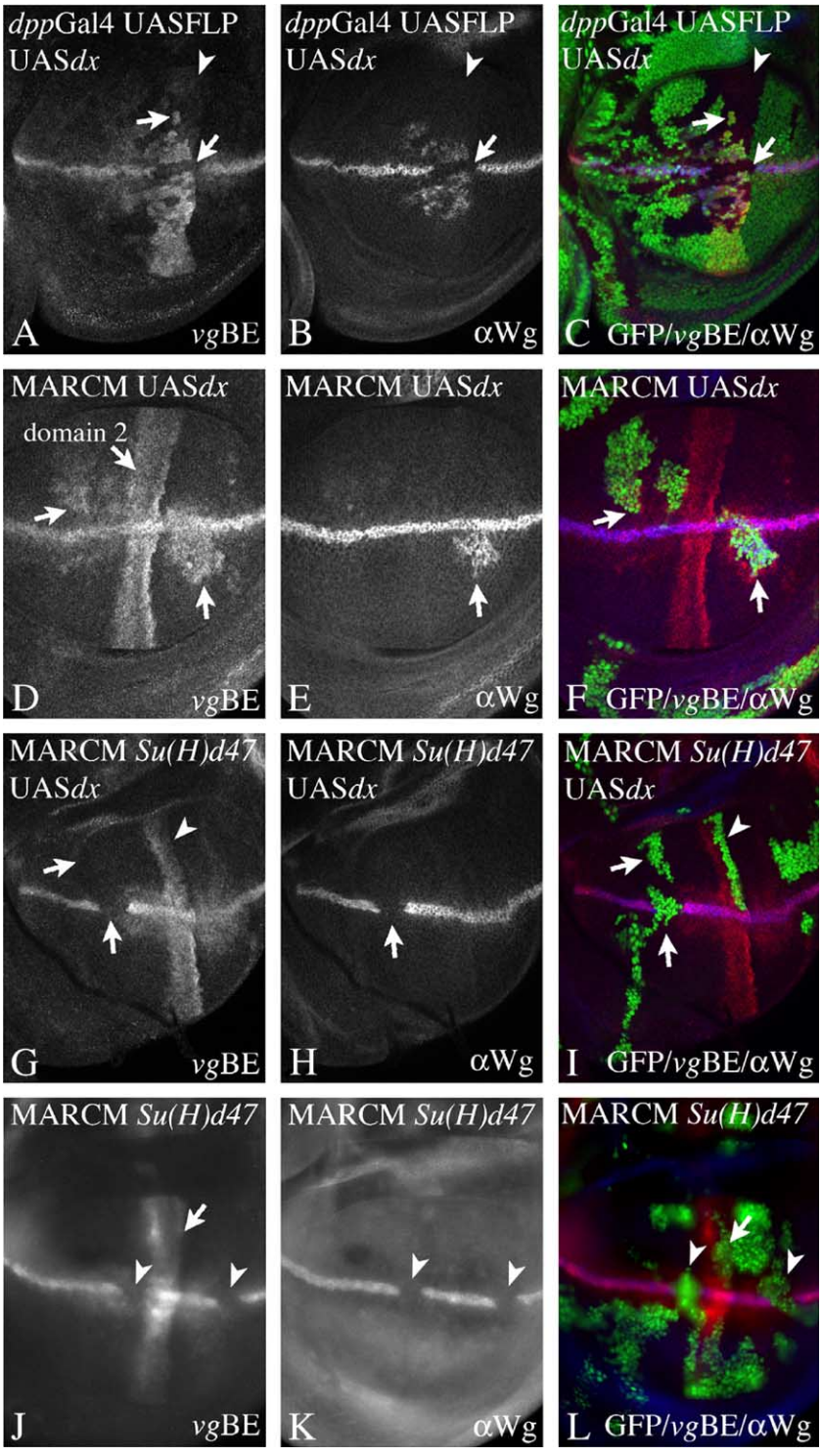
Discussion

In this work, we provide an answer to the observation that the patterning defects of *Su(H)* mutant wing imaginal discs is weaker than anticipated for a gene that encodes a factor that mediates most of the transcriptional activity of the *Notch*-signaling pathway. We further demonstrate that Su(H) is required for the formation of the D/V compartment boundary despite any obvious defect in this process in the absence of its function. In both processes, the explanation for the phenotype of *Su(H)* mutants is the loss of its function as repressor of transcription along with its function as an activator.

We have recently described that loss of function of *Su(H)* leads to an arrest in the development of the sensory organ precursor cell of the bristle sense organ (Koelzer and Klein, 2003). Although we were able to demonstrate genetically that de-repression of expression of some genes of the *Enhancer of split-complex* are responsible for the arrest, we were not able to detect the expression of any of these genes directly. In this work, we could show that de-repression of *vg* is a consequence of loss of *Su(H)* function during wing development. Although this de-repression was weak and transient, it was sufficient to establish more distal elements than in mutants of other genes necessary for *Notch* signaling. Our results are in agreement with two earlier reports that show de-repression of target genes in *Su(H)* mutants in other developmental processes such as mesectoderm specification and bristle development (Morel and Schweisguth, 2000; Koelzer and Klein, 2003). Thus, de-repression of target genes appears to be a common phenomenon during *Drosophila* development, if *Su(H)* function is lost.

Fig. 8. Dx is dependent on the function of *Su(H)*. Wing imaginal discs of the late third larval instar stage are stained with anti-β-Gal- and anti-Wg antibody to reveal their expression patterns. (A, D, G, J) Expression of the *vg*BE; (B, E, H, K) Expression of Wg. (C, F, I, L) Merge of the preceding pictures in the corresponding row, showing expression of GFP in green, that of the *vg*BE in red and that of Wg in blue. (A–C) Expression of the UAS*dx* by *dpp*Gal4. At the same time $Su(H)^{d47}$ mutant cell clones were induced by UASFlp. The mutant territory is labeled by the absence of GFP. The ectopic expression of UAS*dx* leads to the induction of ectopic expression of Wg and the *vg*BE. However, expression of the *vg*BE is strongly reduced or absent in the $Su(H)^{d47}$ mutant territories. Expression of Wg is always abolished. The lower arrow highlights a cell clone at the D/V boundary. Expression of the *vg*BE is reduced to weak background levels similar to other regions close to the D/V boundary. Expression of Wg is abolished. The arrowhead highlights a large mutant area in the dorsal half of the wing pouch that includes part of domain 2 (see also D), where expression of the *vg*BE was reduced to background levels. Note the small patch of wild-type cells that is highlighted by the upper arrow. In this patch, the expression of the *vg*BE is maintained at normal levels. (D–L) MARCM clones of various genotypes positively labeled by GFP. (D–F) MARCM clones that express UAS*dx*. The arrows point to ectopic expression of the two markers induced by ectopic expression of dx. In contrast to the clone marked by the upper arrow, the clone crossing the D/V boundary (lower arrow) expresses Wg in addition to the *vg*BE. This is in agreement with the observation that Dx can activate the *Notch* pathway stronger in the ventral half of the wing pouch (Matsuno et al., 2002), and that induction of expression of Wg requires higher level of *Notch* activity than that of the *vg*BE. (G, H) $Su(H)^{d47}$ mutant MARCM clones that express UAS*dx*. The induction of ectopic expression of the *vg*BE as well as that of Wg is prevented by the loss of Su(H) function in the cell clones (upper arrow). Furthermore, the endogenous expression of both markers along the D/V boundary is also abolished (lower arrow). Note that the $Su(H)^{d47}$ mutant clone at the domain 2 (arrowhead) is only reduced. We observed an identical behaviour of the two markers in MARCM cell clones that are solely mutant for $Su(H)^{d47}$ depicted in panels J–L. Also in this case, the endogenous expression of both markers is interrupted (arrowheads), whereas the expression in domain 2 is only reduced (arrow).

Importantly, we show for the first time that this de-repression can even become strong enough to obscure an involvement of Su(H) in a developmental process, the formation of the D/V compartment boundary. De-repression of target genes upon loss of the repressor function of Su(H) is an attractive explanation for the paradox that loss of *Notch* function during the first larval instar stage is cell lethal, but loss of *Su(H)* function is not (de Celis and García-Bellido, 1994). Presumably, the de-repression of expression of target genes that are required for cell survival guarantees the survival of *Su(H)* mutant cells. In contrast, a similar de-repression cannot occur in *Notch* mutant cells, and the cells undergo apoptosis. Although the repressor function has been initially found in cell culture experiments with the vertebrate ortholog CBF1, reports analyzing the consequences of loss of its repressor function during vertebrate development are missing. Our presented results should encourage researchers to search for such an effect in their vertebrate model systems.



The presented results do further have important implications on the use of various mutants in order to analyze the function of the *Notch* pathway in a particular developmental process. They show that the phenotype of loss of function of *Su(H)*, or its vertebrate ortholog CBF1, is not necessarily identical to that of loss of the *Notch*-signaling activity. It is possible that de-repression of *Notch* target genes occurs upon loss of function of *Su(H)* but not upon inactivation of the pathway by other means. Previous work indicates that only a subset of genes might be de-repressed in a developmental process if Su(H) is absent. For example, we have never observed de-repression of expression of *wg* along the D/V compartment boundary upon loss of *Su(H)* function (Klein et al., 2000; Koelzer and Klein, 2003). The de-repression of only a subset of target genes could cause a phenotype that is difficult to interpret. Thus, it is better to use alleles of genes such as *Psn, kuz* or *nic*, which do not affect the repressor function of Su(H), to determine the function of the *Notch* pathway within a process of interest.

The weaker phenotype of *Su(H)* mutants during wing development was considered an argument for the existence of a *Su(H)*-independent mechanism of *Notch* signal transduction. Our findings strongly argue against the existence of such a mechanism in the analyzed processes. A recent report by Hori et al. (2004) reported further evidence to the existence of a Su(H)-independent Notch-signaling pathway that is mediated by Dx. Since we had excluded the existence of such a pathway in the two other described situations, we wondered whether an alternative explanation might exist for the observations made in the work of Hori et al. (2004). Indeed we found no evidence that Dx participates in a *Su(H)*-independent *Notch* signal during wing development. Our results suggest that in this case, the confusion came from analyzing a domain of the *vg*BE (domain 2) that appears not to be completely dependent on the function of *Su(H)*. Using the MARCM technique to generate Dx expressing *Su(H)* mutant cell clones, we could clearly show that Dx depends on the function of Su(H) to induce target gene expression in ectopic places as well as along the D/V boundary. Thus, our results abolish three arguments for the existence of a Su(H)-independent signal transduction mechanism during wing development. However, this does not imply that such a pathway does not exist. Indeed, evidence exists that during dorsal closure of the embryo, Notch acts independently of Su(H), through the JNK pathway (Zecchini et al., 1999).

Notch signaling during formation of the D/V compartment boundary

Recent work indicates that cell–cell interactions are required for the establishment of both the A/P—as well as the D/V compartment boundaries (reviewed in Dahmann and Basler, 1999). While it is clear that a transcriptional response mediated by the transcription factor Cubitus interruptus (Ci) is necessary to establish the A/P boundary, the situation at the D/V boundary was unclear. The results of Miccheli and Blair (1999) raised the possibility of a *Su(H)*-independent mechanism that is used to establish the D/V boundary. This mechanism might not even require a transcriptional response to the Notch signal. Our results demonstrate that this is not the case: similar to the formation of the A/P boundary compartment boundary, a transcriptional response to the *Notch* signal is required for the segregation of dorsal and ventral cells, and this response is mediated by Su(H). Similar to Ci, Su(H) acts as a transcriptional activator at the D/V boundary, where Notch is active and as a transcriptional repressor in a complex with H, and probably Groucho and dCtBP away from the boundary (Barolo et al., 2002). Our results suggest that the loss of this repressor function results in the de-repression of the relevant target genes in a manner sufficient to allow the formation of the D/V compartment boundary even in absence of Su(H). Overall the scenario at the D/V boundary seems to be very similar to that proposed for the formation of the A/P compartment boundary. In this situation, En endows the posterior fate and regulates the expression of Hedgehog that signals to anterior cells (Dahmann and Basler, 1999). As a response to Hh, the transcription factor Ci is transformed from a repressor to an activator of transcription and activates the expression of target genes in a stripe along the anterior side of the A/P boundary. Our results suggest a similar scenario for the formation of the D/V compartment boundary: similar to En, Ap imposes the dorsal fates on cells and activates the expression of Ser. Ser signals to the ventral cells at the D/V boundary. Similar to Hh transforming Ci from a repressor into an activator of transcription, Ser induced activation of the *Notch* pathway transforms Su(H) from a repressor into an activator. In analogy to En, we found that Ap has a second, Notch-independent function during D/V boundary formation. As in the case for En, an attractive possibility is that Ap acts to repress activation of the relevant target genes of Su(H) in dorsal cells. This repression creates a strong difference in expression of these genes at the D/V boundary and eventually leads to a strong difference in adhesion between the dorsal and ventral cells. This repressor function of Ap would also explain why the compartment boundary can form in the absence of Su(H) function, since the de-repression of target genes of Su(H) would be still restricted to ventral cells leading to a similar, albeit weaker difference in expression of these genes and in adhesion at the D/V boundary. Furthermore, it explains why the formation of the boundary fails in the absence of the function of *ap* and Su(H), since in this case no strong difference in expression of target genes will be created.

It appears that very similar strategies are exploited at both compartment boundaries to achieve segregation of the cell lineages. However, in each situation, a set of different but mechanistically similar acting signaling molecules are used to achieve the segregation of cell populations and formation of a compartment boundary.

Acknowledgments

We would like to thank J. Campos-Ortega, A. Martinez-Arias, M. Fortini, G. Struhl, K. Matsuno, P. Lawrence, G. Boekhoff-Falk, F. Matzusaki, S. Artavanis-Tsakonas and F.

Schweisguth for sending stocks and reagents. We thank Markus Kaspar and Sam Mathew for critical comments on the manuscript. Thanks to the unknown referees for helpful comments that improved the manuscript. The work of TK is supported by the Deutsche Forschungs-Gemeinschaft (DFG) through SFB 572.

References

Artavanis-Tsakonas, S., Rand, M.D., Lake, R.J., 1999. Notch signaling: cell fate control and signal integration in development. Science 284, 770–776 (United States).

Barolo, S., Stone, T., Bang, A.G., Posakony, J.W., 2002. Default repression and Notch signalling: hairless acts as an adaptor to recruit the corepressors Groucho and dCtBP to Suppressor of Hairless. Genes Dev. 16, 1964–1976.

Brennan, K., Tateson, R., Lewis, K., Martinez-Arias, A., 1997. A functional analysis of notch mutations in *Drosophila*. Genetics 147, 177–188.

Cohen, B., McGuffin, M.E., Pfeifle, C., Segal, D., Cohen, S.M., 1992. Apterous, a gene required for imaginal disc development in *Drosophila* encodes a member of the LIM family of developmental regulatory proteins. Genes Dev. 6, 715–729.

Dahmann, C., Basler, K., 1999. Compartment boundaries: at the edge of development. Trends Genet. 15, 320–326.

de Celis, J.F., García-Bellido, A., 1994. Roles of the Notch gene in *Drosophila* wing morphogenesis. Mech. Dev. 46, 109–122.

Delanoue, R., Zider, A., Cossard, R., Dutriaux, A., 2002. Interactions between apterous and early expression of *vestigial* in formation of the dorso-ventral compartments in the *Drosophila* wing disc. Genes Cells 7, 1255–1266.

Diaz-Benjumea, F.J., Cohen, S.M., 1995. Serrate signals through Notch to establish a wingless-dependent organizer at the dorsal/ventral compartment boundary of the *Drosophila* wing. Development 121, 4215–4225.

Furriols, M., Bray, S., 2001. A model response element detects Suppressor of Hairless-dependent molecular switch. Curr. Biol. 11, 60–64.

Gho, M., Lecourtois, M., Géraud, G., Posakony, J.W., Schweisguth, F., 1996. Subcellular localization of Suppressor of Hairless in *Drosophila* sense organ cells during Notch signalling. Development 122, 1673–1682.

Go, M.J., Eastman, D.S., Artavanis-Tsakonas, S., 1998. Cell proliferation control by Notch signaling in *Drosophila* development. Development 125, 2031–2040.

Haines, N., Irvine, K.D., 2003. Glycosylation of Notch signalling. Nat. Rev., Mol. Cell Biol. 10, 786–796.

Halder, G., Polaczyk, P., Kraus, M.E., Hudson, A., Kim, J., Laughon, A., Carroll, S., 1998. The Vestigial and Scalloped proteins act together to directly regulate wing-specific gene expression in *Drosophila*. Genes Dev. 12, 3900–3909.

Hori, K., Fostier, M., Ito, M., Fuwa, T.J., Go, M.J., Okano, H., Baron, M., Matsuno, K., 2004. *Drosophila* deltex mediates Suppressor of Hairless-independent and late-endosomal activation of Notch signaling. Development 131, 5527–5537.

Hu, Y., YE, Y., Fortimni, M., 2002. Nicastrin is required for gamma-secretase cleavage of the *Drosophila* Notch receptor. Dev. Cell 1, 69–78.

Kim, J., Sebring, A., Esch, J.J., Kraus, M.E., Vorwerk, K., Magee, J., Carroll, S.B., 1996. Integration of positional signals and regulation of wing formation and identity by *Drosophila* vestigial gene. Nature 382, 133–138.

Kim, J., Johnson, K., Chen, H.J., Carroll, S., Laughon, A., 1997a. *Drosophila* mad binds to DNA and directly mediates activation of vestigial by decapentaplegic. Nature 388, 304–308.

Kim, J., Magee, J., Carroll, S.B., 1997b. Intercompartmental signaling and the regulation of vestigial expression at the dorsoventral boundary of the developing *Drosophila* wing. Cold Spring Harbor Symp. Quant. Biol. 62, 283–291.

Klein, T., 2001. Wing disc development in the fly: the early stages. Curr. Opin. Genet. Dev. 11, 470–475.

Klein, T., 2002. *Kuzbanian* is required cell autonomously during Notch signalling in the *Drosophila* wing. Dev. Genes Evol. 212, 251–255.

Klein, T., Martinez-Arias, A.M., 1998. Interactions among delta, serrate and fringe modulate notch activity during *Drosophila* wing development. Development 125, 2951–2962.

Klein, T., Martinez-Arias, A.M., 1999. The vestigial gene product provides a molecular context for the interpretation of signals during the development of the wing in *Drosophila*. Development 126, 913–925.

Klein, T., Seugnet, L., Haenlin, M., Martinez-Arias, A., 2000. Two different activities of Suppressor of Hairless during wing development in *Drosophila*. Development 127, 3553–3566.

Koelzer, S., Klein, T., 2003. A *Notch* independent function of Suppressor of Hairless during the development of the bristle sensory organ precursor cell of *Drosophila*. Development 130, 1973–1988.

Koelzer, S., Fuss, B., Hoch, M., Klein, T., 2003. The homeobox transcription factor encoded by the *defective ventriculus* (*dve*) gene is required for pattern formation along the proximo-distal axis, cell proliferation and formation of veins in the *Drosophila* wing. Development 130, 4135–4147.

Kopan, R., 2002. Notch: a membrane-bound transcription factor. J. Cell Sci. 115, 1095–1097.

Lai, E.C., 2002. Keeping a good pathway down: transcriptional repression of Notch pathway target genes by CSL proteins. EMBO Rep. 9, 840–845.

Le Borgne, R., Bardin, A., Schweisguth, F., 2005. THe roles of receptor and ligand endocytosis in regulating Notch signaling. Development 132, 1751–1762.

Lecourtois, M., Schweisguth, F., 1995. The neurogenic Suppressor of Hairless DNA-binding protein mediates the transcriptional activation of the enhancer of split complex genes triggered by Notch signaling. Genes Dev. 9, 2598–2608.

Lee, T., Luo, L., 2001. Mosaic analysis with a repressible cell marker (MARCM) for *Drosophila* neural development. Trends Neurosci. 5, 251–254.

Lieber, T., Kidd, S., Young, M., 2002. Kuzbanian-mediated cleavage of Notch. Genes Dev. 16, 209–221.

Matsuno, K., Ito, M., Hori, K., Miyashita, F., Suzuki, S., Kishi, N., Artavanis-Tsakonas, S., Okano, H., 2002. Involvement of a proline rich motif and RING-H2 finger of Deltex in the regulation of Notch-signaling. Development 129, 1049–1159.

Miccheli, C.A., Blair, S.S., 1999. Dorsoventral restriction in wing imaginal discs requires Notch. Nature 401, 473–476.

Milan, M., Cohen, S.M., 1999. Notch signalling is not sufficient to define the affinity boundary between dorsal and ventral compartments. Mol. Cell 4, 1073–1078.

Morel, V., Schweisguth, F., 2000. Repression by Suppressor of Hairless and activation by Notch are required to define a single row of single-minded expressing cells in the *Drosophila* embryo. Genes Dev. 14, 377–388.

Morel, V., Lecoutois, M., Massiani, M., Maier, D., Preiss, A., Schweisguth, F., 2001. Transcriptional repression by Suppressor of Hairless involves the binding of a Hairless-dCtBP complex in *Drosophila*. Curr. Biol. 11, 789–792.

Mumm, J.S., Kopan, R., 2000. Notch signaling: from the outside in. Dev. Biol. 228, 151–165.

Nakagoshi, H., Hoshi, M., Nabeshima, Y., Matsuzaki, F., 1998. A novel homeobox gene mediates the Dpp signal to establish functional specificity within target cells. Genes Dev. 12, 2724–2734.

Neumann, C.J., Cohen, S.M., 1996. Distinct mitogenic and cell fate specification functions of wingless in different regions of the wing. Development 122, 1781–1789.

Neumann, C.J., Cohen, S.M., 1997. Long-range action of Wingless organizes the dorsal-ventral axis of the *Drosophila* wing. Development 124, 871–880.

Rauskolb, C., Irvine, K.D., 1999. Notch-mediated segmentation and growth control of the *Drosophila* leg. Dev. Biol. 210, 339–350.

Sotillos, S., Roch, F., Campuzano, S., 1997. The metalloprotease-disintegrin Kuzbanian participates in Notch activation and patterning of *Drosophila* imaginal discs. Development 124, 4769–4779.

Speicher, S.A., Thomas, U., Hinz, U., Knust, E., 1994. The Serrate locus of *Drosophila* and its role in morphogenesis of the wing imaginal discs: control of cell proliferation. Development 120, 535–544.

St. Pierre, S., Galindo, M.I., Couso, J.P., Thor, S., 2002. Control of *Drosophila* imaginal disc development by rotund and roughened eye: differentially expressed transcripts of the same gene encoding functionally distinct zinc finger proteins. Development 129, 1273–1281.

Struhl, G., Greenwald, I., 1999. Presenilin is required for activity and nuclear access of Notch in *Drosophila* [see comments]. Nature 398, 522–525.

Wilder, E.L., Perrimon, N., 1995. Dual functions of wingless in the *Drosophila* leg imaginal disc. Development 121, 477–488.

Williams, J.A., Bell, J.B., Carroll, S.B., 1991. Control of *Drosophila* wing and haltere development by the nuclear vestigial gene product. Genes Dev. 5, 2481–2495.

Williams, J.A., Paddock, S.W., Vorwerk, K., Carroll, S.B., 1994. Organization of wing formation and induction of a wing-patterning gene at the dorsal/ventral compartment boundary. Nature 368, 299–305.

Ye, Y., Lukinova, N., Fortini, M.E., 1999. Neurogenic phenotypes and altered Notch processing in *Drosophila* Presenilin mutants [see comments]. Nature 398, 525–529.

Yeh, E., Gustafson, K., Boulianne, G.L., 1995. Green fluorescent protein as a vital marker and reporter of gene expression in *Drosophila*. Proc. Natl. Acad. Sci. U. S. A. 92, 7036–7040.

Zecchini, V., Brennan, K., Martinez-Arias, A., 1999. An activity of Notch regulates JNK signalling and affects dorsal closure in *Drosophila*. Curr. Biol. 9, 460–469.