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

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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 of 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.

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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.
(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 Ubiquitin 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 (vgBE). 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 (Micheli 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 corepressor 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 (Koezler 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 (vgBE). 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)-447 P(B)FRT40A (Morel and Schweisguth, 2000), PsnF3 (Struhl and Greenwald, 1999), PsnF3 (Ye et al., 1999), ntc57 (Hu et al., 2002), kuz2106, kuz2106 (Soilhes et al., 1997), Dv68h8/FRT101 (Brennan et al., 1997); apL5031 and ap-lacZ (ap8206) (Cohen et al., 1992), Su(H)59F and F311 (Lecours and Schweisguth, 1995); vg8387 and the vgBE (Williams et al., 1994).
UAS stocks: UASvg (Kim et al., 1996); UAS GFP (Yeh et al., 1995). UAS H (Go et al., 1998), UAS Su(H) (Klein et al., 2000), UASdx (Matsumo et al., 2002).


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 tubGal4 UAS GFP; FRT 40A tubGal 80 were crossed with w; FRT 40A; UASdx/Tm6b males. To generate cell clones that are mutant for Su(H)d47 and express UASdx, females of the y w hsFlp tubGal4 UASGFP; FRT 40A Gal80 genotype were crossed against w; FRT40A Su(H)d47; UASdx 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)SP78 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 g-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 as the wing pouch. (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 PsnC1 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(ty)d47 mutants (arrowhead in E). The phenotype resembles that of a hypomorphic allelic combination of kuz, kuz1403/kuz1403, 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-hypomorphic 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 PsnC1 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 nicA7 mutant wing discs. However, it can be detected in a kuz-hypomorphic (kuz1405/kuz1405) 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 \( \text{Notch} \) pathway causes the “weak” phenotype of the \( kaz \)-hypomorph 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 \( \text{Notch} \) pathway. Instead, it is caused by the loss of a function of \( Su(H) \) that is independent of the \( \text{Notch} \) pathway.

In the absence of \( \text{Notch} \) 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 \( \text{Notch} \)-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 \( \text{Notch} \) 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 \( \text{vestigial} (vg) \) 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

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Fig. 3. The phenotype of \( Su(H) \) mutants is different from other mutants of other genes involved in the \( \text{Notch} \) pathway because of the loss of a second, \( \text{Notch} \)-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 \( kaz^{1403}; kaz^{1403} \) and \( Psn^{C1} \), indicating that residual activity of the \( \text{Notch} \) pathway was responsible for the expression of both genes in the hypomorphic \( kaz \)-mutant situation. (B, C) Expression of \( Wg/Dve \) (B) and of \( Wg/Dll \) (C) in \( Su(H)^{ex} ; Psn^{C1} \)-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 \( Su(H)^{ex} ; nic^{C1} \)-double mutants (D). These observations suggest that the weaker phenotype of \( Su(H) \) mutants is not caused by a residual activity of the \( \text{Notch} \) pathway but because of the loss of a \( \text{Notch} \)-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.
found that this is the case: forced expression of UASvg in Psn mutant discs with ptcGal4 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. ptcGal4 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 UASvg 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 (vgBE) (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 vgBE 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 vgBE 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 vgBE in H; Psn-double mutant discs and indeed found that the vgBE 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 vgBE 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 vgBE, whose expression cannot be activated by the Notch pathway, because its Su(H)-binding site is mutated (vgBE-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
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...
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 a hypomorphic apGal4/ apUG035 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 vgnull (Delanoue et al., 2002). However, when we generated a vg loss of function situation...
using \(vg^{null}\) in combination with another loss of function allele, \(vg^{83b,27R}\), 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 \(Su(H)\) function. (G–L) Su(H) is involved in the formation of the boundary. (G–K) 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, overexpression 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 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 \(vg\)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.
In order to evaluate these possible explanations, we performed two types of experiments. Similar to Hori et al. (2004), we expressed UAS\(\text{vg}\) with dppGal4 in the first experiment and induced \(\text{Su(H)}\) mutant cell clones. As a measure for the activity of the Notch pathway, we monitored the expression of the \(\text{vgBE}\) as well as Wg in the \(\text{Su(H)}\) mutant territories. In contrast to Hori et al. (2004), we found a clear reduction in or abolition of the expression of the \(\text{vgBE}\) in most \(\text{Su(H)}\) 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 \(\text{vgBE}\) 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 \(\text{Su(H)}\) 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 \(\text{Su(H)}\) mutant clones that concomitantly express UAS\(\text{dx}\). 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 \(\text{vgBE}\) and to a lesser degree that of Wg were induced in control clones that only expressed UAS\(\text{dx}\) (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 \(\text{Su(H)}\) (Figs. 8G–I). In the \(\text{Su(H)}\) mutant cell clones, expression of UAS\(\text{dx}\) was unable to induce cell proliferation or ectopic expression of wg and the \(\text{vgBE}\). 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 \(\text{dx}\) (Figs. 8G–I). In contrast, in clones located in domain 2, we sometimes found only a reduction of the expression of the \(\text{vgBE}\) (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 \(\text{Su(H)}\) but did not express UAS\(\text{dx}\) (Figs. 8J–L). This indicates that the expression of the \(\text{vgBE}\) in domain 2 is not absolutely dependent on \(\text{Su(H)}\) function. Thus, the residual expression of the \(\text{vgBE}\) observed in \(\text{Su(H)}\) mutant cells in this domain is not caused by a \(\text{Su(H)}\)-independent activity of Dx. Since our analysis indicates that outside domain 2 the activity of the \(\text{vgBE}\) is clearly dependent on \(\text{Su(H)}\), irrespective of the presence or absence of over-expression of Dx, we conclude that Dx does not mediate a \(\text{Su(H)}\)-independent signal.

**Discussion**

In this work, we provide an answer to the observation that the patterning defects of \(\text{Su(H)}\) 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 \(\text{Su(H)}\) 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 \(\text{Su(H)}\) 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 \(\text{Su(H)}\) 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 \(\text{Su(H)}\) 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 \(\text{Su(H)}\) 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 \(\text{Su(H)}\) function is lost.
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 Pen, 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 vgBE (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 Micheli 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.

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