Cell proliferation control by Notch signaling in *Drosophila* development

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**SUMMARY**

The Notch receptor mediates cell interactions controlling the developmental fate of a broad spectrum of undifferentiated cells. By modulating Notch signaling in specific precursor cells during *Drosophila* imaginal disc development, we demonstrate that Notch activity can influence cell proliferation. The activation of the Notch receptor in the wing disc induces the expression of the wing margin patterning genes *vestigial* and *wingless*, and strong mitotic activity.

However, the effect of Notch signaling on cell proliferation is not the simple consequence of the upregulation of either *vestigial* or *wingless*. Vestigial and Wingless, on the contrary, display synergistic effects with Notch signaling, resulting in the stimulation of cell proliferation in imaginal discs.

Key words: Notch signaling, *vestigial*, *wingless*, *Drosophila*, Wing development, Cell proliferation, UAS-GAL4 system

**INTRODUCTION**

Multicellular development depends on the coordinate implementation of cellular differentiation and proliferation programs. However, little is known about how cell fate controlling mechanisms link differentiation to proliferation. The Notch signaling pathway defines an evolutionarily conserved cell interaction mechanism that controls cell fate choice decisions in a very broad spectrum of precursor cells throughout development (reviewed in Artavanis-Tsakonas et al., 1995). We describe experiments aimed to modulate Notch signaling activity in specific precursor cells during *Drosophila* imaginal disc development in an attempt to examine possible links between differentiation and proliferation cues mediated by Notch.

Although the biochemical nature of Notch signaling is not well defined, several elements of the pathway have been identified through genetic and molecular analyses (reviewed in Artavanis-Tsakonas et al., 1995). *Delta* (*Dl*) and *Suppressor of Hairless* (*Soh*) encode a transcription factor that acts as a downstream effector of Notch signaling (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). *Hairless* (*Hl*) encodes a negative regulator of Notch signaling, which is thought to act through direct association with *Su(H)* (Baily and Posakony, 1995; Bang et al., 1995; Lyman and Yedvodnick, 1995). Recent studies suggest that *Su(H)* may not be the only effector of Notch signaling (Lecourtois and Schweisguth, 1995; Shawber et al., 1996; Matsuno et al., 1997; Wang et al., 1997). Developmentally, Notch appears to control local cell interactions in a general way. Cell interaction events resulting in the segregation of precursors into distinct cell types, are often formally distinguished either as lateral inhibition/specification or as inductive signaling. Extensive studies have implicated Notch in both the mechanisms that control the transition of a precursor cell to a more differentiated cell (reviewed in Simpson, 1994; Artavanis-Tsakonas et al., 1995).

Here, we examine the consequences of modulating Notch activity during imaginal disc development, especially in wing morphogenesis, using the UAS-GAL4 system (Brand and Perrimon, 1993). We explore the relationship between Notch signaling and the wing margin patterning genes *vestigial* (*vg*) and *wingless* (*wg*), whose expression depends on Notch activation (Couso et al., 1995; Diaz-Benjumea and Cohen, 1995; Kim et al., 1995, 1996; Rulifson and Blair, 1995; de Celis et al., 1996; Doherty et al., 1996). We find that Notch activity not only controls cell differentiation, but also can influence cell proliferation.

Activation of Notch signaling induces strong mitotic activity in the wing disc in a *Su(H)*-dependent manner. We present evidence indicating that the effect of Notch signaling on cell proliferation is indirect and is not the simple consequence of either *vg* or *wg* induction. In fact, we find misexpression of *Vg* in the wing pouch results in small wing discs and loss of *wg* expression, a phenotype opposite to that associated with activation of the Notch receptor. However, we demonstrate that either *Vg* or *Wg* display synergistic effects with Notch signaling affecting cell proliferation during wing morphogenesis.

**MATERIALS AND METHODS**

**Fly strains**

Fly cultures and crosses were performed according to standard procedures at 25°C unless otherwise noted. All mutant fly strains in this study are described in Lindsley and Zimm (1992).
For misexpression experiments, we used UAS-Dl (Doherty et al., 1996), UAS-Per (Speicher et al., 1994), UAS-Vg (Kim et al., 1996) and UAS-Wg (Lawrence et al., 1995), which were kindly provided by Y. N. Jan, E. Knust, S. Carroll and S. Cohen, respectively.

The GAL4-driven strains used in this study were kindly provided by N. Perrimon (71B, 30A), N. Perrimon and J. Urban (T113), S. Cohen (decapentaplegic [dpp]-GAL4), T. Xu (patched [ptc]-GAL4), S. Varadarajan and K. V. Raghavan (vg-GAL4), K. Wharton and D. Brower (766 and 719a), and K. Wharton and C. S. Goodman (A9).

The vg-GAL4 injected with the helper plasmid described by Spradling (1986). The constructs (1 mg/ml) were from late third instar larvae in PBS, fixed in 4% paraformaldehyde in limited), and the video images were assembled in Adobe Photoshop.

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RESULTS

To explore the consequences of Notch signaling modulation during Drosophila development, we have used the UAS-GAL4 system (Brand and Perrimon, 1993). Loss-of-function phenotypes were elicited through the expression of either a truncated, dominant negative form of the Notch receptor (d.n.N) lacking the intracellular domain (see Materials and Methods section; Rebay et al., 1993), or the H protein, a negative regulator of Notch signaling (Bang and Posakony, 1992; Maier et al., 1992; Brou et al., 1994; Schweisguth and Posakony, 1994; Bang et al., 1995; Bailey and Posakony, 1995; Lyman and Yedwodnich, 1995). Gain-of-function phenotypes were induced by expressing a constitutively activated form of the Notch receptor (act.N) (see Materials and Methods section; Fortini et al., 1993; Lieber et al., 1993; Struhl et al., 1993).

S2 cell experiments and β-galactosidase assays

Drosophila S2 cells were cultured in complete M3 media (Sigma) containing 12.5% FCS (Gibco) at 25°C. S2 cells were transfected using lipofectin (Gibco-BRL) as previously described (Diedrich et al., 1994). Briefly, S2 cells were transfected with 2 mg of p12xFu(H)bs-lacZ alone, or co-transfected with 2 mg of Su(H) or/and act.N (ICN) under the control of the inducible metallothionien promoter, and β-galactosidase (β-gal) activity was assayed as described (Maniatis et al., 1989). Units of β-gal activity were determined as the OD420 spectrophotometric reading divided by the protein concentration.

Adult flies were processed for scanning electron microscopy (SEM) by hydration in an ethanol series, followed by critical point drying and mounting on stubs, and viewed on an ISI-SS40.

Su(H)-dependent Notch activity is suppressed by H in vivo

To examine the link between the H misexpression phenotypes and Su(H)-dependent Notch activity, we generated transgenic animals carrying a lacZ reporter construct driven by the fusion between multimerized Su(H)-binding sites and an E(spl)m7 promoter, a known Su(H) target (Fig. 1A). In contrast to the constructs described in Bailey and Posakony (1995), this construct consists almost exclusively of engineered Su(H)-binding sites. In the cell culture based reporter assay developed by Eastman et al. (1997), the expression from the reporter construct was induced by the simultaneous expression of Su(H) and act.N, while the expression of any one construct alone failed to induce transcription (Fig. 1B).

Fig. 1C shows the expression pattern of the same reporter construct in vivo. Strong lacZ expression was detected in the posterior part of the eye disc of late third instar transgenic larva (Fig. 1C, arrow). This expression was effectively suppressed by misexpression of H using the GAL4 line T113 (see Fig. 6C for the expression pattern of T113 in the eye disc) and resulted in small eye discs (Fig. 1D, arrow), indicating that H overexpression can suppress Su(H)-dependent Notch signaling in vivo.

The adult eye phenotype associated with misexpression of H using the GAL4 line T113 is shown in Fig. 1F. Consistent with the above observation, the size of the eye was significantly affected and, in extreme cases, the eye was missing (Fig. 1F). Fig. 2A shows another example of the phenotypes induced by misexpression of H with the GAL4 line T113. In addition to

Constructs and germline transformation

The act.N construct (ICN) in this study is described as Notch nucl in Fortini et al. (1993), in which the extracellular domain including the signal peptide and transmembrane domain is deleted. The d.n.N construct in this study is described as ECN in Rebay et al. (1993), in which the extracellular domain including the signal peptide and transmembrane domain is deleted. The d.n.N constructs were kindly provided by A. Preiss. All three constructs were cloned into pCaspAUG-gal.

For the construction of p12xFu(H)bs-lacZ, the PCR product, which contained the mTF basal promoter as well as 2 Su(H) binding sites (Eastman et al., 1997), was cloned into the pBluescript vector to obtain pmP12×bs. In order to obtain pmP12×bs, the 10×Su(H) binding site oligonucleotide, which had been generated by annealing 10 tandem Su(H) consensus binding sites, (1) AATTTGACCTACGTTGGAACGGAAGCGTGGAGAAGCTTGGAACGAGGACTGAGCAAGGCTGGAACGACGTGGAGAAGCTTGGAACGAGGACTGAGCAAGGCTGGAACGACGT

Histochemistry and histology

Imaginal discs and central nervous systems (CNSs) were dissected from late third instar larvae in PBS, fixed in 4% paraformaldehyde in PBS for 20 minutes on ice and washed in PBS. For X-gal staining, the reaction was developed in 3 mM K4[Fe(CN)6], 3 mM K3[Fe(CN)6], 1 mM MgCl2, 150 mM NaCl and 0.25% X-gal in phosphate buffer (pH 7.2) containing 0.1% Saponin. For immunohistochemistry, the tissues were blocked with 0.1% bovine serum albumin (BSA), 1% normal goat serum and washed with PBS containing 0.1% Saponin. The incubation for primary antibodies was done overnight at 4°C and for 2 hours at room temperature for secondary antibodies. The rabbit polyclonal antibody to the Wg protein (a generous gift from R. Nusse) was used at 1/100 dilution, and the mouse monoclonal antibody to 5-bromo-2’-deoxyuridine (BrdU) (Amersham or Becton Dickinson) at 1/50 dilution. For HRP staining, the reaction was developed in 0.5 mg/ml DAB in phosphate buffer (pH 7.2) containing 0.06% H2O2. BrdU incorporation was done in essentially the same way as described in de Nool and Hariharan (1995).

Adult fly wings were removed and mounted in Aquamount (BDH Limited), and the video images were assembled in Adobe Photoshop.
small eyes, we observed small wings and halteres as well as more typical Notch loss-of-function phenotypes, such as extra thoracic bristles. The ‘small eye’ phenotype induced by H expression was not associated with severe eye roughness (Fig. 2A). This ‘small eye’ phenotype, together with the wing and haltere abnormalities, is reminiscent of Ser loss-of-function mutations (Speicher et al., 1994).

To further explore the possibility that the observed eye phenotype reflects Ser-dependent Notch signaling, we examined the genetic interactions with Beaded of Goldschmidt (BdG), a dominant negative mutation of Ser known to affect wing margin development (Hukriede and Fleming, 1997). A weak UAS-H line driven by the GAL4 line T113 did not display visible phenotypes (Fig. 2B). However, in combination with BdG, strong synergistic effects were observed displaying phenotypes characteristic of Ser, such as small eyes, wings and halteres (Fig. 2C, arrows). Therefore, H misexpression can mimic Ser loss-of-function mutations, raising the possibility that Ser/Notch signaling may control eye morphogenesis. This notion is also consistent with the finding that misexpression of a dominant negative, secreted form of Ser can produce a similar effect (Sun and Artavanis-Tsakonas, 1997). Misexpression of d.n.N with the T113 driver also showed a similar effect (data not shown). In contrast, misexpression of act.N with T113 resulted in slightly enlarged eyes (data not shown).

**Modulation of Notch activity during wing morphogenesis**

To further investigate the role Notch signaling plays in morphogenesis, we expressed our transgenes at the d/v compartment boundary of the wing disc using the vg-GAL4 driver. Misexpression of either H or d.n.N resulted in similar phenotypes, which ranged from wing margin notches to rudimentary wings (Fig. 3B,C). The effect of H misexpression can be suppressed by expressing act.N and vice versa (Baily and Posakony, 1995; Lyman and Yedvodnick, 1995). For example, the lethality associated with misexpression of act.N is suppressed by simultaneous expression of H. Conversely, the phenotypes elicited by H misexpression were largely suppressed by act.N (Fig. 3D). This mutual suppression was observed with other GAL4 lines as well. Given that the actions of act.N and H seem to be manifested through Su(H) (Brou et al., 1994; Fortini and Artavanis-Tsakonas, 1994; Bailey and Posakony, 1995; Jarriault et al., 1995; Lecourtois and Schweisguth, 1995; Tamura et al., 1995), it is likely that the mutual suppression of act.N and H is also mediated by Su(H). It is noteworthy that, even though both H and d.n.N act as antagonists of Notch signaling and the phenotypes associated with their expression are similar, their interactions with act.N are different. While act.N is an effective suppressor of the phenotypes induced by H misexpression, we found that it fails to suppress the effects of d.n.N.
We further examined the relationship between Notch signaling and the expression of \( \text{vg} \) and \( \text{wg} \), since the induction of both genes is considered to be essential for wing morphogenesis (reviewed in Brook et al., 1996; Cohen, 1996). Consistent with previous reports (Rulifson and Blair, 1995; Kim et al., 1996), when either d.n.N or H was misexpressed along the anterior/posterior (a/p) boundary using the \( \text{ptc-GAL4} \) line, expression from the \( \text{vg} \) d/v boundary enhancer, as well as the \( \text{wg} \) enhancer, was effectively repressed near the intersection between the a/p and d/v boundaries (arrow in Fig. 3E for \( \text{vg} \), and data not shown for \( \text{wg} \)). In contrast, the \( \text{vg} \) quadrant enhancer, which is normally silent at the intersection between a/p and d/v boundaries, was induced by the identical constructs (arrow in Fig. 3F). Essentially the opposite effect was observed when act.N was misexpressed (data not shown; see also Fig. 5A,E), demonstrating that Notch signaling has the opposite effects on two distinct enhancers of \( \text{vg} \) (see also Kim et al., 1996).

**Notch signaling induces mitotic activity in the wing disc**

The wing phenotypes elicited by misexpression of act.N were similar to those induced by \( \text{Abruptex} \) (Ax) mutations, which are Notch gain-of-function alleles associated with point mutations in the extracellular domain of the protein (Hartley et al., 1987; Kelly et al., 1987). In this study, we used the heteroallelic combination of two Ax alleles, Ax\text{16} and Ax\text{28} (Foster, 1975; Portin, 1975). Consistent with the notion that this heteroallelic Ax combination results in the activation of Notch signaling, the expression of Notch downstream genes was induced. For instance, we found ectopic \( \text{wg} \) expression around the d/v boundary, in agreement with a previous report (data not shown; de Celis et al., 1996). We also observed induction of the \( \text{vg} \) d/v boundary enhancer (Fig. 3G) and repression of the \( \text{vg} \) quadrant enhancer around the d/v boundary (Fig. 3H), similar to the effect of expression of act.N.

Activation of Notch signaling around the d/v boundary of the wing disc through either misexpression of act.N (Fig. 4B) or the Ax mutations (Fig. 3G,H) resulted in a substantial enlargement of the disc. BrdU incorporation experiments indicate that these phenotypes are associated with an elevated mitotic activity. Fig. 4I shows the wild-type BrdU incorporation pattern where incorporation is relatively uniform except in the area around the d/v boundary, which displays lower mitotic activity (O’Brochta and Bryant, 1985; Schubiger and Palka, 1987). Fig. 4C and D show the BrdU incorporation in the discs expressing act.N along the d/v and a/p boundaries, respectively, while Fig. 4J shows an Ax\text{16}/Ax\text{28} disc. In all cases, BrdU incorporation is stimulated and was particularly obvious in the peripheral region of the wing pouch, suggesting that the periphery was more responsive than other regions.

Misexpression of act.N in other parts of the wing disc also resulted in the stimulation of mitotic activity. Fig. 4E shows the mitotic pattern when act.N was expressed in the wing pouch (\( \text{GAL4} \) line 71B). The disc has grown in such a way that the characteristic folded structures of the wing pouch are pushed to the periphery (arrows in Fig. 4E). Conversely, Fig. 4F (arrows) shows the same structures ‘pushed’ toward the d/v boundary when act.N was expressed in the periphery (\( \text{GAL4} \) line 30A). When act.N was misexpressed in a discrete pattern in the periphery using the \( \text{GAL4} \) line 766 (Fig. 4G), we observed a regional correspondence between Notch signaling activation and high mitotic activity, demonstrating a local effect of Notch activity on cell proliferation in the periphery (Fig. 4H). However, as is particularly evident when the Notch receptor is activated around the d/v boundary (Fig. 4C,J), the region of Notch signaling activation does not coincide with the region of the highest mitotic activity. We therefore conclude that the effect of Notch signaling on cell proliferation must be indirect.

As shown above, the effects of act.N misexpression and the Ax mutations on cell proliferation are similar. Since the Ax alleles are thought, on the basis of genetic evidence (Heitzler and Simpson, 1993), to act in a ligand-dependent fashion, it was likely that the effects of the Notch receptor activation by its ligands DI and Ser on mitosis would be similar as well. Indeed when either DI or Ser was misexpressed along the a/p boundary (\( \text{dpp-GAL4} \) or \( \text{ptc-GAL4} \)), the wing pouch was enlarged and cells in the peripheral region of the wing pouch were actively dividing (Fig. 4K for DI, and data not shown for Ser), similar to the observations described above. In contrast, when \( \text{Wg} \) was expressed along the a/p boundary, the notum and hinge regions were substantially enlarged instead of the wing pouch (Fig. 4L). This indicates that the effect of Notch signaling activation on cell proliferation is not the simple consequence of \( \text{wg} \) induction.
The effect of Notch activity on cell proliferation is not the simple consequence of Vg induction

Since Vg is a direct target of Su(H)-dependent Notch signaling (Kim et al., 1996), it is possible that the mitogenic effect of Notch is mediated by the upregulation of Vg. In this case, misexpression of Vg would be expected to result in phenotypes similar to those elicited by act.N.

Misexpression of act.N in the dorsal side of the wing pouch, using the GAL4 line A9, induced expression from the Vg d/v boundary enhancer (Fig. 5A) as well as the wg enhancer (data not shown; Neumann and Cohen, 1996b). The dorsal side of the wing pouch region appeared enlarged (Fig. 5A, arrow). In contrast, when Vg was misexpressed in the same region, as shown in Fig. 5B, the dorsal side of the wing pouch became much smaller than the ventral side, while wg expression in the periphery of the dorsal side was suppressed (Fig. 5B, arrow). The loss of dorsal wing pouch induced by Vg misexpression was significantly rescued by expressing Wg simultaneously (Fig. 5C). This is consistent with the notion that the observed phenotype caused by misexpression of Vg is due to the repression of wg, whose expression in the wing pouch is more uniform at earlier stages (Couso et al., 1993; Phillips and Whittle, 1993; Williams et al., 1993; Ng et al., 1996). Fig. 5D shows that misexpression of Wg alone in the dorsal side, unlike the misexpression of act.N, does not have a significant effect on cell proliferation in the wing pouch (see also Neumann and Cohen, 1996a).

These results indicate that the effect of act.N expression on mitosis is separable from Vg induction. In addition, they indicate that Vg is capable of repressing wg expression in the wing pouch, but not at the d/v boundary. We further explored this relationship using other GAL4 lines to drive Vg expression in the wing disc. When act.N was misexpressed along the a/p boundary using dpp-GAL4, expression from both the Vg d/v boundary (Fig. 5E) and the wg (data not shown) enhancers was upregulated. On the contrary, when Vg was expressed along the a/p boundary using the same dpp-GAL4 driver, wg expression in the peripheral region was suppressed while the expression at the d/v boundary remained unaffected (arrows in Fig. 5F).

Consistent with the notion that Vg is capable of downregulating wg outside the d/v boundary, we were able to induce small wing disc phenotypes associated with the loss of wg expression in the peripheral region, simply by misexpressing the Vg protein in the wing pouch (GAL4 line 719a or T113) (Fig. 5G,H). The effect of Vg misexpression in the wing pouch thus appears to be the opposite to that of Notch signaling activation.

Synergism between Notch and Vg on wg expression and cell proliferation

To further study the effects of Notch signaling activation or Vg misexpression on cell proliferation, we expressed act.N or Vg in other discs by using two GAL4 lines, T113 and dpp-GAL4. Both lines allow expression of the transgenes in imaginal discs in different expression patterns. The expression pattern of T113 in the eye disc of late third instar larvae is shown in Fig. 6C. Unlike dpp-GAL4, in which GAL4 is expressed posterior to the morphogenetic furrow, GAL4 is also expressed in cells anterior to the furrow in the T113 line (see Kim et al., 1996 for dpp-GAL4 expression).

When act.N was expressed with the T113 driver line, the
wing discs were enlarged and accompanied by \( \text{vg} \) and \( \text{wg} \) induction (data not shown). The effect on the eye disc was relatively small but the leg discs also became noticeably abnormal and were associated with \( \text{wg} \) induction (arrow in Fig. 6A). It is noteworthy that expression of act.N in the leg discs was found to induce \( \text{wg} \) but not \( \text{vg} \) (data not shown), demonstrating that the induction of \( \text{wg} \) and \( \text{vg} \) by Notch signaling can be separated depending on the developmental context.

We have shown that misexpression of \( \text{Vg} \) compared to act.N has opposite effects in the wing disc. Thus, \( \text{Vg} \) misexpression in the wing disc induces \( \text{wg} \) downregulation and small disc. In contrast, misexpression of \( \text{Vg} \) in the eye discs upregulates \( \text{wg} \) and results in a clear enlargement of the discs (arrows in Fig. 6B), demonstrating \( \text{Vg} \) can either repress or induce \( \text{wg} \) expression in a context-dependent manner.

The observed context-dependent effect of \( \text{Vg} \) on \( \text{wg} \) expression raised the possibility that Notch signaling may be capable of modulating the way \( \text{Vg} \) affects \( \text{wg} \) expression. This was of particular interest in view of the possibility that \( \text{Vg} \) does not suppress \( \text{wg} \) expression at the d/v boundary because of the existing high level of Notch signaling activity. In fact, the simultaneous expression of act.N and \( \text{Vg} \) revealed a striking synergistic effect on cell proliferation. Fig. 6D,E show the effect of simultaneous expression of act.N and \( \text{Vg} \) using the T113 line. Most notable were the eye discs where tissue expressing the two proteins showed striking overgrowth associated with strong \( \text{wg} \) induction. The other discs were also clearly affected displaying cellular overgrowth, but the effects were far less dramatic than the eye discs (data not shown). This overgrowth phenotype was also evident when act.N and \( \text{Vg} \) misexpression was driven by \( \text{dpp-GAL4} \) (data not shown), even though the synergistic effect was less dramatic. In contrast, the effect of misexpression of \( \text{Vg} \) with \( \text{dpp-GAL4} \) on \( \text{wg} \) induction and cell proliferation in the eye discs was, in some cases, significantly suppressed by simultaneous expression of \( \text{H} \) (data not shown). Although the degree of this suppression was variable, it appeared significant considering misexpression of \( \text{Vg} \) alone never failed to show overgrowth of the eye discs. We also note that misexpression of \( \text{H} \) with \( \text{dpp-GAL4} \) alone has no visible effect on the eye development.

As described above, when \( \text{Wg} \) alone was expressed with \( \text{dpp-GAL4} \), the wing disc displayed a weak and distinct overgrowth in the wing disc (Fig. 4L). We found the eye discs to be also enlarged (data not shown). The simultaneous expression of \( \text{Wg} \) and \( \text{Vg} \) failed to show any synergistic effects on cell proliferation either in the eye or wing disc. However, when \( \text{Wg} \) was expressed together with act.N, there was a clear synergistic effect on cell proliferation in the imaginal discs, although the effect was weaker than that resulting from the synergy between \( \text{Vg} \) and act.N (data not shown).

These experiments demonstrate that the proliferative
potential of certain tissues can be modulated by the synergistic action of Notch with other genes. Moreover they identify Notch signaling as an important factor in the way Vg affects wg expression and cell proliferation at the d/v boundary during wing morphogenesis (Fig. 7).

DISCUSSION

Numerous studies have lead to the notion that the Notch signaling pathway defines a fundamental mechanism that controls a common step in the progression of a precursor cell to the next developmental stage (reviewed in Artavanis-Tsakonas et al., 1995; Fleming et al., 1997). Expression and phenotypic analyses of genes integrated in the Notch pathway have demonstrated that the action of this signaling mechanism is highly pleiotropic, affecting cell fates in a broad spectrum of tissues. The experiments that we described here show that, in addition to controlling cell fates, Notch signaling can also affect cell proliferation.

Early expression studies in Drosophila have pointed to a correlation between mitotically active cells and the expression of the Notch transcript (Markopoulou and Artavanis-Tsakonas, 1989). However, not all mitotic cells express the Notch protein and vice versa (Fehon et al., 1991). In this

Fig. 5. Effect of Vg on wg expression in the wing pouch. X-gal staining of lacZ reporter lines for vg (A,E) and wg (B-D, F-H) in the wing discs. The act.N protein (A), Vg (B), both Vg and Wg (C) and Wg (D) were misexpressed in the dorsal wing pouch using GAL4 line A9. (A) Expression from the vg d/v boundary enhancer was induced in the dorsal pouch. The expansion of the dorsal wing pouch is obvious compared with the ventral pouch because of cell proliferation (white arrow). (B) The dorsal wing pouch and wg expression in the dorsal hinge basically disappeared (white arrow). (C) The small dorsal wing pouch phenotype by Vg misexpression was significantly suppressed by simultaneous expression of Wg. (D) Misexpression of Wg alone did not show a significant effect on cell proliferation in the wing pouch. (E) The act.N protein was misexpressed along the a/p boundary with dpp-GAL4. Expression from the vg d/v enhancer was induced along the a/p boundary. (F-H) Vg was misexpressed in various patterns, such as along the a/p boundary (with dpp-GAL4 in F) and in the pouch (with 719a in G and with T113 in H). (F) Expression from the wg enhancer in the periphery around the a/p boundary (arrows) was repressed, whereas the expression at the d/v boundary remained unaffected. (G,H) Small wing disc phenotypes associated with the loss of wg expression in the periphery were observed. Essentially the same results were obtained by using antibody to the Wg protein (data not shown). Anterior is at left, dorsal is at top.

Fig. 6. Synergistic effects of Notch and Vg on cell proliferation. (A,B) X-gal staining of lacZ reporter line for wg in CNSs. The act.N protein (A) and Vg (B) were misexpressed using GAL4 line T113. (A) Significant overproliferation in the leg discs, associated with wg induction, was observed (arrow), although the effect on the eye disc was relatively small. (B) Strong effect of Vg expression on cell proliferation in the eye disc, associated with wg induction, was observed (arrows). (C) X-gal staining of GAL4 expression for T113 in the eye disc. Note GAL4 is expressed in cells both anterior and posterior to the morphogenetic furrow (arrow). (D,E) Antibody staining for the Wg protein. Both act.N and Vg were misexpressed simultaneously with T113. In some cases, dramatic synergistic effects on cell proliferation associated with wg induction, especially in the eye disc, was observed. E is a high magnification of D. Note that the eye disc appeared to have continued growing in an uncoordinated manner. Note the size of the CNSs in A, B, D is basically the same, which serves as an internal control.

Fig. 7. Schematic illustration of the proposed Notch signaling activity during wing morphogenesis. Events at the d/v boundary of the wing disc are shown on the right. The Notch receptor (N), which is activated by the ligands Dl and Ser, induces the expression of both vg (vg) and wg (wg; top two horizontal arrows). The Vg protein induces or maintains wg expression at the d/v boundary in the presence of high Notch signaling activity. Notch seems capable of inducing wg expression independent of Vg as well. Finally Notch (bottom arrow) is involved in additional inductive events including, for example, the induction of cut. In the wing pouch outside the d/v boundary, Vg represses wg expression. Note that Wg at the d/v boundary is responsible for vg expression in the wing pouch. See text for details.

In the study, the expression of a truncated constitutively activated form of the Notch receptor clearly links Notch signaling to the induction of mitotic activity in imaginal discs. This link is maintained when the Notch receptor is activated in a ligand-dependent fashion. The effect on cell proliferation can be suppressed by expressing H; it thus seems that Notch affects cell proliferation through the effector Su(H).

Since the region displaying the highest Notch activity does not correspond to the region of the highest mitotic activity (see Fig. 4C,J), we conclude that the effect of Notch signaling on cell proliferation is indirect. This apparent cell non-autonomous effect is also consistent with the findings of de Celis and Bray (1997), which demonstrated that cell outgrowths induced by misexpression of act.N in clonal patches are mixtures of cells expressing act.N and wild-type cells. It is also evident that the ability of Notch to stimulate mitotic activity in a particular cell depends on the developmental context. For instance, cells in the peripheral region of the wing pouch can be stimulated by Notch to enter mitosis more readily than cells in other regions. This is also worth pointing out that Wg may be required to make a zone of non-proliferating cells around the d/v boundary quiescent, implying that Wg, in this context, is a negative regulator of cell proliferation (Phillips and Whittle, 1993). This could explain seemingly less-proliferating cells around the d/v boundary where wg is induced by Notch signaling activation (Fig. 4C,J). We also note that, even though the GAL4 drivers that we have used do express the transgenes in the CNS (e.g. Fig. 4), Notch signaling activation did not elicit a detectable proliferative response in the cells of the CNS.

By inducing wing margin patterning genes such as vg and wg, Notch signaling plays a central role in the formation of the d/v boundary, which serves as an organizing center for wing morphogenesis (reviewed in Brook et al., 1996; Irvine and Vogt, 1997). The subsequent control of vg expression in the wing pouch from both the d/v and a/p boundaries is considered to be a key step in wing morphogenesis, in which the growth-controlling activity of the two patterning systems is integrated (Cohen, 1996). Although it seems that the induction of vg and wg is, at least partly, responsible for the effect of Notch signaling activation on cell proliferation, we demonstrated that this effect cannot be explained simply by the induction of either vg or wg.

The effect of Notch signaling activation on cell proliferation in the wing disc is quite distinct from the phenotype associated with the misexpression of Wg. In terms of proliferation, cells in the notum and hinge regions are much more responsive to wg signaling activation than cells in the wing pouch (Fig. 4L) (Neumann and Cohen, 1996a; de Celis and Bray, 1997). Moreover misexpression of Wg along the a/p boundary in the wing disc leads to wing duplication instead of the significant overproliferation in the wing pouch seen in the case of act.N expression (Fig. 4L) (Neumann and Cohen, 1996b; Ng et al., 1996). The mitogenic effect of Notch signaling can also be separated from the induction of wg, which has been shown to be a direct target of Su(H) (Kim et al., 1996). The phenotype elicited by Vg misexpression in the wing pouch, consisting of small wing pouch and downregulation of wg expression, was the opposite to that of Notch signaling activation (Fig. 5).

Our study revealed some noteworthy relationships between Notch, vg and wg during wing morphogenesis (Fig. 7). The Vg misexpression phenotype is likely to be caused by the downregulation of wg, since it was rescued by simultaneous expression of Wg. This, in turn, raises the possibility that the previously documented vg induction by Wg through the vg quadrantal enhancer (Zecca et al., 1996; Neumann and Cohen, 1997) may, as a final outcome, repress wg expression in the wing pouch (Fig. 7). This downregulation of wg by Vg may underlie the postulated self-refinement mechanism of wg expression at the d/v boundary, according to which Wg, directly or indirectly, represses wg expression in adjacent cells (Rulifson et al., 1996).

The function of the Vg protein is not clear but its action seems to be dependent on the developmental context, since its presence can repress wg expression in the wing disc (Fig. 5) while induce it in the eye disc (Fig. 6), which normally does not express vg. Consistent with the notion that the differential action of Vg can be modulated by Notch signaling activity, we observed a dramatic synergistic effect between Vg and Notch activation on cell proliferation in the eye disc (Fig. 6). It is possible that the synergy between Notch and Vg underlies the maintenance of high levels of wg expression at the d/v boundary of the wing disc and the effect on cell proliferation (Fig. 7), while the initial expression of wg at this boundary may not require Vg (Neumann and Cohen, 1996b).

In addition to Vg, Wg can also affect cell proliferation through synergy with Notch activation. The synergy between Notch and either Vg or Wg was not mimicked by co-expression of Vg and Wg. Therefore, the synergy between Notch and Vg on cell proliferation is likely to be caused by simultaneous activation of Notch and wg signaling, presumably through additional inductive events by Notch (Fig. 7). This interpretation is also consistent with the observation that the simultaneous activation of Notch and wg signaling results in synergistic effects inducing the formation of ectopic wing (Couso et al., 1995). The Notch/wg synergistic effect on cell...
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proliferation is particularly interesting considering that abnormal activation of Notch or wg signaling in mammals has been associated with neoplasias (Ellisen et al., 1991; Jhappan et al., 1992; Nussle and Varmus, 1992; Robbins et al., 1992; Uyttendaele et al., 1996; Gallahan and Gallahan, 1997; Moon and Miller, 1997). However, we do not expect that the ectopic activation of Notch alone in mammalian precursor cells will always result in oncogenic conditions. As we found here, the effect of Notch on cell proliferation depends on the developmental context. A similar behavior has also been documented in mammalian tissue culture cells where the activation of the Notch receptor results in focus formation only in the presence of the adenovirus oncogene gene E1A (Capobianco et al., 1997).

We have proposed before that the developmental role of Notch is to control the progression of precursor cells to the next developmental state (Artavanis-Tsakonas et al., 1995; Fleming et al., 1997). Developmental analyses indicate that Notch does not transmit specific developmental signals but rather modulates the ability of precursor cells to respond to such signals. Several studies have demonstrated that Notch signaling activity affects the response of a precursor cell to differentiation signals, thus controlling cell fates. The present work provides evidence that broadens the role of Notch signaling in development, showing that, in addition to cell differentiation, it can profoundly influence cell proliferation.

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