Development 135, 3995-4001 (2008) doi:10.1242/dev.027789

Notch signalling coordinates tissue growth and wing fate specification in *Drosophila*

Neus Rafel and Marco Milán*

During the development of a given organ, tissue growth and fate specification are simultaneously controlled by the activity of a discrete number of signalling molecules. Here, we report that these two processes are extraordinarily coordinated in the *Drosophila* wing primordium, which extensively proliferates during larval development to give rise to the dorsal thoracic body wall and the adult wing. The developmental decision between wing and body wall is defined by the opposing activities of two secreted signalling molecules, Wingless and the EGF receptor ligand Vein. Notch signalling is involved in the determination of a variety of cell fates, including growth and cell survival. We present evidence that growth of the wing primordium mediated by the activity of Notch is required for wing fate specification. Our data indicate that tissue size modulates the activity range of the signalling molecules Wingless and Vein. These results highlight a crucial role of Notch in linking proliferation and fate specification in the developing wing primordium.

KEY WORDS: Notch, Wingless, EGF receptor, Wing imaginal disc

INTRODUCTION

Growth and fate specification of a given organ are two regulated processes that have to be tightly coupled to generate a correctly shaped and sized structure (Lecuit and Le Goff, 2007). Uncoupling these two processes has disastrous consequences in development and disease. In the past few years, much has been learnt about the regulation of growth and fate specification. However, very little is known about how these processes are coupled. The *Drosophil*a wing imaginal disc provides a well-studied model system for analyzing at a genetic, cellular and molecular level these processes during development (Cohen, 1993).

The wing primordium contains the progenitors of both the adult body wall and the wing (Bryant, 1975). The developmental decision between wing and body wall is made early in development and is defined by the opposing activities of two secreted signalling molecules, Wingless (Wg) and the EGFR ligand Vein (Vn), in the most ventral and dorsal sides of the wing primordium, respectively (Ng et al., 1996; Wang et al., 2000; Zecca and Struhl, 2002). Genetic manipulations that increase Notch activity or activate Notch at ectopic sites result in increased growth, and also in cancer (Ferres-Marco et al., 2006; Moberg et al., 2005; Radtke and Raj, 2003; Thompson et al., 2005; Vaccari and Bilder, 2005). Here, we present evidence that growth of the early wing primordium mediated by the activity of Notch modulates the cellular response to Wingless and facilitates wing fate specification.

MATERIALS AND METHODS

Drosophila strains

UAS-N^{dsRNA} (Presente et al., 2002); *UAS-notum* (Giraldez et al., 2002); *UAS-Ser*^{TM} and *UAS-Dl*^{TM} (Herranz et al., 2006); *UAS-cycD UAS-cdk4* (Datar et al., 2000; Meyer et al., 2000); *UAS-dmyc* (Johnston et al., 1999); *UAS-hippo* (Harvey et al., 2003; Wu et al., 2003); *UAS-PTEN* (Goberdhan et al., 1999). Other stocks are described in FlyBase (http://flybase.org/).

ICREA and Institute for Research in Biomedicine (IRB), Parc Científic de Barcelona, Baldiri Reixac 10, 08028 Barcelona, Spain.

*Author for correspondence (e-mail: marco.milan@irbbarcelona.org)

Accepted 21 October 2008

Antibodies

Mouse anti-Wg (4D4, Developmental Hybridoma Bank); mouse anti-Nubbin (Ng et al., 1995); rat anti-Hth (Wu and Cohen, 1999). Other antibodies used are commercially available.

BrdU incorporation and tissue size measurements

Flies were allowed to lay eggs for 12 hours. Larval size was used to select the same developmental stage in all genotypes analyzed. The number of BrdU-labeled cells was counted in wild-type, *sd-gal4; UAS-Ser*^{TM} and *sd-gal4; UAS-Ser*^{TM}*; UAS-CycE* early second instar wing discs (48 hours after egg laying) that had been raised in the same conditions and dissected simultaneously. BrdU staining was performed as described by Milan et al. (Milan et al., 1996). Using Image J software, the area of these discs was also measured. The area of the *sd-gal4; UAS-Ser*^{TM} and *sd-gal4; UAS-Ser*^{TM}*; UAS-CycE* discs were always compared with wild-type discs raised in the same conditions. Final area measurements were normalized to the wild-type ones and are presented in arbitrary units. Using Microsoft-Excel Software, the average size and standard deviation of wing discs were calculated, and *t*-test analysis was carried out.

Temperature shifts

We used the Gal4/UAS system (Brand and Perrimon, 1993) combined with the thermo-sensitive version of Gal80 [Gal80^{ts} (McGuire et al., 2004)], a repressor of Gal4 protein activity, to precisely control, in time and space, gene expression. Adult flies carrying a Gal4 driver, the Gal80^{ts} construct and an UAS-transgene were allowed to lay eggs over a period of 24 hours at 18°C. The progeny were then raised at 18°C to maintain the Gal4/UAS system in a switched-off state but transferred to 29°C for different periods of time during larval development to induce Gal4/UAS-dependent gene expression.

RESULTS AND DISCUSSION

Early requirement of Notch in wing fate specification

During the second larval stage, the antagonistic activities of Wg and Vn specify wing versus body wall fate. Notch activity has been proposed to have a role in this process because the loss of Notch during this developmental stage leads to a failure in the induction of wing fate with a concomitant duplication of body wall structures (Couso and Martinez Arias, 1994). We decided to further analyze the role of Notch in this process, not only in the adult fly, but also in the developing wing primordium, by using the corresponding wing and body wall molecular markers.

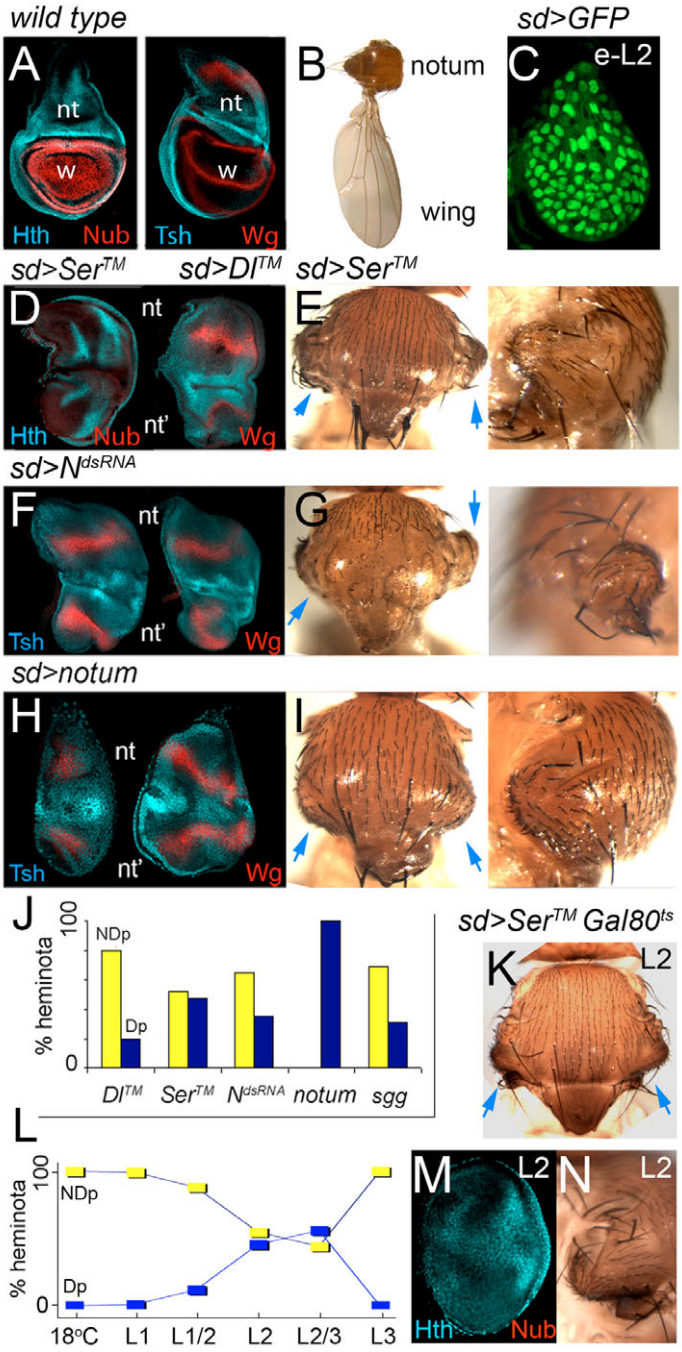

Fig. 1. Failure to specify wing fate in the absence of Notch activity. (**A**,**B**,**D-I**) Late third instar wing discs (A,D,F,H) and resulting adult wings or nota (B,E,G,I) of the following genotypes: wild type (A,B), *sd-Gal4; UAS-Ser*^{TM} or *sd-Gal4; UAS-Dl*^{TM} (D,E), *sd-Gal4; UAS-N*^{dsRNA} (F,G) and *sd-Gal4; UAS-notum* (H,I). Two wing discs per genotype are shown and labelled to visualize Nubbin (Nub, red) or Wingless (Wg, red), and Homothorax (Hth, blue) or Teashirt (Tsh, blue) protein expression. Wing territory (w), endogenous nota (nt) and duplicated nota territories (nt') are marked in A,D,F,H. Magnifications of the adult duplicated heminota (blue arrows) are shown in the right panels in E,G,I. (**C**) *sd-Gal4; UAS-GFP* early second instar wing disc. Note expression in the whole wing disc. (**J**) Histogram showing the percentage duplicated (Dp, blue) and non-duplicated (NDp, yellow) hemi-nota in different genotypes. Number of scored heminota: *Dl*^{TM}, *n*=398; *Ser*^{TM}, *n*=211; *N*^{dsRNA}, *n*=69; *notum*, *n*=40; *sgg*, *n*=312. (**K**,**M**,**N**) *sd-Gal4; UAS-Ser*^{TM}*Gal80*^{ts} late third instar wing disc (M) and resulting duplicated adult nota (K,N) of larvae raised at 18°C and shifted to 29°C during the second instar (48-72 hours after egg-laying, AEL). Wing disc in M was labelled to visualize Nubbin (Nub, red) and Homothorax (Hth, blue) protein expression. (**L**) Histogram showing the percentage of duplicated (Dp, blue) and non-duplicated (NDp, yellow) hemi-nota in *sd-Gal4; UAS-Ser*^{TM}*Gal80*^{ts} adult flies raised at 18°C and shifted to 29°C during different developmental stages: first instar (L1, 24-48 hours AEL), first/second instar (L1/2, 36-60 hours AEL), second instar (L2, 48-72 hours AEL), second/third instar (L2/3, 60-84 hours AEL) and third instar (L3, 72-96 hours AEL). Number of scored heminota: 18°C, *n*=148; L1, *n*=118; L1/2, *n*=314; L2, *n*=230; L2/3, *n*=74; L3, *n*=142.

We first blocked the activity of Notch and analyzed the resulting adult flies (Fig. 1). To block Notch, we expressed either dominant-negative forms of the Notch ligands Delta and Serrate [Dl^{TM} and Ser^{TM}, intracellular deletions of Delta and Serrate well known to repress Notch activity (Sun and Artavanis-Tsakonas, 1996)], or a Notch RNA interference construct [*N*^{dsRNA}, known to reduce Notch protein levels and to lead to strong Notch loss-of-function phenotypes in *Drosophila* tissues (Presente et al., 2002)] (see Fig. S1 in the supplementary material). We used the *scalloped-Gal4* (*sd-Gal4*) driver because it is expressed in the wing disc from early larval stages (Fig. 1C). The expression of *sd-Gal4* in early wing discs was not affected in conditions of reduced Notch activity (see Fig. S2 in the supplementary material). In adult flies, wings were either vestigial or absent (data not shown), and body wall structures were often duplicated (Fig. 1E,G,J). In the developing wing imaginal disc, expression of the homeodomain protein Homothorax (Hth) and the zinc-finger transcription factor Teashirt (Tsh) was restricted to the presumptive body wall, while the homeodomain protein Nubbin (Nub) was expressed in the presumptive wing territory (Ng et al., 1996; Wu and Cohen, 2002) (Fig. 1A). Wg was expressed in the body wall and wing territories of late third instar discs in a characteristic pattern (Fig. 1A). We then analyzed and compared the expression of these molecular markers in mature wing discs in which Notch activity had been compromised. Nub was absent, and the characteristic expression of Hth, Tsh and Wg in the notum showed a mirror-image duplication (Fig. 1D,F). The characteristic expression pattern of *sd-Gal4* in the resulting late third instar wing discs also showed mirror-image duplication (see Fig. S2 in the supplementary material). These results confirm the requirement for Notch in wing fate specification.

We next decided to temporally control the reduction in Notch activity by using the Gal4/Gal80^{ts} system (see Materials and methods for details). Reducing Notch activity for a period of 24 hours during the second instar stage (in *sd-gal4; UAS-Ser*^{TM} *Gal80*^{ts} larvae) gave rise to wing imaginal discs and adult flies in which wings were absent and body wall structures were duplicated (Fig. 1K-N). These effects were not observed when Notch activity was reduced during the first or third instar stages (Fig. 1L), thereby confirming the requirement of Notch in wing fate specification during the second larval stage, as proposed by Couso and Martinez Arias (Couso and Martinez Arias, 1994).

Notch acts upstream of Wg in wing fate specification

Similar defects in wing fate specification were obtained when the activity of Wg protein (by overexpression of the Wg antagonist Notum) or its signalling pathway (by overexpression of the

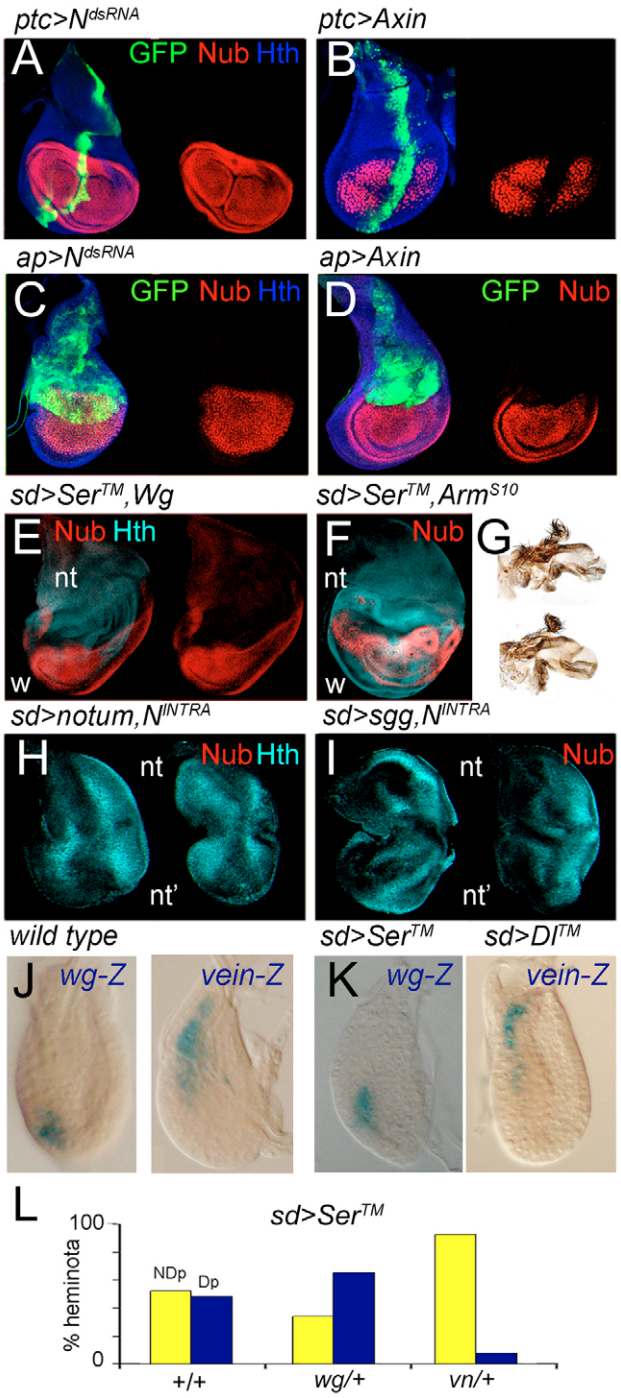

Fig. 2. Notch acts through Wg to induce wing fate specification. (**A-I**) Late third instar wing discs and adult wings of the following genotypes: *UAS-N^{dsRNA}/+; ptc-Gal4·UAS-GFP/+* (A), *ptc-Gal4·UAS-GFP/+; UAS-Axin/+* (B), *UAS-N^{dsRNA}/+; ap-Gal4·UAS-GFP/+* (C), *ap-Gal4·UAS-GFP/+; UAS-Axin/+* (D), *sd-Gal4; UAS-Ser^{TM}, UAS-Wg* (E), *sd-Gal4; UAS- Ser^{TM}, UAS-Arm^{S10}* (F,G), *sd-Gal; UAS-notum, UAS-N^{INTRA}* (H), *sd-Gal4; UAS-sgg, UAS-N^{INTRA}* (I), and labelled to visualize GFP (green, A-D), Homothorax (Hth, blue) and Nubbin (Nub, red) protein expression. The penetrance of the phenotype was 100% in E-I. Number of scored wing discs was: E, 29; F, 17; H, 9; I, 13. Wing territory (w), endogenous nota (nt) and duplicated nota territories (nt') are marked in E-I. (**J**,**K**) Wild-type (J) and *sd-Gal4, UAS-Ser^{TM}* or *sd-Gal4, UAS-Dl^{TM}* (K) early second instar wing discs labelled to visualize *vn-lacZ* and *wg-lacZ* expression. (**L**) Histogram showing the percentage of duplicated (Dp, blue) and non-duplicated (NDp, yellow) hemi-nota in *sd-Gal4; UAS-Ser^{TM}* adult flies in a +/+, *wg^{r0727}*/+ or *vn^{10567}*/+ mutant backgrounds. Number of scored heminota: +/+, 211; *wg^{r0727}*/+, 108; *vn^{10567}*/+, 108.

antagonist of the Wg pathway, the kinase Shaggy/Gsk3) were compromised in the *sd* domain (Fig. 1H-J; data not shown) (Giraldez et al., 2002; Morata and Lawrence, 1977; Sharma and Chopra, 1976), or when a temperature-sensitive mutant allele of *wg* was used to block its function for a period of 24 hours during the second instar (Couso and Martinez Arias, 1994). Thus, Notch might either control the expression or activity of Wg, or collaborate with the Wg pathway during wing fate specification. Consistent with this, reducing the amount of Wg protein, in a *wg* heterozygous background, increased the frequency of duplicated nota (Fig. 2L). However, only in the case of collaboration between Notch and Wg pathways might Notch be required in a cell-autonomous manner. Therefore, to choose between these two alternatives, we decided to block the Notch or Wg signalling pathways in a subset of cells within the presumptive wing primodium and examine the expression of Nub. Unfortunately, classical clonal analysis cannot be used to address this issue, as clones of cells lacking Notch or Wg activity cannot be recovered in the wing primordium, probably because of impaired cell proliferation or viability (de Celis and Garcia Bellido, 1994; Giraldez and Cohen, 2003; Johnston and Sanders, 2003). For this reason, we decided to use the Gal4/UAS system to compromise Notch activity in discrete territories within the wing primordium. Blocking Notch activity in a stripe along the anteroposterior compartment boundary (in *patched-Gal4; UAS-N^{dsRNA}* larvae) or throughout the dorsal compartment (in *apterous-Gal4; UAS-N^{dsRNA}* larvae) did not result in the loss of Nub expression (Fig. 2A,C). By contrast, blocking the response to Wg by the overexpression of Axin or Shaggy/Gsk3 [two antagonists of the Wg pathway (Logan and Nusse, 2004)] in the same domains induced the loss of Nub expression in the anterior (Fig. 2B; data not shown) or dorsal (Fig. 2D) compartments. These results indicate that Wg signalling is required in a cell-autonomous manner to induce wing fate specification, as previously shown (Ng et al., 1996). By contrast, the requirement of Notch signalling in this process is not cell-autonomous and it might be mediated by the activity of Wg. Indeed, epistatic analysis confirmed this hypothesis. The expression of Wg ligand or the activation of the Wg pathway in wing discs in which Notch activity had been compromised rescued Nub expression (Fig. 2E,F) and adult wing specification (Fig. 2G). By contrast, activation of the Notch pathway in wing discs in which Wg activity had been compromised did not rescue the expression of this protein (Fig. 2H,I), nor adult wing specification (data not shown).

The most plausible explanation for this epistatic relationship is that Notch activity positively regulates Wg expression. However, the following results indicate that this might not be the case. Early in wing development, Wg is expressed in a ventral wedge of cells located in the anterior compartment (Fig. 2J) (Couso et al., 1993). Blocking Notch activity in these cells throughout development (in *patched-Gal4; UAS-N^{dsRNA}* larvae) did not result in the loss of Nub expression (Fig. 2A), and Wg expression was not affected in early wing discs in which Notch activity had been compromised (in *sd>Ser^{TM}* larvae, Fig. 2K). Wg is required to repress Vn expression in the most dorsal region of the early disc (Wang et al., 2000). Vn

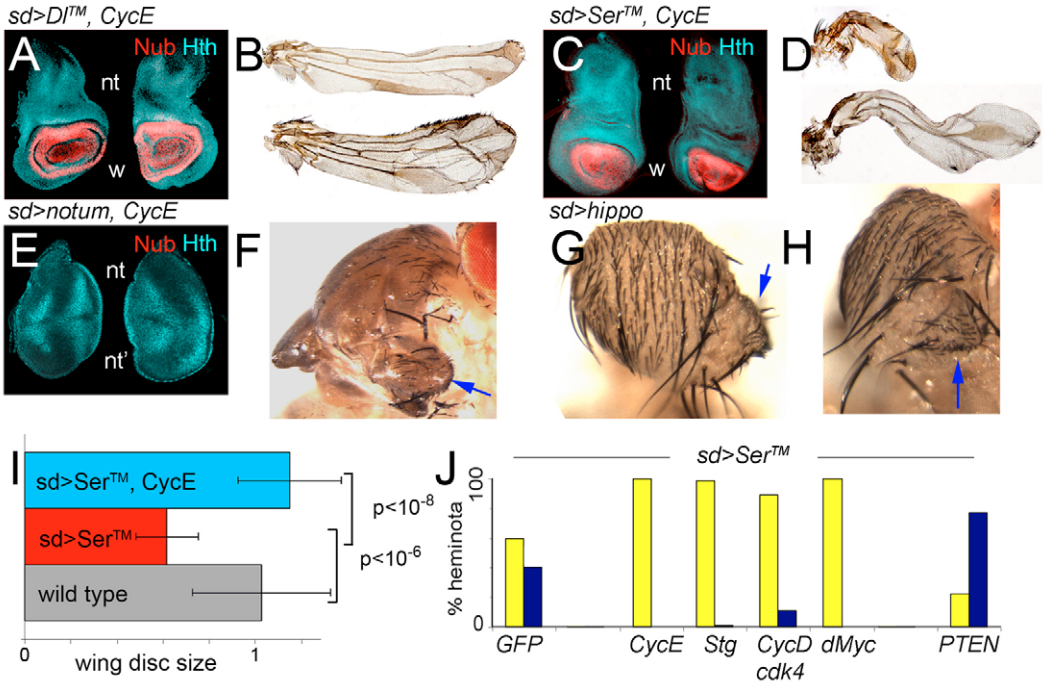

Fig. 3. Growth is essential for wing fate specification. (**A-H**) Late third instar wing discs and adult wing or nota of the following genotypes: *sd-Gal4, UAS-Dl^{TM}, UAS-CycE* (A,B), *sd-Gal4, UAS-Ser^{TM}, UAS-CycE* (C,D), *sd-Gal4, UAS-notum, UAS-CycE* (E,F) and *sd-Gal4, UAS-hippo* (G,H). Wing discs were labelled to visualize Nubbin (Nub, red) and Homothorax (Hth, blue) protein expression. The penetrance of the phenotype was 100% in A-F. Number of scored wing discs ranged between 7 and 11. Wing territory (w), endogenous nota (nt) and duplicated nota territories (nt') are marked in A,C,E. Blue arrows point to duplicated nota in F-H. (**I**) Histogram showing the size (in arbitrary units) of wild type, *sd-Gal4, UAS-Ser^{TM}* and *sd-Gal4, UAS-Ser^{TM}, UAS-CycE* early second instar wing discs raised under the same conditions. The average wing disc sizes and standard deviations were 1±0.3 (wild type), 0.67±0.14 (*sd>Ser^{TM}*) and 1.13±0.36 (*sd>Ser^{TM}, CycE*). *sd>Ser^{TM}* wing discs were significantly smaller than wild-type discs ($P<10^{-6}$) and expression of CycE was able to rescue wing disc size when compared with *sd>Ser^{TM}* wing discs ($P<10^{-8}$). Number of scored discs: wild type, 22; *sd>Ser^{TM}*, 40; *sd>Ser^{TM}, CycE*, 21. (**J**) Histogram showing the percentage of duplicated (Dp, blue) and non-duplicated (NDp, yellow) hemi-nota in *sd-Gal4; UAS-Ser^{TM}* adult flies expressing UAS-GFP (n=236), UAS-CycE (n=36), UAS-Stg (n=136), UAS-CycD,UAS-Cdk4 (n=64), UAS-dMyc (n=94) or UAS-PTEN (n=40).

antagonizes wing development and specifies body wall fate. An alternative mechanism by which Notch might affect Wg activity would be by interfering with the ability of Wg to repress Vn expression. Indeed, we noted that reducing the amount of Vn protein, in a *vn* heterozygous background, decreased the frequency of duplicated nota caused by compromised Notch activity (Fig. 2L). However, Vn expression was not affected by blocking Notch activity (compare Fig. 2J and 2K). Taken together, these results indicate that Notch activity is required upstream of Wg in the process of wing fate specification, but that Notch does not regulate the relative expression patterns of Wg and Vn.

Tissue growth promoted by Notch is required for wing fate specification

Notch is thought to promote growth in the early wing imaginal disc (de Celis and Garcia Bellido, 1994), and growth induced by Notch is required for specification of the eye within the *Drosophila* eye-antenna primordium (Kenyon et al., 2003). We therefore hypothesized that the requirement of Notch in wing fate specification is because of its control of tissue growth.

We first measured the size and analyzed the proliferation dynamics of early second instar wing discs after blocking Notch activity. Early second instar wing discs expressing the dominant-negative form of Serrate (Ser^{TM}) in the *sd-Gal4* domain were on average 34% smaller than were wild-type primordia raised in the same conditions (Fig. 3I). The average wing disc sizes, in arbitrary units, were 1±0.3 (wild type) and 0.67±0.14 (*sd>Ser^{TM}*; number of scored discs: wild type, n=22; *sd>Ser^{TM}* n=40; $P<10^{-6}$). The number of cycling cells, monitored by BrdU incorporation, was also reduced. The number of BrdU-positive cells in wild type and *sd>Ser^{TM}* wing discs was 14±4 and 6±1, respectively (wild type, n=7; *sd>Ser^{TM}*, n=11).

We next tested whether overexpressed cell cycle regulators or growth promoters were able to rescue tissue growth and wing fate specification in conditions of reduced Notch activity. Overexpression of cell cycle regulator Cyclin E [which drives G1–S transition (Neufeld et al., 1998)] in wing discs in which Notch activity had been compromised was able to restore the size of the wing primordia (Fig. 3I). The average size of *sd>Ser^{TM},CycE* discs (1.13±0.36, n=21) was significantly bigger than that of *sd>Ser^{TM}* discs (0.67±0.14, n=40, $P<10^{-8}$) grown in the same conditions. Similarly, the number of proliferating cells was also restored (an average of 13±3 BrdU-positive cells, n=7 discs). Interestingly, Nub expression (Fig. 3A,C) and adult wing specification (Fig. 3B,D,J) were restored in these conditions (in *sd>Ser^{TM},CycE* larvae and flies). Cyclin E did not show this capacity in the absence of Wg activity (Fig. 3E,F). Consistent with this, the size of the wing discs was not reduced after blocking Wg activity (the average wing disc sizes were 1±0.3 for wild type and 1.04±0.2 for *sd>notum*; P=0.77; n=8 and 5 discs, respectively). Adult fate specification was also rescued when the cell cycle regulator String [previously known as Dcdc25, which drives G2–M transition (Neufeld et al., 1998)] was expressed in conditions of reduced Notch activity (Fig. 3J). It is interesting to note that, in late third instar wing discs, overexpression of CycE and String has been reported to drive G1–S and G2–M transitions without causing any increase in tissue size (Neufeld et al., 1998). We therefore wondered whether the overexpression of these cell cycle regulators was able to induce tissue growth in second instar discs. Interestingly, the average size of *sd>CycE* (1.7±0.4, n=39) and *sd>Stg* (1.13±0.22, n=33) early second instar wing discs was significantly bigger than that of wild-type (1±0.22, n=47) discs grown in the same conditions ($P<10^{-13}$ and P=0.01, respectively;

see Fig. S3 in the supplementary material). These results suggest that the ability of CycE and String to rescue wing fate specification is a consequence of increased tissue growth. Alternatively, the increased cell cycling caused by these cell cycle regulators might interfere with the ability of the cells to transduce Notch signalling. We therefore analyzed the ability of Ser^{TM} to block Notch signalling in the presence of high levels of CycE or String. Similarly, we analyzed the ability of a dominantly active form of the Notch receptor [N^{intra} (Struhl and Adachi, 1998)] to activate the expression of Notch target genes in the presence of high levels of CycE or String. As shown in Fig. S4 (see supplementary material), the activity of the Notch pathway is not affected by the overexpression of these cell cycle regulators.

The growth promoters Cyclin D and Cdk4 (Datar et al., 2000), or dMyc (Dm – FlyBase) (Johnston et al., 1999), were also able to rescue wing fate specification under conditions of reduced Notch activity (Fig. 3J). Conversely, expression of the growth repressor PTEN (Goberdhan et al., 1999) increased the frequency of duplicated nota under conditions of reduced Notch activity (Fig. 3J), and blocking growth by means of overexpression of the tumor suppressor gene *hippo* (Harvey et al., 2003; Wu et al., 2003) in the early wing primordium induced failure in wing fate specification with a concomitant duplication of body wall structures (Fig. 3G,H). Taken together, these results indicate that growth induced by Notch activity is essential for the Wg-dependent induction of wing fate.

Concluding remarks

The expression of Wg in the most ventral part of the wing disc specifies the wing field at the same time as restricting Vn expression to the most dorsal part (Fig. 4A). Vn is required to block the responsiveness of body wall cells to Wg. Thus, the relative concentration of the diffusible proteins Wg and Vn (Neumann and Cohen, 1997; Schnepp et al., 1996; Zecca et al., 1996) experienced by disc cells directs their wing versus body wall fate. It is interesting to note that the expression of these two molecules is established long before the wing field is induced in the presumptive wing primordium (Wu and Cohen, 2002). Wg expression starts long before wing field specification takes place, as revealed by the later induction of Nub expression and the reduction in the expression of the body wall cell marker Tsh (Fig. 4D-G). We therefore propose that tissue growth modulates the cellular response to these signalling molecules and controls, in time, wing fate specification. In the early wing primordium, Vn might reach every wing cell, thereby blocking responsiveness to Wg and repressing wing fate specification. Growth induced by Notch activity might pull the sources of Wg and Vn apart and, thus, most ventral cells might not sense sufficient Vn levels, so Wg would be able to induce wing fate. Interestingly, the overexpression of Wg or overactivation of its signalling pathway is able to bypass the requirement of growth in this process (Fig. 2E,F), which indicates that the cells sense the relative levels of Wg and Vn. Once the wing field has been specified, Wg starts to be expressed along the presumptive wing margin, where it exerts a fundamental function in the maintenance of the Notch-dependent organizing center along the DV boundary (Buceta et al., 2007; Couso et al., 1994; Rulifson and Blair, 1995). Note that the organizing activity of Notch at the DV boundary takes place long after the early function of Notch revealed in this work, which is involved in promoting growth and facilitating wing fate specification. As revealed by the expression of the Notch target *E(spl)m-β*, it is not until late in the second instar that the expression of Notch is restricted to the DV boundary (see Fig. S1 in the supplementary material). During the process of wing fate specification that takes place during second instar, it is uniformly expressed in the whole wing disc (Fig. S1 in the supplementary material). These results imply that growth also facilitates the reiterative use of signalling molecules, such as Wg and Notch, to exert different functions during the development of a multicellular organ like the wing primordium.

At the same time that wing and body wall fate specification takes place in the wing primordium, Vn is involved in the induction of *apterous* expression in the dorsal region (Wang et al., 2000; Zecca and Struhl, 2002) (see also Fig. S6 in the supplementary material). Consistent with the model proposed above, the activity of Vn, as

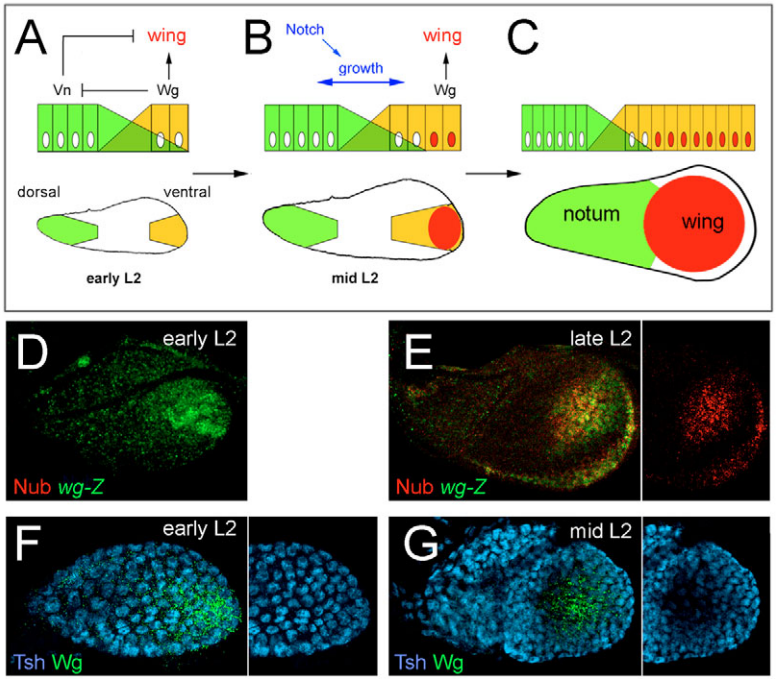

Fig. 4. Coupling growth and wing fate specification. (**A-C**) Illustration of the subdivision of the wing imaginal disc into wing (red) and body wall (notum, green) primordia by the signalling molecules Wingless (Wg, orange) and Vein (Vn, green). Ventrally expressed Wg induces the expression of wing fate marker genes, whereas dorsally expressed Vn blocks this activation. Wg represses Vn expression restricting it to the dorsal region. (A) Vn is expressed by a subset of cells (green), but it diffuses and reaches every wing cell in the early wing primordium. In this situation, ventral cells (orange) are not able to respond to Wg and do not differentiate as wing tissue. (B,C) Growth of the tissue induced by the activity of Notch pulls the sources of Wg and Vn apart. Now, ventral cells (orange) do not sense sufficient levels of Vn, so they respond to Wg and differentiate as wing tissue (red nuclei). (**D-G**) Early (D,F), mid (G) and late (E) second instar wing discs labelled to visualize *wg-lacZ* (antibody to β-Gal, green) and Nubbin protein expression (Nub, red; D,E), or Wg (green) and Teashirt (Tsh, blue; F,G) protein expression.

monitored by the expression of *apterous*, was modulated by tissue growth (see Figs S5, S6 in the supplementary material). In the absence of Notch activity, even though Vn expression is not affected (Fig. 2K), Vn appears to reach every wing cell, as *apterous* expression was expanded ventrally (see Fig. S5 in the supplementary material). Increased levels of Wg expression or growth promoted by CycE appear to re-establish the dorsally restricted range of activity of Vn, as *apterous* expansion was blocked under these circumstances (see Fig. S5 in the supplementary material).

Growth promoted by Notch has also been shown to be directly involved in the specification of the eye within the *Drosophila* eye-antenna primordium (Kenyon et al., 2003), a process that also depends upon the opposing activities of two secreted signalling molecules, in this case Dpp and Wg. Thus, Notch coordinates in a very elegant manner both eye and wing primordia tissue growth and eye/wing specification, by modulating the response of the cells to the activities of signalling molecules. These results indicate that the same mechanism might be commonly used in animal development to coordinate tissue growth and fate specification.

The evolution of wings was crucial in the process of adaptation, allowing insects to escape predators or colonize new niches. It has recently been shown that the loss and recovery of wings has occurred during the course of evolution (Whiting et al., 2003). This finding would suggest that wing developmental pathways are conserved in wingless insects and are being re-used. According to our results, we speculate that adaptative changes in animal size could modulate the cellular response to signalling molecules such as Wg, thereby helping to drive some of these extraordinary reversible transitions.

We thank N. Azpiazu, S. Cohen, B. Edgar, D. Hipfner, L. Johnston, M. Furriols, J. P. Vincent, the Bloomington Stock Center and the Developmental Studies Hybridoma Bank for flies and reagents, and I. Becam, S. Cohen, H. Herranz and three anonymous reviewers for comments on the manuscript. N.R. was funded by a predoctoral fellowship from the Ministerio de Educación y Ciencia, Spain, and M.M.'s laboratory was funded by Grants from Dirección General de Investigación Científica y Técnica (BFU2004-00167/BMC and BFU2007-64127/BMC) and Generalitat de Catalunya (2005 SGR 00118), intramural funds and the EMBO Young Investigator Programme.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/24/3995/DC1

References

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Bryant, P. J. (1975). Pattern formation in the imaginal wing disc of *Drosophila melanogaster*: fate map, regeneration and duplication. *J. Exp. Zool.* **193**, 49-77.

Buceta, J., Herranz, H., Canela-Xandri, O., Reigada, R., Sagues, F. and Milan, M. (2007). Robustness and stability of the gene regulatory network involved in DV boundary formation in the Drosophila wing. *PLoS ONE* **2**, e602.

Cohen, S. M. (1993). Imaginal disc development. In *Drosophila Development*. Vol. 2 (ed. A. Martinez-Arias and M. Bate), pp. 747-841. Cold Spring Harbor: Cold Spring Harbor Press.

Couso, J. P. and Martinez Arias, A. (1994). Notch is required for *wingless* signalling in the epidermis of Drosophila. *Cell* **79**, 259-272.

Couso, J. P., Bate, M. and Martinez Arias, A. (1993). A *wingless*-dependent polar coordinate system in the imaginal discs of Drosophila. *Science* **259**, 484-489.

Couso, J. P., Bishop, S. and Martinez Arias, A. (1994). The wingless signalling pathway and the patterning of the wing margin in Drosophila. *Development* **120**, 621-636.

Datar, S. A., Jacobs, H. W., de La Cruz, A. F., Lehner, C. F. and Edgar, B. A. (2000). The drosophila cyclin D-cdk4 complex promotes cellular growth [in process citation]. *EMBO J.* **19**, 4543-4554.

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

Ferres-Marco, D., Gutierrez-Garcia, I., Vallejo, D. M., Bolivar, J., Gutierrez-Avino, F. J. and Dominguez, M. (2006). Epigenetic silencers and Notch collaborate to promote malignant tumours by Rb silencing. *Nature* **439**, 430-436.

Giraldez, A. J. and Cohen, S. M. (2003). Wingless and Notch signaling provide cell survival cues and control cell proliferation during wing development. *Development* **130**, 6533-6543.

Giraldez, A. J., Copley, R. R. and Cohen, S. M. (2002). HSPG modification by the secreted enzyme Notum shapes the Wingless morphogen gradient. *Dev. Cell* **2**, 667-676.

Goberdhan, D. C., Paricio, N., Goodman, E. C., Mlodzik, M. and Wilson, C. (1999). Drosophila tumor suppressor PTEN controls cell size and number by antagonizing the Chico/PI3-kinase signaling pathway. *Genes Dev.* **13**, 3244-3258.

Harvey, K. F., Pfleger, C. M. and Hariharan, I. K. (2003). The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. *Cell* **114**, 457-467.

Herranz, H., Stamataki, E., Feiguin, F. and Milan, M. (2006). Self-refinement of Notch activity through the transmembrane protein Crumbs: modulation of gamma-Secretase activity. *EMBO Rep.* **7**, 297-302.

Johnston, L. A. and Sanders, A. L. (2003). Wingless promotes cell survival but constrains growth during Drosophila wing development. *Nat. Cell Biol.* **5**, 827-833.

Johnston, L. A., Prober, D. A., Edgar, B. A., Eisenman, R. N. and Gallant, P. (1999). Drosophila myc regulates cellular growth during development. *Cell* **98**, 779-790.

Kenyon, K. L., Ranade, S. S., Curtiss, J., Mlodzik, M. and Pignoni, F. (2003). Coordinating proliferation and tissue specification to promote regional identity in the Drosophila head. *Dev. Cell* **5**, 403-414.

Lecuit, T. and Le Goff, L. (2007). Orchestrating size and shape during morphogenesis. *Nature* **450**, 189-192.

Logan, C. Y. and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.* **20**, 781-810.

McGuire, S. E., Mao, Z. and Davis, R. L. (2004). Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in Drosophila. *Sci STKE* **2004**, pl6.

Meyer, C. A., Jacobs, H. W., Datar, S. A., Du, W., Edgar, B. A. and Lehner, C. F. (2000). Drosophila cdk4 is required for normal growth and is dispensable for cell cycle progression [in process citation]. *EMBO J.* **19**, 4533-4542.

Milan, M., Campuzano, S. and Garcia-Bellído, A. (1996). Cell-cycling and patterned cell proliferation in the wing primordium of *Drosophila*. *Proc. Natl. Acad. Sci. USA* **93**, 640-645.

Moberg, K. H., Schelble, S., Burdick, S. K. and Hariharan, I. K. (2005). Mutations in erupted, the Drosophila ortholog of mammalian tumor susceptibility gene 101, elicit non-cell-autonomous overgrowth. *Dev. Cell* **9**, 699-710.

Morata, G. and Lawrence, P. A. (1977). The development of wingless, a homeotic mutation of Drosophila. *Dev. Biol.* **56**, 227-240.

Neufeld, T. P., de la Cruz, A. F., Johnston, L. A. and Edgar, B. A. (1998). Coordination of growth and cell division in the Drosophila wing. *Cell* **93**, 1183-1193.

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

Ng, M., Diaz-Benjumea, F. J. and Cohen, S. M. (1995). *nubbin* encodes a POU-domain protein required for proximal-distal patterning in the *Drosophila* wing. *Development* **121**, 589-599.

Ng, M., Diaz-Benjumea, F. J., Vincent, J. P., Wu, J. and Cohen, S. M. (1996). Specification of the wing by localized expression of wingless protein. *Nature* **381**, 316-318.

Presente, A., Shaw, S., Nye, J. S. and Andres, A. J. (2002). Transgene-mediated RNA interference defines a novel role for notch in chemosensory startle behavior. *Genesis* **34**, 165-169.

Radtke, F. and Raj, K. (2003). The role of Notch in tumorigenesis: oncogene or tumour suppressor? *Nat. Rev. Cancer* **3**, 756-767.

Rulifson, E. J. and Blair, S. S. (1995). *Notch* regulates *wingless* expression and is not required for reception of the paracrine *wingless* signal during wing margin neurogenesis in *Drosophila*. *Development* **121**, 2813-2824.

Schnepp, B., Grumbling, G., Donaldson, T. and Simcox, A. (1996). Vein is a novel component in the Drosophila epidermal growth factor receptor pathway with similarity to the neuregulins. *Genes Dev.* **10**, 2302-2313.

Sharma, R. P. and Chopra, V. L. (1976). Effect of the wingless (wg1) mutation on wing and haltere development in Drosophila melanogaster. *Dev. Biol.* **48**, 461-465.

Struhl, G. and Adachi, A. (1998). Nuclear access and action of Notch *in vivo*. *Cell* **93**, 649-660.

Sun, X. and Artavanis-Tsakonas, S. (1996). The intracellular deletions of Delta and Serrate define dominant negative forms of the Drosophila Notch ligands. *Development* **122**, 2465-2474.

Thompson, B. J., Mathieu, J., Sung, H. H., Loeser, E., Rorth, P. and Cohen, S. M. (2005). Tumor suppressor properties of the ESCRT-II complex component Vps25 in Drosophila. *Dev. Cell* **9**, 711-720.

Vaccari, T. and Bilder, D. (2005). The Drosophila tumor suppressor vps25 prevents nonautonomous overproliferation by regulating notch trafficking. *Dev. Cell* **9**, 687-698.

Wang, S. H., Simcox, A. and Campbell, G. (2000). Dual role for Drosophila epidermal growth factor receptor signaling in early wing disc development. *Genes Dev.* **14**, 2271-2276.

Whiting, M. F., Bradler, S. and Maxwell, T. (2003). Loss and recovery of wings in stick insects. *Nature* **421**, 264-267.

Wu, J. and Cohen, S. M. (1999). Proximodistal axis formation in the Drosophila leg: subdivision into proximal and distal domains by Homothorax and Distal-less. *Development* **126**, 109-117.

Wu, J. and Cohen, S. M. (2002). Repression of Teashirt marks the initiation of wing development. *Development* **129**, 2411-2418.

Wu, S., Huang, J., Dong, J. and Pan, D. (2003). hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. *Cell* **114**, 445-456.

Zecca, M. and Struhl, G. (2002). Subdivision of the Drosophila wing imaginal disc by EGFR-mediated signaling. *Development* **129**, 1357-1368.

Zecca, M., Basler, K. and Struhl, G. (1996). Direct and long-range action of a Wingless morphogen gradient. *Cell* **87**, 833-844.