Wingless and Notch signaling provide cell survival cues and control cell proliferation during wing development

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Summary

Tissue growth during animal development depends on the coordination of cell proliferation and cell death. The EGF-receptor/MAPK, Hedgehog, Dpp, Wingless (Wg) and Notch signaling pathways have been implicated in growth control in the developing *Drosophila* wing. In this report, we examine the effects of Notch and Wg on growth in terms of cell proliferation and cell survival. Reduction of Wg signaling impaired compartment and clonal growth, and increased cell death. Inhibition of apoptosis in cells deficient for Wg signaling only partially rescued the clone growth defect, suggesting that Wg is also required to promote cell proliferation. This is supported by the finding that ectopic expression of Wg caused over-proliferation of cells in the proximal wing. Localized activation of Notch had non-autonomous effects on cell proliferation. However, only part of this effect was attributable to Notch-dependent induction of Wg, suggesting that other Notch-inducible signaling molecules contribute to the control of cell proliferation in the wing.

Supplemental data available online

Key words: Cell proliferation, Apoptosis, Pattern formation

Introduction

Growth of tissues and organs requires coordination of the rates of cell proliferation and cell death (reviewed by Conlon and Raff, 1999; Edgar et al., 2001; Johnston and Gallant, 2002). Cell proliferation depends on signals to stimulate cell growth and cell division. In addition, cells compete for survival cues that are required to prevent them from undergoing apoptosis. To date, relatively little is known about how these cellular processes are coordinated with pattern formation, although genes linking these processes are beginning to be identified (Brennecke et al., 2003).

Secreted signaling proteins of the Hedgehog, Wingless (Wg) and Dpp families organize spatial pattern in the imaginal discs of *Drosophila*. These signaling proteins have also been implicated in control of imaginal disc growth. Hedgehog and Dpp activate cell proliferation in a region specific manner in the imaginal discs (Duman-Scheel et al., 2002; Martin-Castellanos and Edgar, 2002). Dpp signaling also supports cell survival (Moreno et al., 2002a). Notch and Wg have been implicated in both growth and cell survival signaling. Reduction of Notch or Wg activity leads to a reduction in the size of the wing (Couso et al., 1994; de Celis and Garcia Bellido, 1994; Neumann and Cohen, 1996a; Neumann and Cohen, 1997; Go et al., 1998; Klein and Martinez-Arias, 1998; Baonza and Garcia-Bellido, 1999; Chen and Struhl, 1999; Thompson et al., 2002). Ectopic activation of Notch induces high levels of Wg expression and causes tissue overgrowth during wing development (Diaz-Benjumea and Cohen, 1995; Neumann and Cohen, 1996b; Go et al., 1998). Toward the end of the third larval instar these signals cause cells near the dorsoventral (DV) boundary to exit proliferation before cells in other regions of the disc do so (Phillips and Whittle, 1993; Johnston and Edgar, 1998).

Notch and Wg regulate expression of each other at the DV boundary of the wing disc (Rulifson and Blair, 1995; Diaz-Benjumea and Cohen, 1995; de Celis et al., 1996; Neumann and Cohen, 1996b; Micchelli et al., 1997; de Celis and Bray, 1997). Previous reports have not adequately distinguished the individual contributions of the two pathways to control of cell proliferation and survival. In this report, we examine the effects of reducing Wg activity in ways that do not compromise the signaling centers. We compare the effects of ectopically expressing Wg with those of ectopic activation of the Notch pathway under conditions where Wg cannot be activated. We present evidence that Wg serves as both a proliferation signal and a cell survival signal, and that it does so to different degrees in different regions of the wing pouch. These effects can be distinguished from the effects of Wg on cell fate specification outside the wing pouch. Notch acts through a signal relay mechanism to stimulate cell proliferation non-autonomously. Some of the effects of Notch can be attributed to the induction of Wg expression; however, we present evidence that Notch also acts through another relay signal. We suggest that Wg and Notch act synergistically to control tissue growth and cell survival.

Materials and methods

Drosophila strains

UAS-Notum^{GT} expresses a Golgi-tethered active form of Notum; *UAS-Notum^{S237A}* expresses an inactive form of Notum (Giraldez et al., 2002). *UAS-nkd-myc^{II}* (Wharton et al., 2001), FRT42D *arrow^2*

(Wehrli et al., 2000), $pygo^{S28}$ (Thompson et al., 2002) and puc^{E69} (*puc-lacZ*) (Martin-Blanco et al., 1998) have all been described previously. Other stocks are described at http://flybase.bio.indiana.edu/. Crosses were carried out at 25°C unless otherwise indicated.

Genotypes used to generate mosaic analysis

$hsFLP^{122}$/+; FRT42D arm-lacZ/FRT42D Ubi-GFP (Fig. 1C);

$hsFLP^{122}$/+; FRT42D $arrow^2$/FRT42D Ubi-GFP (Fig. 1D, Fig. 2A);

$hsFLP^{122}$/+; FRT82B $pygo^{S28}$/FRT82B arm-lacZ (Fig. 1E, Fig. 2B);

$hsFLP^{122}$/+; FRT42D $arrow^2$/FRT42D Ubi-GFP; puc^{E69}/+ (Fig. 3);

$hsFLP^{122}$/+; FRT82B arm-lacZ/FRT82B hsP(πmyc), $M(3)w^{124}$ (Fig. 5A,C,F);

$hsFLP^{122}$/+; FRT82B $pygo^{S28}$/FRT82B hsP(πmyc), $M(3)w^{124}$ (Fig. 5B,C,G);

$hsFLP^{122}$/+; FRT42D arm-lacZ/FRT42D hsP(πmyc) $M(2)53^1$ (Fig. 5D);

$hsFLP^{122}$/+; FRT42D $arrow^2$/FRT42D hsP(πmyc) $M(2)53^1$ (Fig. 5E);

$hsFLP^{122}$/+; FRT42D $arrow^2$/FRT42D Ubi-GFP; FRT82B $pygo^{S28}$/FRT82B arm-lacZ (Fig. 5H-K);

$hsFLP^{122}$ UAS-CD8-GFP/+; FRT42D arm-lacZ/FRT42D Gal80; UAS-p35/tubGal4 (Fig. 4A, Fig. 7F);

$hsFLP^{122}$ UAS-CD8-GFP/+; FRT42D $arrow^2$/FRT42D Gal80; UAS-p35/tub^{Gal4} (Fig. 4C-E, Fig. 7F);

$hsFLP^{122}$ UAS-CD8-GFP/+; FRT42D arm-lacZ/FRT42D Gal80; UAS-puc/tub^{Gal4} (Fig. 4F);

$hsFLP^{122}$ UAS-CD8-GFP/+; FRT42D $arrow^2$/FRT42D Gal80; UAS-puc/tub^{Gal4} (Fig. 4H-J);

$hsFLP^{122}$ UAS-CD8-GFP/+; FRT40A arm-lacZ/FRT40A Gal80; UAS-Notch^{Intra}/tub^{Gal4} (Fig. 7B,E; Fig. 8B; Fig. 9A,E);

$hsFLP^{122}$ UAS-CD8-GFP/+; FRT40A wg^{cx4}/FRT40A Gal80; UAS-Notch^{Intra}/tub^{Gal4} (Fig. 7C,E; Fig. 9B,C,E);

$hsFLP^{122}$ UAS-CD8-GFP/+; FRT40A arm-lacZ/FRT40D Gal80; +/tub^{Gal4} (Fig. 7A,E; Fig. 8A); and

$hsFLP^{122}$ UAS-CD8-GFP/+; tub^{Gal4}/+; FRT82B Axin^{S044230}/FRT arm-lacZ (Fig. 7D,E; Fig. 8C).

Measurement of clone and compartment size

For area measurements on compartment size, larvae expressing the transgenes were grown in the same vial with the control larvae expressing only the Gal4 driver. An average of 10 female larvae of each genotype were analyzed 120 hours after egg laying (AEL). Larvae were genotyped by antibody staining. Using Wg and Nubbin expression as landmarks, the area of the dorsal (D) versus ventral (V) wing blade was measured using NIH image software. En-Gal4 UAS-GFP was used to distinguish anterior (A) and posterior (P) compartments. Crosses with UAS-Notum^{GT} and UAS-DFz2-GPI were kept at 22°C.

For control, *arrow* and *pygopus* mutant clones, embryos were collected for 12 hours. Larvae were heat-shocked at 60±6 hours AEL, or 66±6 hours, for 20 minutes at 37°C, and dissected at 116±6 hours AEL. For Minute$^+$ (M$^+$) clones, embryos were collected for 6-8 hours. The wild-type and the mutant larvae were combined in the same vial 24 hours AEL to provide identical growth conditions. Larvae were heat-shocked at 76±4 hours AEL for 20 minutes at 37°C, and dissected at 160±4 hours AEL. Mutant and wild-type clone areas were measured from confocal images using NIH image. The size of wild-type clones was quantified in the apical surface, whereas in the *arrow* clones it was quantified in the basal region, were most of the *arrow* mutant cells were found; the section used for the quantification was that of maximal clone area. In M$^+$ experiments, some clones were obviously fused; however, there was usually a clear border between them, which allowed an approximation of the contribution of the separate clones. The presence of a few heterozygous cells in the middle of a clone was indicative of two fused clones, and thus these were quantified as two separate clones.

To measure tissue overgrowth due to expression of Notch^{Intra} in wild-type, wg^{cx4} or vg^{83b27R} mutant cells, we generated positively marked clones using the Gal80 system (Lee and Luo, 1999). Embryos were collected for 8 hours. Larvae were heat-shocked at 48±4 hours AEL for 20 minutes at 37°C, and dissected at 118±4 hours AEL. Clones were scored for obvious tissue overgrowth, including alteration of shape in the surrounding tissue (bulging), compared with clones that did not produce obvious overgrowth. Three different areas of the wing disc were considered separately: (1) distal, adjacent to the DV boundary defined by the Wg staining (3-5 cell diameters); (2) medial, between the proximal and the distal region; and (3) proximal, adjacent to the fold that delimitates the wing pouch (7-10 cell diameters). All three areas of the wing pouch express Nubbin and Vestigial protein. The 'hinge and pleura' was defined as the area outside the fold that defines the wing pouch, coincident with Wg expression in the two rings and Nubbin expression. These cells do not normally express Vg protein.

BrdU incorporation

Larvae were dissected in Grace's insect medium (Sigma), incubated for 1 hour in medium containing 0.2 mg/ml of BrdU (Sigma), washed and fixed for labeling with rabbit anti-GFP and anti-BrdU antibodies (Usui and Kimura, 1992). To compare BrdU incorporation in clones expressing Notch^{Intra} in the presence or the absence of Wg, we generated positively marked clones in larvae of genotype hsFLP^{122}, UAS-CD8-GFP; FRT40A arm-lacZ (or FRT40A, wg^{cx4})/FRT40A Gal80; UAS-Notch^{Intra}/tub^{Gal4}. Embryos were collected for 8 hours. Larvae were heat-shocked at 48±4 hours AEL for 20 minutes at 37°C, and dissected at 118±4 hours AEL. Larvae were labeled with anti-GFP, anti-BrdU and DAPI. Figs 6 and 7 show projections of four single sections taken at 4 µm intervals for the BrdU channel. The GFP and the DAPI channel are single sections.

Flow cytometry

To analyze the effect of the Notch and the Wingless pathway on the cell cycle profile and on cell size, we generated positively marked clones using the Gal80 system (Lee and Luo, 1999). Embryos were collected for 8 hours. Larvae were heat-shocked at 48±4 hours AEL for 1 hour at 37°C, and dissected at 105±4 hours (+p35) AEL, or 115±4 hours AEL. Discs were dissociated using trypsin, and stained with Hoechst 33342 as described (Neufeld et al., 1998). GFP content, cell cycle profile, and forward scatter were analyzed. Experiments were repeated twice with equivalent results.

Antibodies

Mouse anti-BrdU was used at a dilution of 1:50 (PharMingen). Rabbit anti-activated caspase-3 was used at 1:20 (Cell Signaling Technology). Rabbit anti c-Myc (A-14) was used at 1:200 (Santa Cruz Biotechnology). Guinea pig anti-Notum were used at 1:100 (Giraldez et al., 2002). Mouse anti-Cut was used at 1:100 (Micchelli et al., 1997). Rat anti-Dll was used at 1:200 (Wu and Cohen, 1999). Rat anti β-gal was used at 1:500 (Strigini and Cohen, 1997). Rabbit anti-Vg was used at 1:200 (Williams et al., 1993). Monoclonal mouse anti-Wg was used at 1:10 (Brook et al., 1996). Mouse anti-Nubbin was used at 1:100 (Ng et al., 1996). Mouse anti-Cyclin D was used at 1:5; and rabbit anti-Cyclin E (#3433) was used at 1:25 (Duman-Scheel, 2002).

Results

Decreased Wg signaling reduces growth of the wing

Inhibitors of Wg signaling were used to selectively reduce Wg activity in the dorsal (D) compartment. By expressing the inhibitors only in D cells, the activity of the DV signaling center was not compromised. Consequently, effects on D compartment size could be compared with ventral (V)
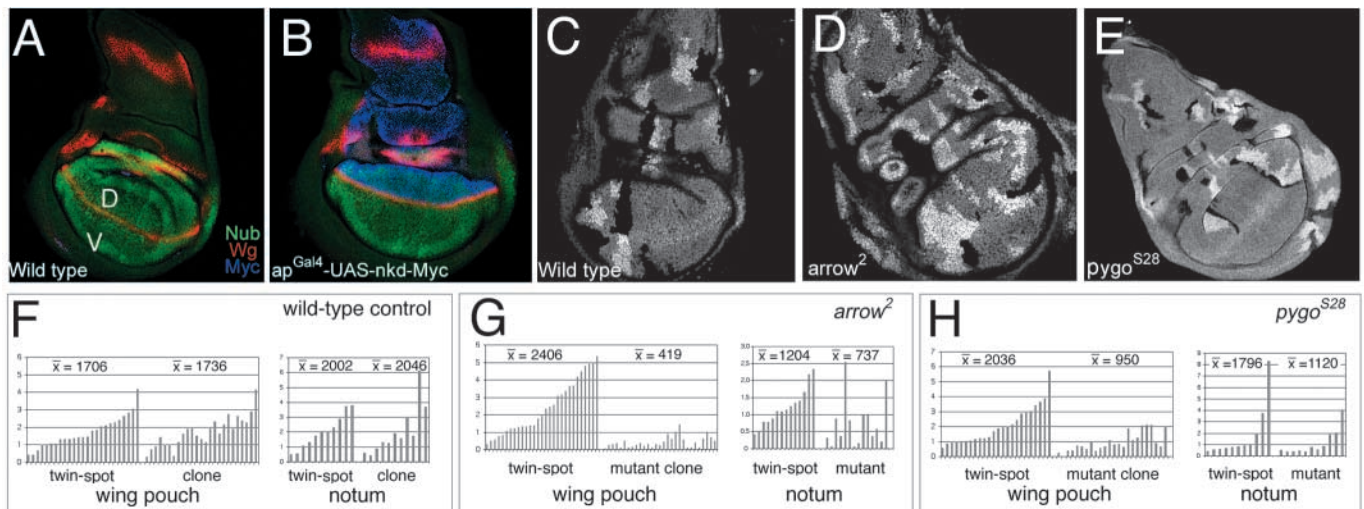
Fig. 1. Wg signaling influences growth during wing development. (A,B) Confocal sections of third instar wing imaginal discs labeled with antibodies to visualize Wg (red), Nubbin (green) and the Myc epitope tag (blue) (A) Wild type. D and V indicate dorsal and ventral compartments. (B) Reduction of Wg signaling in the D compartment of an ap-Gal4 Uas-nkd-myc (II) wing disc. (C-E) Wing discs with multiple marked clones. Clones were marked by the absence of nuclear GFP (C,D) or β-galactosidase (E). The twin-spots express two copies of the marker and appear brighter. (C) Control clones genotype: hsFLP^{122}/+; FRT42D arm-lacZ/FRT42D Ubi-GFP. (D) Genotype: hsFLP^{122}/+; FRT42D arrow^2/FRT42D Ubi-GFP. (E) Genotype: hsFLP^{122}/+; FRT82B pygo^{S28}/FRT82B arm-lacZ. (F-H) Histograms presenting the areas of mutant and twin spot clones (x axis, thousands of pixels). Clones in the wing pouch and notum are shown separately. Individual pairs of twin spots and mutant clones are in the same order (shown according to the increasing sizes of the twin). (F) Wild-type clone average area was 1736 versus 1706 pixels for the twin spots (n=25) in with wing pouch. In the notum, average areas were 2046 and 2002 pixels (n=11). (G) arrow twin versus clone, 2406 versus 419 pixels (n=29) in the wing pouch, and 1204 versus 737 pixels (n=14) in the notum. (H) pygo^{S28} twin versus clone areas were 2036 versus 950 pixels (n=24) in the wing pouch, and 1796 versus 1120 pixels (n=14) in the notum.

compartment size in the same disc. We measured the area of the presumptive D and V compartments using Nubbin and the rings of Wg expression as markers for the D and V wing pouch. *naked* encodes an EF-hand protein that interacts with Dishevelled to reduce Wg signaling (Wharton et al., 2001; Zeng et al., 2000). Expression of UAS-nkd-myc^{II} in D cells under apterous-GAL4 control strongly reduced the relative size of the D compartment (Fig. 1A,B). These flies survived to adulthood and had wings with a reduced dorsal surface, which curled upwards (not shown). Note that reduction of Wg responsiveness in D cells did not eliminate the Wg expression stripe at the DV boundary (see also Micchelli et al., 1997; Giraldez et al., 2002). Comparable results were obtained using other inhibitors of Wg signaling, including a Golgi-tethered form of the Notum protein, UAS-Dally-like and UAS-Dfz2-GPI (Bhanot et al., 1996; Baeg et al., 2001; Giraldez et al., 2002) (see supplemental Fig. S1 online at http://dev.biologists.org/supplemental/) (data not shown). Comparable effects on compartment growth were obtained when UAS-Dfz2-GPI was used to reduce Wg signaling in the P compartment (see Brennecke et al., 2003). These observations suggest that the level of Wg signaling activity contributes to determining the size of the wing pouch.

Cell autonomous requirement for Wg activity

To determine whether these phenotypes reflected a property of the compartment as a whole, or whether they reflected a cell-autonomous requirement for Wg activity, FLP-induced mitotic recombination was used to produce clones of homozygous mutant cells and sister clones that were homozygous wild type. Each pair of clones derives from a single cell division.

Consequently, relative growth can be compared by measuring the areas of the individual pairs of mutant and wild-type twin clones after a period of time. Mutations in two components of the Wg signaling pathway were examined. *arrow* encodes the Wg co-receptor (Wehrli et al., 2000). The *arrow^2* allele truncates the protein before the transmembrane domain and should be a null allele. *pygopus* encodes a nuclear protein required for Wg signaling (Kramps et al., 2002; Thompson et al., 2002). The *pygo^{S28B}* allele truncates the protein before the PHD domain and appears to be a strong hypomorphic mutant.

Clones generated before 48 hours after egg laying (AEL) were poorly recovered at 120 hours (Thompson et al., 2002; Wehrli et al., 2000), therefore we induced recombination at 60±2 hours AEL and examined the clones at 120 hours. For wild-type control clones, there was no difference in average clone and twin-spot areas in wing pouch or in the notum (Fig. 1C,F; pouch, P=0.9; notum, P=0.94, using T-test). By contrast, *arrow^2* and *pygo^{S28}* clones were on average considerably smaller than their wild-type twins in the pouch (Fig. 1D,E,G,H). These differences were statistically significant (*arrow*, P=1.6×10^{-7}; *pygo*, P<0.001). In the notum, the *arrow^2* and *pygo^{S28}* mutant clones were not significantly smaller than their twins (*arrow*, P=0.07; *pygo*, P=0.41). These results indicate that Wg signaling is required cell-autonomously for clonal growth in the wing pouch. Reduced clone size could reflect either a decrease in the rate of cell proliferation (cell growth and division) or an increase in cell death.

Wg signaling provides a survival cue

To determine whether the reduced area of *arrow^2* and *pygo^{S28}* clones could be attributed to increased apoptosis, we examined
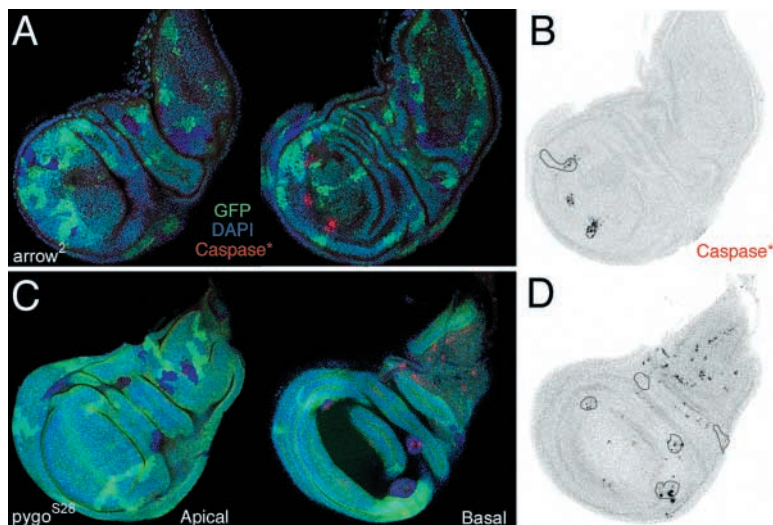
Fig. 2. Apoptosis in cells with reduced Wg signaling. (A,B) wing disc with *arrow²* mutant clones. (A) Disc labeled to visualize apoptotic cells with an antibody to activated caspase-3 (red). Clones were marked by the absence of GFP (green). Nuclei were labeled with DAPI (blue). Apical (left) and basal (right) optical sections are shown for the same disc. (B) Projection of four sections from the disc in A taken at 5 μm intervals, showing anti-capsase labeling. Clones are outlined. (C) *pygo^S28* clones marked by the absence of β-Gal (green). (D) Projection of four sections from the disc in C.

mutant clones for activation of caspase-3 (Yu et al., 2002). High levels of activated caspase-3 were found toward the basal surfaces of *arrow²* mutant clones, indicating apoptosis (Fig. 2A,B). A smaller increase in the number of apoptotic cells was found in *pygo^S28* mutant clones (Fig. 2C,D). The difference in the proportion of apoptotic cells seen in *arrow²* and *pygo^S28* mutant clones was consistent with the differences in their average size (Fig. 1G,H), suggesting that at least part of the growth defect an be attributed to reduced cell survival.

The Jun kinase (JNK) pathway is activated during apoptotic cell death in wing disc (Adachi-Yamada et al., 1999; Adachi-Yamada and O'Connor, 2002; Moreno et al., 2002). *puckered* (*puc*) encodes a dual-specificity phosphatase that is transcriptionally upregulated by JNK pathway activation (Martin-Blanco et al., 1998). A *puc-lacZ* reporter gene is induced in cells undergoing developmentally triggered apoptosis; for example, due to reduced Dpp signaling (Adachi-Yamada et al., 1999; Adachi-Yamada and O'Connor, 2002; Moreno et al., 2002). To test whether the JNK pathway was activated in cells with reduced Wg signaling, we examined *puc-lacZ* expression in *arrow²* mutant clones. *puc-lacZ* was induced in 17/28 clones examined (Fig. 3A). *puc-lacZ* was observed in apical sections. In more basally located optical sections, activated caspase-3 was observed in the same clones (Fig. 3B). In addition, *arrow²* mutant clones showed a genetic interaction with *puckered*. Recovery of *arrow²* mutant clones was reduced in the *puc-lacZ*/+ genetic background compared with an otherwise wild-type background (10/25 versus 21/29 clones/twins examined). These results indicate a contribution of JNK-mediated amplification to apoptosis in cells deprived of Wg signaling.

Clone growth defect is only partly due to increased cell death

To assess the contribution of cell death to the reduced size of arrow mutant clones, we made use of the FRT/Gal80 MARCM system (Lee and Luo, 1999) to direct Gal4-dependent expression of UAS-p35 transgene in FLP/FRT generated mutant clones. The viral caspase inhibitor protein p35 (Hay et al., 1994) was expressed and clone areas were measured (Fig. 4A-D). Expression of p35 allowed the recovery of *arrow²*

mutant cells that accumulated activated caspase-3. Many of these cells sorted out below the disc epithelium, but did not undergo apoptosis (Fig. 4D,E). Suppression of cell death only partially rescued clonal growth. *arrow²* clones expressing p35 were on average 56% of the size of wild-type clones expressing p35 in the wing pouch (Fig. 4B; $P<0.001$), compared with 17% wild-type clone size for *arrow²* mutant clones. As a second way of reducing apoptosis in the mutant clones, we expressed *puckered* to reduce JNK pathway activity. This led to reduced activation of Caspase-3 and fewer cells with pyknotic nuclei (Fig. 4H-J). Reducing JNK-mediated apoptosis partially rescued of the growth defect of *arrow²* clones compared with comparable wild-type clones (Fig. 4G). *arrow²* clones expressing *puckered* were ~32% the size of control clones expressing *puckered* ($P=3\times10^{-6}$), compared with 17% of the wild-type clone size for *arrow²* alone. As blocking apoptosis only partially suppressed the *arrow²* mutant clonal growth deficit, we conclude that reduced Wg signaling also
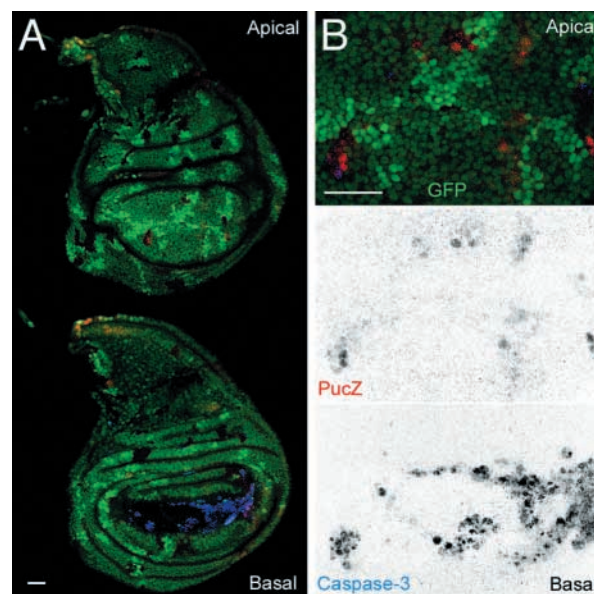

Fig. 3. JNK pathway activation in *arrow²* mutant clones. (A) Apical and basal optical sections of a wing disc with *arrow²* mutant clones. Clones were marked by the absence of GFP (green). Apoptotic cells were labeled using an antibody to activated caspase-3 (blue). *puc-LacZ* expression was visualized with anti-β-Gal (red).
(B) Magnification of the central region of the wing pouch of the disc in A. Lower panels show *puc-lacZ* and cleaved caspase-3. Scale bar: 25 μm.
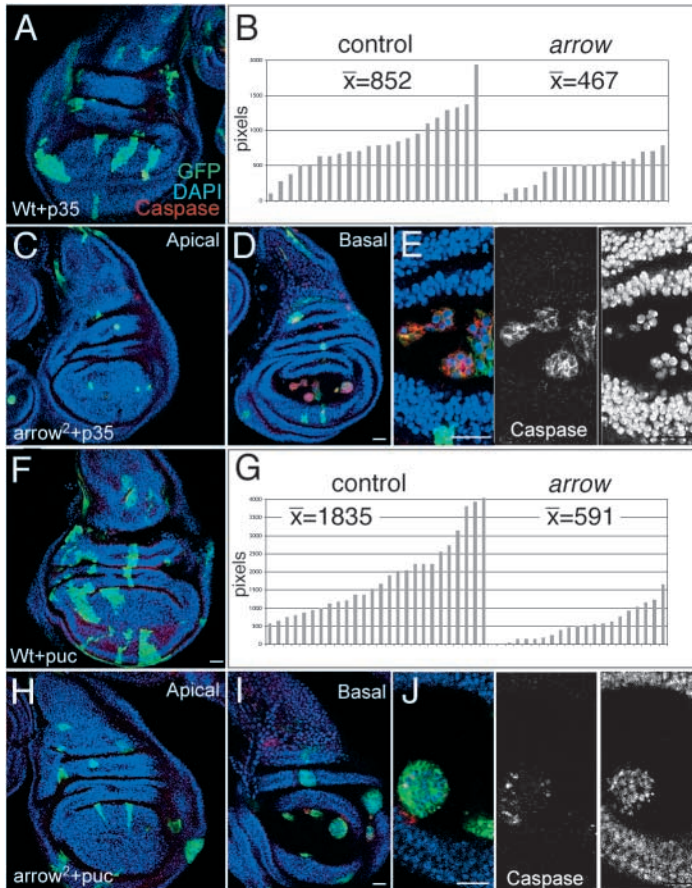
reduces cell proliferation. Thus Wg signaling appears to be required autonomously to support cell proliferation in the wing disc.

Cell competition

Slower-growing cells can be eliminated by competition with faster-growing cells in the developing wing disc (Morata and Ripoll, 1975; Simpson, 1979; Simpson et al., 1981). If cell survival in a clone is reduced as a result of cell competition, providing the mutant cells with a relative growth advantage by impairing the growth of the other cells can reduce cell death in the mutant clone (e.g. Moreno et al., 2002). To assess the contribution of cell competition, we produced clones of $arrow^2$ M^+ and $pygo^{S28}$ M^+ at 76±4 hours AEL in discs heterozygous for both mutations. Because homozygous M mutant cells die, we generated wild-type $M^{+/+}$ clones in a $M^{+/-}$ background as a control. The experimental and control larvae were grown in the same vial at low density to ensure comparable growth conditions. Recovery of $pygo^{S28}$ M^+ mutant clones was comparable to control M^+ clones (Fig. 5A,B), but average clone area was 59% of the controls (Fig. 5C; $P=4\times10^{-10}$). We found similar levels of activated caspase-3 in control M^+ and $pygopus^{S28}$ M^+ mutant cells (Fig. 5F,G). Thus, the reduced clonal growth observed under these conditions cannot be attributed to elevated cell death.

Even when provided with a growth advantage, recovery of $arrow^2$ M^+ clones was low compared with control M^+ clones (Fig. 5D,E). Caspase activation was high in $arrow^2$ M^+ and we

observed many pyknotic nuclei (Fig. 5E, inset). The fact that $arrow$ mutant cells died despite having been given a growth advantage suggests that the reduction of Wg signaling might be stronger in the $arrow^2$ mutant than in the $pygo^{S28}$ mutant clones. To test this, we generated genetic mosaics for both mutants in the same disc, and assessed the level of expression of Distal-less (Dll), a Wg target gene that is sensitive to the level of Wg activity (Neumann and Cohen, 1997; Zecca et al., 1996). Dll protein levels were reduced to a greater extent in $arrow^2$ mutant cells than in $pygo^{S28}$ mutant cells, indicating that $arrow^2$ compromises Wg signaling more severely that $pygo^{S28}$. Thus we can conclude that the lower levels of Wg signaling in $arrow^2$ mutants resulted in apoptosis, even when clones were given a growth advantage. The level of Wg signaling activity in $pygo^{S28}$ mutants reduced clonal growth but not primarily as a result of cell death. These findings suggest that Wg serves both as a survival factor and to stimulate cell proliferation during wing development. Thus Wg activity appears to be comparable to that of Dpp with respect to proliferation and cell survival (Martin-Castellanos and Edgar, 2002; Moreno et al., 2002).

Wg promotes cell proliferation

In view of the foregoing results, we examined the ability of Wg to promote cell proliferation in the wing disc. We used the FRT/Gal80 MARCM system (Lee and Luo, 1999) and Dpp-Gal4 to ectopically activate Wingless signaling, and used BrdU incorporation to label cells that had undergone DNA replication during a one-hour-labeling period. Cells in wing discs from control dpp-Gal4 UAS-GFP larvae incorporated BrdU in a uniformly random pattern (Fig. 6A; except for the normal zone of reduced proliferation near the DV boundary known as the ZNC) (Phillips and Whittle, 1993; Johnston and Edgar, 1998). Discs expressing Wg showed considerable expansion of the proximal wing pouch, as well as hinge and notum regions (Fig. 6C). BrdU incorporation was elevated in the proximal part of the wing pouch, indicating an increased rate of cell division. This was accompanied by an increase in the relative area of the proximal wing pouch, so we infer that Wg stimulated a net increase in cell proliferation (cell growth and division) in the proximal part of the wing. We noted a lower level of BrdU incorporation in the center of the wing pouch compared with control discs, which may reflect an expansion of the normal ZNC [which depends on Wg signaling from the DV boundary (Johnston and Edgar, 1998)]. The reduced proliferation observed in the center of the wing pouch presumably reflects an expansion of the endogenous ZNC, which contrasts with the increased cell proliferation observed toward the proximal edge of the wing pouch.

Fig. 5. Effects of reduced cell competition. (A,B) Wild type and pygo^{S28} Minute$^{+/+}$ in a Minute$^{+/-}$ heterozygous background. Clones were labeled by the absence of β-gal. (C) Histogram comparing sizes of the control and *pygo* mutant clones. Average areas were 2096 pixels (*n*=140) and 1236 pixels (*n*=55), respectively. (D,E) Wild-type and arrow^2 Minute$^{+/+}$ in a Minute$^{+/-}$ heterozygous background. Clones were labeled by the absence of Myc. The inset in E shows pyknotic nuclei in the basal region of the arrow^2 clone labeled with DAPI. (F,G) Wild-type and pygo^{S28} Minute$^{+/+}$ in a Minute$^{+/-}$ heterozygous background. Activated caspase-3 is shown in red in a projection of six single sections taken at 3.5 μm intervals. The clones are shown as a single channel of an apical section of the same image in green. Note the similar levels of cleaved caspase-3 in wild-type and pygo^{S28} mutant clones. Apoptotic cells were mainly found in the Minute$^{+/-}$

heterozygous cells adjacent to Minute$^{+/+}$ wild-type cells, as a result of cell competition. (H-K) Wing disc with both arrow^2 and pygo^{S28} mutant clones. Clones were labeled by the absence of GFP and β-gal. Dll is shown in blue. Note the lower levels of Dll present in arrow^2 clones (green) compared with pygo^{S28} clones (red).
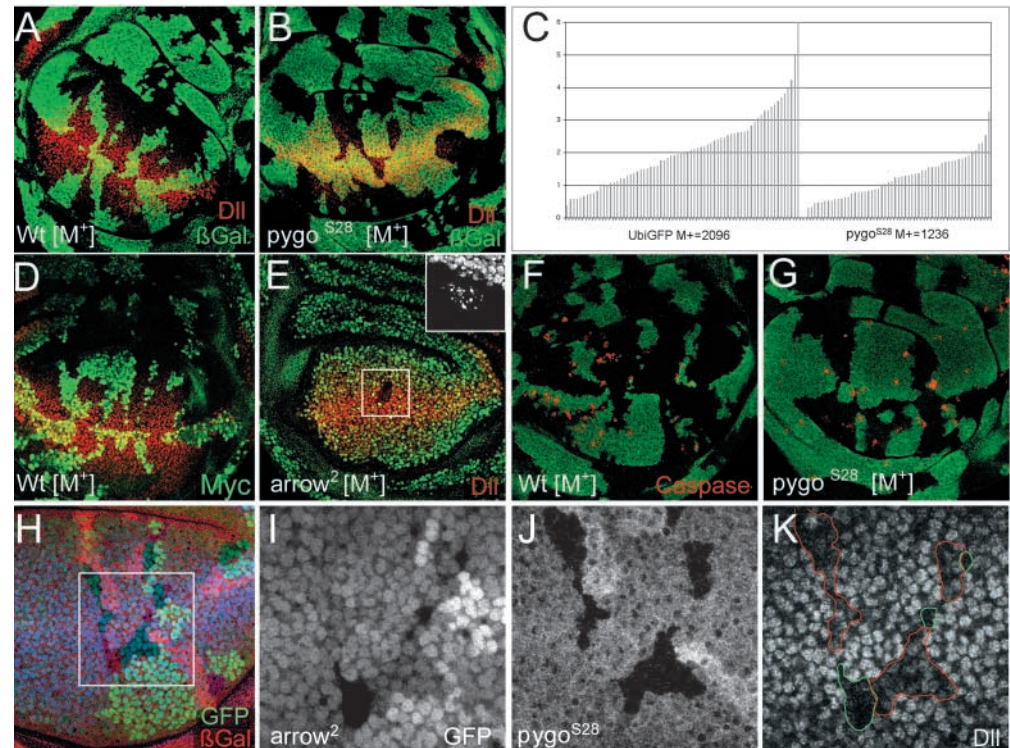
To further examine the contribution of Wg signaling to proliferation, clones of cells were generated in which the Wg pathway was autonomously activated using mutants for the
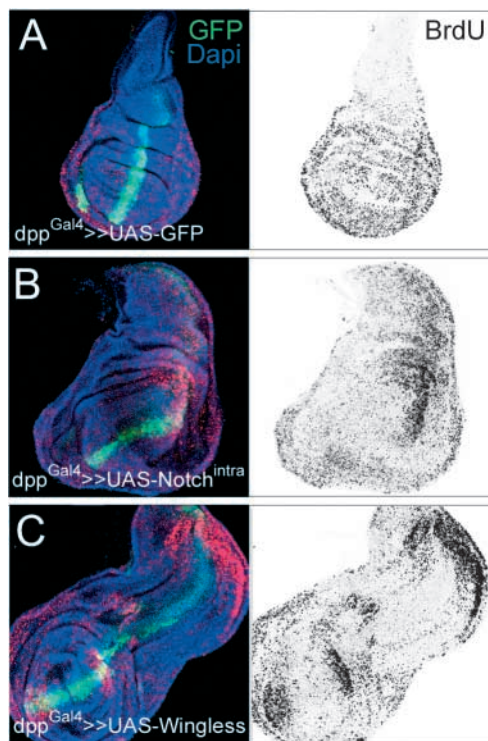
repressor protein *axin* (Hamada et al., 1999; Willert et al., 1999). *axin* mutant clones incorporated more BrdU than nearby cells in the pulse label, indicating that their proliferation rate was increased. This effect was cell autonomous, and stronger in the portions of the clones located at the proximal part of the wing pouch and in the hinge region (Fig. 7D, red arrow). Flow cytometry analysis showed fewer *axin* mutant cells in G1 compared with control cells in the same discs (at 115±4 hours AEL; Fig. 7E), and we observed a reduction in the levels of cyclin E in *axin* mutant cells in the wing pouch (Fig. 8C). As cyclin E regulates G1/S during imaginal disc development (Knoblich et al., 1994; Duman-Scheel et al., 2002), the effect of Wg in the proximal wing pouch may be to accelerate G1/S transition leading to an increase in S phase (increased BrdU incorporation) and to an accumulation of cells in G2. We also noted that *axin* mutant clones in the center of the wing pouch did not show elevated BrdU incorporation (similar to dpp-Gal4 UAS-Wg discs).

To examine cell cycle profiles for cells mutant for components of the Wg signaling pathway, we compared cell cycle profiles of control cells expressing GFP and the apoptosis

Fig. 6. Notch and Wg induce cell proliferation. (A-C) Wing imaginal discs labeled by incorporation of BrdU to mark cells that have gone through S phase (red). Nuclei were labeled with DAPI (blue). Cells expressing dpp-Gal4 and UAS-GFP were labeled with anti-GFP (green). (A) dpp-Gal4 UAS-GFP disc, (B) dpp-Gal4, UAS-GFP and UAS-Notch^{intra}, and (C) dpp-Gal4 UAS-GFP and UAS-Wg. Note the non-autonomous induction of BrdU incorporation near the GFP-expressing cells in B and C.
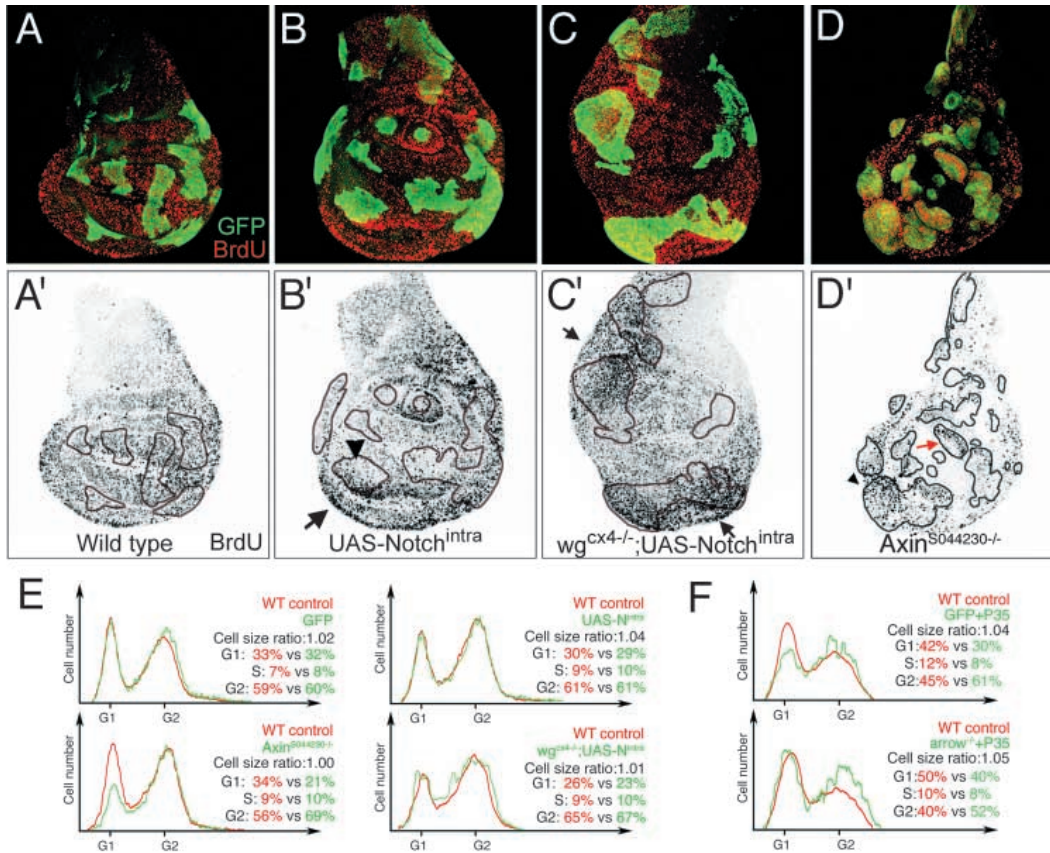
Fig. 7. Notch and Wg effects on cell proliferation and cell cycle phasing. (A-D) Wing imaginal discs labeled by incorporation of BrdU (red) to mark cells that have gone through S phase (projection of four optical sections taken at 5 μm intervals). BrdU channel is shown separately below. Clones were marked by the expression of GFP (green). (A) Wild-type clones expressing CD8-GFP. (B) Clones expressing Notch^{Intra} and CD8-GFP. (C) Clones expressing Notch^{Intra} and CD8-GFP, and mutant for *wg*. (D) *axin* mutant clones marked by expression of GFP. (E) Flow cytometric analysis of cells of the genotypes shown in A-D. Co-expression of GFP was used to sort mutant cells. (F) Flow cytometric analysis of cells expressing p35 and mutant for *arrow*.

inhibitor p35 with those of *arrow* mutant cells expressing GFP and p35. Expression of p35 increased the fraction of cells in G2 at expense of G1, compared with control cells in the same disc. Taking this into account, *arrow* mutant p35 expressing cells showed an increase in the percentage of cells in G1 at expense of G2 (Fig. 7F).

These observations suggest that Wg signaling induces net cell proliferation in the proximal wing pouch, perhaps by accelerating the G1 phase of the cell cycle, whereas blocking Wg signaling leads to an increase of cells in G1. Similar observations on the effects of Wg signaling on cell cycle phasing were recently reported by Johnston and Sanders, who also noted that the cells with elevated Wg signaling resemble cells from younger discs (Johnston and Sanders, 2003). In our view, this reflects Wg induced proliferation during the rapid phase of disc growth. As illustrated in Figs 6 and 7, Wg signaling continues to be able to induce net proliferation in the proximal part of the wing pouch, whereas the high levels of Wg activity near the DV boundary directs cells to pause in the G2 phase of the cell cycle (Johnston and Edgar, 1998). The basis for the proximodistal difference in the cellular response to Wg is not known, but parallels the normal formation of the ZNC in the distal wing pouch.
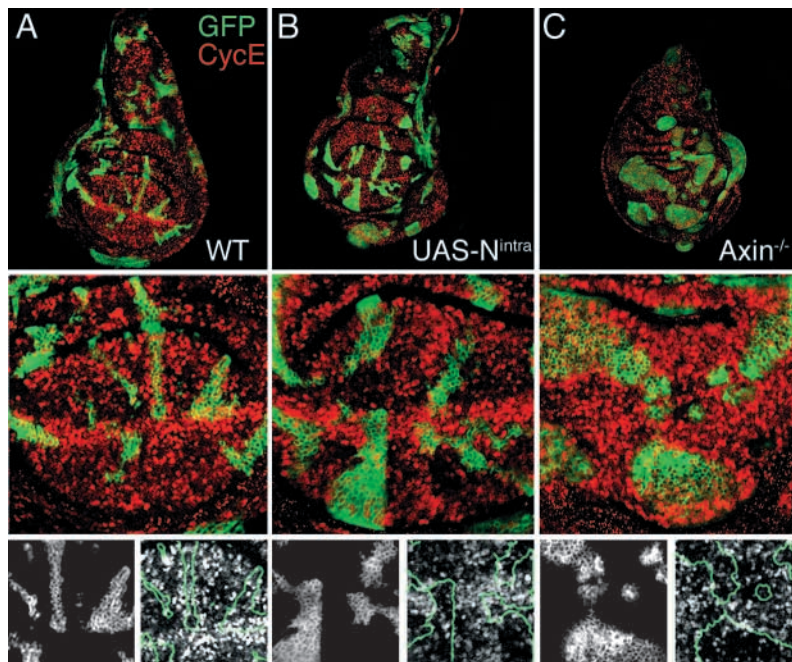

Fig. 8. Notch and Wg effects on cyclin E expression. (A) Cyclin E expression (red) in a wild-type disc containing clones of cells expressing GFP (green). (B) Reduced cyclin E expression in clones expressing activated Notch and GFP. (C) Reduced cyclin E expression in clones mutant for *axin* and expressing GFP. Lower panels show the GFP and cyclin E channels separately.

Notch promotes cell proliferation via Wg-dependent and Wg independent signals

Notch activity has also been implicated in the control of growth in the wing disc. Notch activation induces Wg expression in the wing pouch (Rulifson and Blair, 1995; Diaz-Benjumea and Cohen, 1995), so we expected that part of the effect of Notch could be mediated by induction of Wg expression. We have therefore assessed the effects of Notch activation on cell proliferation and tissue growth in the wing disc under conditions that allow Wg expression, and under conditions where Wg cannot be produced. Expression of the constitutively active form of Notch (Notch^{Intra}), under dpp-Gal4 control, resulted in overgrowth of the wing pouch, and an increase in BrdU incorporation in the proximal pouch and hinge regions (Fig. 6B), indicating increased cell proliferation. Activated Notch differs from Wg in that it also causes more proliferation throughout the notum. Clones of cells expressing Notch^{Intra} produced comparable results, causing extra cell proliferation in the proximal part of the wing pouch, as well as in hinge and notum (Fig. 7A,B). Within the wing pouch proximal cells proliferated more, whereas cells closer to the endogenous DV boundary proliferated less. Notch^{Intra}-expressing clones induce Wg expression and, when induced early enough, caused considerable overgrowth in the proximal wing pouch, as well as in the hinge and the notum (Fig. 9A,E; see Fig. S2 at http://dev.biologists.org/supplemental/). We noted that cells adjacent to Notch^{Intra}-expressing clones in the proximal wing pouch also over-proliferated (Fig. 7B; Fig. 9A), perhaps due to their ability to induce Wg, which can induce proliferation of proximal wing pouch cells (Fig. 6). Interestingly, N^{Intra} expression in wg^{cx4} mutant cells was still able to cause a non-autonomous increase in cell proliferation, measured by BrdU incorporation (Fig. 7C). However, we noted that in the absence of Wg expression, Notch-induced proliferation was mostly restricted to the wing hinge and pleura, with little proliferation observed in the proximal part of the wing pouch (Fig. 7C and Fig. 9B,C). In the proximal wing pouch the induction of ectopic growth was considerably reduced. Although N^{Intra}-expressing cells (wild type for the *wg* gene) induced overgrowth in the proximal pouch in 75% of the cases, only 20% of N^{Intra}-expressing clones (mutant for *wg*) caused overgrowth (Fig. 9E).

FACS analysis showed no difference in the cell cycle parameters of control cells and cells expressing N^{Intra} in the same disc, whether or not they can produce Wg (Fig. 7E). We observed reduced levels of Cyclin E close to the DV boundary in N^{Intra}-expressing cells (Fig. 8B). These observations suggest three conclusions. (1) Wg expression contributes significantly to the ability of Notch to induce tissue growth in the wing pouch. (2) Notch appears to be able to induce another signaling protein that contributes to non-autonomous induction of tissue growth in the proximal parts of the wing pouch, and in the hinge and pleura regions. (3) As Notch activity drives balanced cell proliferation without causing cells to accumulate in G2, the second Notch dependent signal may promote G2/M transition, so that, when expressed with Wg, cells do not accumulate in G2 (as they do when Wg is expressed alone).

Discussion

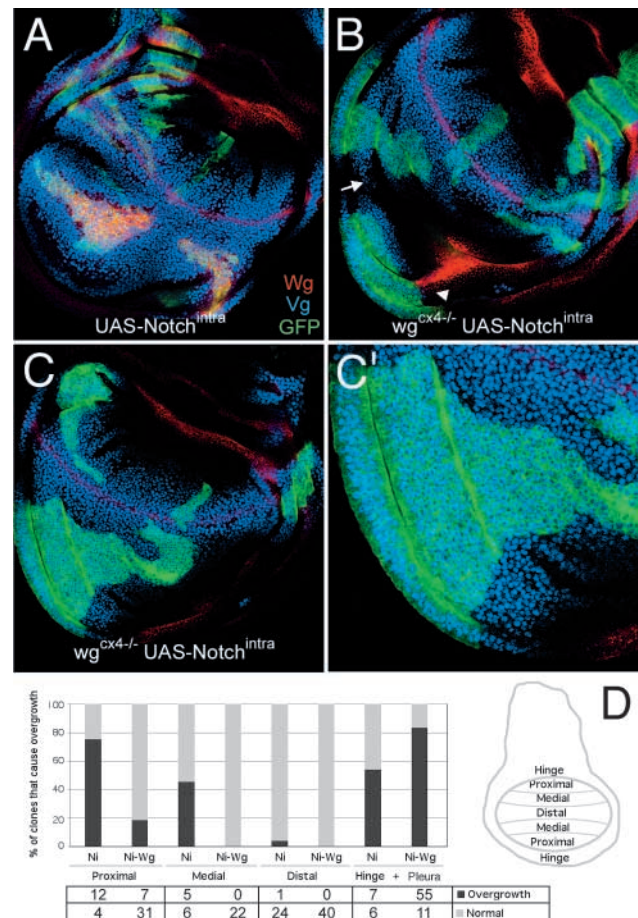In this study we present evidence that (1) Wg is required for

Fig. 9. Effects of Notch on growth, dependent and independent of Wg. (A-C) Clones expressing UAS-Notch^{intra} labeled with CD8-GFP (green), produced using the Gal4/Gal80 system. Wg protein (red); Vestigial (Vg) protein (blue). (A) Otherwise wild-type clones. (B,C) Clones also mutant for wg^{cx4}. Clones expressing Notch^{intra} were not able to induce overgrowth in the wing pouch in the absence of Wg expression, but did induce overgrowth in the wing hinge and pleura. (C′) Detail of panel C. (D) Histogram comparing the percentage of Notch^{intra}-expressing clones able to induce tissue overgrowth in different areas of the wing pouch and in the wing hinge in the presence or the absence of *wg*. Their ability to induce tissue growth was dependent on their position. The dark gray bar indicates clones that induced overgrowth. The number of clones examined is shown in below. The diagram to the right indicates the different areas used for quantification.

cell survival, and for cell proliferation and tissue growth, in the developing wing primordium; and (2) Notch and Wg act synergistically to promote tissue growth, and that Notch also acts through another non-autonomous signal. Interestingly, the effects of Wg and Notch change as the wing disc matures. In the following discussion we distinguish between their proliferation-inducing effects during the rapid phase of disc growth and their ability to promote proliferation exit in distal wing cells at later stages (Johnston and Edgar, 1998).

Wingless

Our findings suggest that Wg signaling is required to support cell survival and to promote cell proliferation during the rapid

phase of wing disc growth. Cells deprived of Wg signaling die as a result of cell competition and apoptosis. In the absence of cell competition, loss of Wg signaling still results in cell death, thus supporting the idea that Wg functions as a survival factor to the cells in the wing. In addition, two types of evidence suggest that Wg is required to support a normal rate of cell proliferation and tissue growth. (1) Clones of cells deprived of Wg signaling grow and divide more slowly than normal cells, even when cell death is prevented. In our hands, *arrow* mutant clones expressing p35 or *puckered*, to block cell death, were always smaller than control clones (Fig. 4). In this respect, our findings differ from those of Johnston and Sanders, who reported that clones expressing a dominant-negative form of TCF and p35 to prevent cell death were larger than control clones (Johnston and Sanders, 2003). (2) We have also presented evidence that Wg expression or activation of Wg signaling in *axin* mutant clones increased cell proliferation in the wing, as measured by BrdU incorporation. In mature third instar discs, Wg increased cell proliferation in the proximal part of the wing, while at the same time inhibiting proliferation distally, near the endogenous ZNC. This was also true of *axin* mutant clones, which were very small distally, but proliferated more rapidly than control cells proximally. Thus, we suggest that Wg-induced proliferation reflects the effects of Wg during the rapid growth phase of the disc. Indeed, reduced Wg function during second and early third instar can reduce the size of the entire wing pouch (Couso et al., 1994; Neumann and Cohen, 1996b; Neumann and Cohen, 1997).

At later stages the role of Wg becomes more complex. High levels of Wg signaling during the last 30 hours of the larval period promote a band of distal cells to exit proliferation and pause in G2 to form the ZNC (Phillips and Whittle, 1993; Johnston and Edgar, 1998). In this context, Wnt signaling has been shown to contribute to regulation of the *bantam* miRNA, which controls the rate of cell proliferation in the ZNC (Brennecke et al., 2003). Our findings suggest that proximal and distal cells continue to respond differently to Wg signaling even at this stage of wing disc development. The basis for this proximal-distal difference in the cellular response to Wg is not known. We suggest that Wg contributes to controlling survival and proliferation during the phase of rapid disc growth, and that the opposing effects seen in distal cells in the late third instar reflects a distinct process. Dpp signaling has a similar profile of effects on cell growth and survival. Ectopic activation of the Dpp pathway autonomously induces cell proliferation in the wing (Martin-Castellanos and Edgar, 2002). As for Wg, cells responded differently to according to their position. Cells far from the normal source of Dpp respond more strongly than cells close to the source. Dpp signaling also provides survival cues (Moreno et al., 2002).

Notch

Activation of Notch has similar effects on wing disc growth to Wg expression. In particular, we note that Notch activity stimulated cell proliferation proximally, and not evenly, in all cells of the clones expressing activated Notch. This observation is difficult to reconcile with the idea that Notch activity per se drives proliferation. We provide evidence that the non-autonomous effects of Notch are mediated in part through Wg and in part through at least one other signaling protein. We observed two different responses to Notch activation. (1)

Notch[Intra] induced non-autonomous overproliferation that was dependent on the ectopic expression of Wg in the wing pouch. (2) Notch[Intra] induced non-autonomous overgrowth in the wing hinge and the notum independently of Wg production. These cells still required a Wnt signal as *arrow²* mutant clones expressing Notch[Intra] did not overgrow (not shown). These observations support a model in which the Notch and Wg pathways act synergistically to induce cell proliferation and tissue growth during early larval stages. We propose that Wg activity may stimulate cells to enter S phase, and thus may contribute to Notch induced proliferation. The other Notch-induced signal may promote mitosis.

Wnt signaling proliferation in development and cancer

Comparable roles for the Wnt pathway in controlling tissue growth have been reported in vertebrate development. Wnt1-mediated signaling stimulates cell proliferation in the developing CNS (Dickinson et al., 1994) and in the pituitary gland (Treier et al., 1998). The Wnt pathway also controls cell proliferation and self-renewal of the intestinal epithelia through β-catenin/Tcf4-mediated transcription of the oncogene Myc (van de Wetering et al., 2002). The Wnt pathway has been shown to act through the transcription factor Pitx2 to promote cell proliferation in the developing pituitary and heart (Kioussi et al., 2002). These few examples illustrate that Wnt signaling plays a role in developmentally controlled cell proliferation in vertebrates.

Mutations that activate the Wnt pathway have been associated with uncontrolled proliferation in humans and mice leading to cancer (reviewed by Polakis, 2000; Taipale and Beachy, 2001). Mutations seem to be preferentially associated with specific types of tumors. Notably, APC mutations occur frequently in colorectal cancer. Humans with germ-line mutations in APC develop familial adenomatous polyposis coli referred to as Gardner Syndrome. These patients mainly develop colorectal cancer and hepatoblastoma, as well as jaw and sebaceous cysts. Other tissues are more weakly affected (see http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db= OMIM). This might reflect cell-type specific growth responses to altered Wnt signaling, perhaps analogous to the region-specific effects of Wg that we observed in the wing disc.

The dependence of cells on extrinsic survival factors provides a powerful mechanism to regulate cell number during development (reviewed by Conlon and Raff, 1999). Although cell death does not seem to play a major role in regulating tissue size during wing development, cell number is controlled by apoptosis in other contexts, such as the *Drosophila* eye and nervous system. In a subset of midline glial cells, activation of the EGFR pathway controls cell number by suppression of the pro-apoptotic protein Hid (Bergmann et al., 2002). Interestingly, cells in the intestinal epithelia far away from the domain of Wnt activation undergo apoptosis and/or extrusion into the lumen (van de Wetering et al., 2002). Given our observation that Wg provides survival cues in the wing disc, it seems worth considering whether APC/β-catenin mutations might contribute to the development of colorectal carcinoma by making intestinal cells refractory to apoptotic signals.

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