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The function and regulation of *cut* expression on the wing margin of *Drosophila*: Notch, Wingless and a dominant negative role for Delta and Serrate

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SUMMARY

We have investigated the role of the Notch and Wingless signaling pathways in the maintenance of wing margin identity through the study of *cut*, a homeobox-containing transcription factor and a late-arising margin-specific marker. By late third instar, a tripartite domain of gene expression can be identified about the dorsoventral compartment boundary, which marks the presumptive wing margin. A central domain of *cut*- and *wingless*-expressing cells are flanked on the dorsal and ventral side by domains of cells expressing elevated levels of the Notch ligands *Delta* and *Serrate*. We show first that *cut* acts to maintain margin *wingless* expression, providing a potential explanation of the *cut* mutant phenotype. Next, we examined the regulation of *cut* expression. Our results indicate that Notch, but not Wingless signaling, is autonomously required for *cut* expression. Rather, Wingless is required indirectly for *cut* expression; our results suggest this requirement is due to the regulation by *wingless* of *Delta* and *Serrate* expression in cells flanking the *cut* and *wingless* expression domains. Finally, we show that *Delta* and *Serrate* play a dual role in the regulation of *cut* and *wingless* expression. Normal, high levels of Delta and Serrate can trigger *cut* and *wingless* expression in adjacent cells lacking *Delta* and *Serrate*. However, high levels of Delta and Serrate also act in a dominant negative fashion, since cells expressing such levels cannot themselves express *cut* or *wingless*. We propose that the boundary of Notch ligand along the normal margin plays a similar role as part of a dynamic feedback loop that maintains the tripartite pattern of margin gene expression.

Key words: pattern formation, cell signaling, *Drosophlia* wing imaginal disc, *Notch*, *wingless*, *cut*, *Delta*, *Serrate*, *disheveled*, *shaggy-zeste white 3*

INTRODUCTION

The specification of distinct cell types within the growing wing disc epithelium depends in large part on interactions between adjacent cells or cell populations. Many of these interactions subdivide the disc in a step-wise fashion. Thus, the disc is initially divided into a small number of lineage compartments, between which cells will not mix. The wing disc is first divided into anterior and posterior (A/P) compartments, and later into dorsal and ventral (D/V) compartments. Interactions between cells in adjacent compartments can then locally define specific cells at compartment boundaries; for instance, Hedgehog secreted by posterior cells signals to cells just to the anterior of the A/P boundary. Finally, boundary cells can themselves subdivide into smaller boundary-specific regions and signal to cells further from the boundary (reviewed in Blair, 1995; Lawrence and Struhl, 1996).

The Notch (N) and Wingless (Wg) signaling pathways play important roles during the development of imaginal discs (pathways reviewed in Artavanis-Tsakonas et al., 1995; Klingensmith and Nusse, 1994). In the wing, these pathways mediate ongoing patterning processes, which overlap in space and time. It is thought that, early in development, N and/or Wg are responsible for the reciprocal signaling between dorsal and ventral compartments, which defines cells near the D/V boundary (Couso et al., 1995; Kim et al., 1995, 1996; Rulifson and Blair, 1995; Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996; de Celis et al., 1996a,b; Neumann and Cohen, 1996; Jonsson and Knust, 1996). Later in development these signals help subdivide the region near the D/V boundary into a number of distinct subregions, and maintain those subdivisions during growth and metamorphosis (Phillips and Whittle, 1993; Couso et al., 1994; Rulifson and Blair, 1995; Rulifson et al., 1996). In this paper, we investigate the distinct roles of N and Wg in establishing and maintaining margin-specific regions of gene expression, concentrating especially on the function and regulation of *cut* (*ct*).

ct encodes a homeodomain transcription factor (Blochlinger et al., 1988) with significant structural and functional similarity to several vertebrate proteins (see Ludlow et al., 1996). *ct* has a well-established role specifying neuronal cell fates within the embryonic peripheral nervous system (PNS), where *ct* is

expressed in external sensory organs (Blochlinger et al., 1990). Removing *ct* transforms external sensory organs into chordotonal organs (Bodmer et al., 1987), while ectopic expression of *ct* causes chordotonal organs to differentiate as external sensory organs (Blochlinger et al., 1991). Thus, *ct* can function as a bimodal switch during cell fate decisions.

ct also plays a distinct role during the development of the D/V boundary in the wing imaginal disc, the site of the future margin of the wing blade. The D/V boundary is first established in mid-second instar at the junction between dorsal, *apterous*-expressing and ventral, non-expressing cells (reviewed in Blair, 1995). *ct* is one of several 'margin-specific' genes expressed in response to the *apterous* boundary (Blair, unpublished data). *ct* is initially expressed in a narrow row of cells, 2-5 cells wide, along the presumptive wing margin beginning at mid to late third instar (Jack et al., 1991; Blochlinger et al., 1993). This row of cells straddles the D/V boundary, and is largely coincident with the region which, slightly earlier in development, expresses *wg* and the *vestigial* (*vg*) intron 2 enhancer (Blair, 1993, 1994; Williams et al., 1994). These 'edge' cells (Couso et al., 1994) delineate a distinct subregion of margin cells located between the dorsal and ventral rows of margin bristle precursors; while the bristle precursors, like other external sensory organs, eventually express *ct*, they only begin doing so several hours after pupariation (Blair, 1993). Loss of *ct* expression from the edge cells results in the loss not only of the *ct*-expressing edge cells and bristles, but also of adjacent epithelial cells, which do not express *ct*; the cell loss is apparently due to cell death during subsequent pupal stages (Jack et al., 1991; Dorsett, 1993).

Thus, the *ct* transcription factor is required for a long-range signal or process that maintains cells both in and adjacent to the region of *ct* expression. The wing defects observed in *ct* mutants are similar to those caused by reductions in Wg signaling. Removing *ct* from both sides of the D/V boundary results in extensive notching of the margin (Jack et al., 1991; Dorsett, 1993), while reducing *ct* function on one side only occasionally induces notching (Santamaria and Garcia-Bellido, 1975; see Discussion). Similarly, removing Wg or the ability to receive the Wg signal from both sides of the D/V boundary results in notching of the margin; removing the ability to receive Wg from one side of the D/V results in the autonomous loss of margin bristles and proneural gene expression without notching (Baker, 1988b; Couso et al., 1994; Diaz-Benjumea and Cohen, 1995; Axelrod et al., 1996; Rulifson et al., 1996). Removing Wg function during mid to late third instar, using temperature-sensitive alleles, also results in incomplete formation and/or loss of wing margin structures (Phillips and Whittle, 1993; Couso et al., 1994; Diaz-Benjumea and Cohen, 1995). We will show below that *ct* is in fact required for the maintenance of margin *wg* expression; this loss of *wg* may play a role in the *ct* wing notching phenotype (see Discussion).

However, it is unclear how *ct* expression is established and maintained. The evidence to date suggests that *ct* expression is regulated either by *N*, *wg*, or both. *N* is required during the third instar for the formation and maintenance of adult wing margin structures, and the expression at late third instar of many margin-specific genes; reduction or loss of the N ligands encoded by *Delta* (*Dl*) or *Serrate* (*Ser*) also induces margin notching in adult wings and the loss or reduction of margin-specific gene expression (Shellenbarger and Mohler, 1978; Jack and DeLotto, 1992; Parody and Muskavitch, 1993; Speicher et al., 1994; de Celis and Garcia-Bellido, 1994a; Thomas et al., 1995; Rulifson and Blair, 1995; Kim et al., 1995; Diaz-Benjumea and Cohen; 1995; Couso et al., 1995; Doherty et al., 1996; de Celis et al., 1996a; Jonsson and Knust, 1996). *ct* expression is reduced in *N* hypomorphs and by the dominant allele of *Ser* (Jack and DeLotto, 1992; Thomas et al., 1995; de Celis et al., 1996a), and is lost from *Suppresser of Hairless* (*Su(H)*) clones (Neumann and Cohen, 1996). We will show below that *ct* is also lost from N^- clones. *N* gain-of-function mutations, or overexpression of N or N ligands, can induce ectopic *ct* expression (Thomas et al., 1995; Doherty et al., 1996; de Celis et al., 1996a; Neumann and Cohen, 1996; Jonsson and Knust, 1996). However, manipulations that cause gain or loss of N signaling also result in the ectopic expression or loss of *wg*, respectively (Thomas et al., 1995; Rulifson and Blair, 1995; Kim et al., 1995, 1996; Couso et al., 1995; Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996; de Celis et al., 1996a; Neumann and Cohen, 1996; Jonsson and Knust, 1996). Since removal of Wg function using either a temperature-sensitive allele or null clones results in a loss of margin *ct* expression (Couso et al., 1994; Neumann and Cohen, 1996), it is possible that these N-mediated effects are indirect.

Therefore, we have used clonal analysis and temperature-sensitive mutants to directly test the role of *N* and *wg* in regulating *ct* expression on the presumptive wing margin. Our results indicate that *ct* is a direct target of N but not Wg. *N* function was required autonomously during mid through late third instar for *ct* expression on the margin. In contrast, *ct* expression was observed in clones that are unable to receive Wg signal. Our evidence will further suggest that *wg* acts indirectly to establish or maintain margin *ct* expression by directing expression of high levels of the N ligands *Dl* and *Ser* in cells adjacent to the *ct* and *wg*-expressing cells.

However, this presents an apparent paradox, as *ct* is not expressed in cells expressing high levels of *Dl* and *Ser*; *Dl* and *Ser* expression is highest in cells to either side of the edge cells and drops abruptly within the edge cells themselves. We will demonstrate that cells expressing high levels of *Dl* and *Ser* cannot express *ct* and *wg*, but are capable of triggering margin-like levels of *ct* and *wg* in adjacent cells lacking *Dl* and *Ser*. Thus, high levels of Dl and Ser appear to act in a cell autonomous dominant negative fashion. We propose that the boundary between flanking cells, which express high levels of *Dl* and *Ser*, and the edge cells, which express much lower levels, directs or maintains *ct* and *wg* expression within the edge cells.

MATERIALS AND METHODS

All genetics, clone generation, gene overexpression, immunohistochemistry, in situ hybridization, and light and confocal microscopy were as previously described (Rulifson and Blair, 1995; Rulifson et al., 1996), with the following additions.

Primary antisera: 1/400 rabbit anti-Dsh (kindly provided by R. Nusse), 1/1000 guinea pig anti-Dl (kindly provided by M. Muskavitch), 1/1000 rabbit anti-Ser (Speicher et al., 1994; kindly provided by E. Knust), 1/2000 rabbit anti-Cut (kindly provided by K. Blochlinger), 1/1000 rat anti-Apterous (Lundgren et al., 1995; kindly provided by J. Thomas).

Mutant stocks: ct^{C145} is a lethal amorphic allele (Bodmer et al.,

1987) that eliminates anti-Cut staining (not shown); we generated the ct^{C145} FRT^{18A} line. ct^{2s} was kindly provided by P. Morcillo. Dl^{rev10} is a null allele (Doherty et al., 1996); the FRT^{82B} Dl^{rev10} line was kindly provided by D. Doherty. We used Dl^{RF}/Dl^{B2} for Dl^{ts} experiments; Dl^{RF} is a temperature-sensitive allele (Parody and Muskavitch, 1993) and Dl^{B2} is an amorphic allele, both kindly provided by M. Muskavitch. Ser^{RX82} and Ser^{RX106} are null alleles (Thomas et al., 1991; Spreicher et al., 1994). We generated the FRT^{82B} Ser^{RX106} line; the FRT^{82B} Ser^{RX82} Dl^{rev10} was kindly provided by G. Struhl. dsh^{-} clones used svb^{YP17b} dsh^{v26} FRT^{101} or *y w* dsh^{75} FRT^{101}, which gave identical results. N^{-} clones used N^{55e11} FRT^{18A}, N^{-} dsh^{-} clones used N^{1081} svb^{YP17b} dsh^{v26} FRT^{101}, wg^{-} used FRT^{42D} wg^{CX4} and *sgg-zw3*$^{-}$ used *sgg-zw3*^{D127} FRT^{18A}. Each was crossed to appropriate πM-FRT-FLP stocks.

RESULTS

Gene expression on the developing wing margin

The expression patterns of *ct*, *wg* and the N ligands *Dl* and *Ser* change during development of the wing disc. From mid to late second instar (60-48 hours before pupariation, BP) *Ser* is expressed throughout the dorsal compartment (Couso et al., 1995; Diaz-Benjumea and Cohen, 1995); *Dl* is expressed in both dorsal and ventral compartments (Doherty et al., 1996; de Celis et al., 1996a); *wg* is expressed in a ventral domain that overlaps slightly into the dorsal compartment (Couso et al., 1993, 1994; Williams et al., 1993; Phillips and Whittle, 1993; Ng et al., 1996). At early to mid third instar (48-24 hours BP), this pattern changes. *Dl* and *Ser* both are expressed at higher levels near the D/V boundary. *wg* is expressed throughout the prospective wing blade and, at higher levels, in a wide stripe concentrated near the margin; our results indicate that this stripe is initially stronger on the ventral side of the D/V boundary (Fig. 1A), coincident with the higher expression of *Dl* (Fig. 1B).

From mid to late third instar (24-12 hours BP), this pattern further refines (Fig. 1B-D). *wg* becomes expressed almost exclusively in a narrow stripe of cells straddling the D/V boundary termed the 'edge' cells (Baker, 1988a; Blair, 1993, 1994; Couso et al., 1993, 1994). *Dl* and *Ser* are expressed at higher levels immediately flanking this stripe but levels are lowered within the edge cells (Kooh et al., 1993; Speicher et al., 1994). *Dl* and *Ser* are also expressed at high levels along broad 'prevein' regions, and *Ser* retains a dorsal emphasis at late third instar. *ct* also becomes expressed in the edge cells (Jack et al., 1991; Blochlinger et al., 1993; Blair, 1993) at approximately the same stage as when the decrease in Dl and Ser levels within the edge cells first becomes apparent (Fig. 1B,C). Thus, by late third instar (12-0 hours BP), gene expression defines a tripartite domain of cells about the D/V boundary: the edge cell region, approximately 2-5 cells wide, and the two flanking regions (Fig. 1D). The flanking regions in the anterior also express high levels of members of the *achaete-scute* complex (Romani et al., 1989), and thus approximate the 'proneural' regions from which dorsal and ventral rows of margin sensory bristles arise. *wg* is also expressed in two rings surrounding the presumptive wing blade and in a stripe in the notal region of the disc. *N* expression at late third instar varies spatially (Fehon et al., 1991; Hing et al., 1994), but is at significant levels throughout the disc (see Fig. 4B in Rulifson and Blair, 1995).

cut is autonomously required to maintain margin *wingless* expression

During late third instar, *ct* and *wg* share a common domain of expression along the presumptive wing margin and many aspects of their mutant phenotypes appear consistent (see Discussion). We have therefore tested whether *ct* mutations alter margin *wg* expression.

In ct^{2s}, a small deletion specifically disrupts the function of the *ct* wing margin enhancer; this enhancer is necessary and sufficient to drive *ct* expression along the wing margin (Jack et al., 1991; Mogila et al., 1992; Dorsett, 1993). We examined the distribution of both *wg* protein and RNA in ct^{2s} wing discs at mid-late third instar and white prepupal (WPP, 0 hour BP) stages. The mid-late third instar discs displayed a nearly wild-type distribution of *wg* transcript and protein along the margin (Fig. 2B). In WPP discs, however, there was significant reduction of *wg* transcript levels, varying from thinning of the *wg*-expressing stripe to its complete loss (Fig. 2C). Similar results were observed using anti-Wg (not shown).

This *ct* requirement is cell autonomous. ct^{-} clones that intersected the edge cells showed cell autonomous reductions or loss of *wg* (Fig. 2D,E). While ct^{-} cells in some younger discs

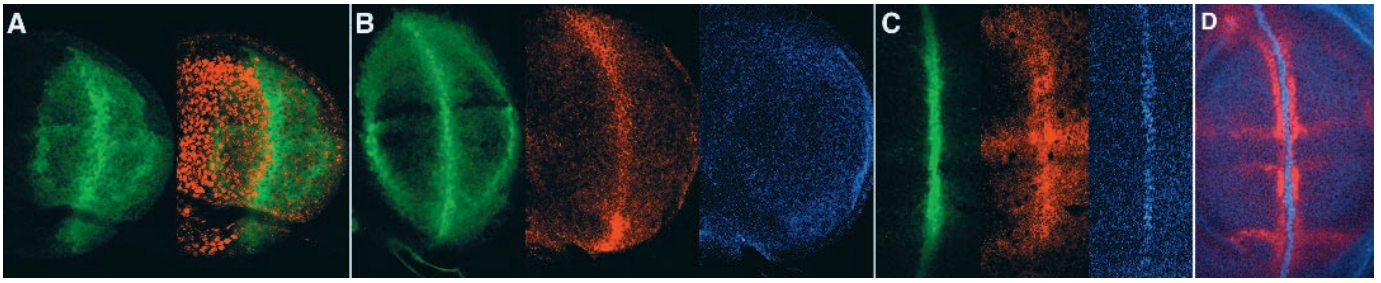

Fig. 1. Expression patterns in *wg-LacZ* wing discs, stained with anti-β-gal (green). In this and subsequent figures, anterior is up and dorso-proximal left; A-C are at the same magnification; D is at lower magnification. (A) Early-mid third instar disc, stained also with anti-Ap to show dorsal compartment (red). Note *wg-lacZ* expression throughout the prospective wing blade and heightened expression along the margin, largely on the ventral side. (B) Mid third instar, stained also with anti-Dl (red) and anti-Ct (blue). Heightened *Dl* expression follows the heightened margin expression of *wg-lacZ*. No *ct* expression is detectable. (C) Mid-late third instar. The region of margin *wg-lacZ* expression has narrowed to the 'edge' cells, and most or all of the wing blade expression is lost. *Dl* expression has begun to fade from the edge cells, and is heightened in flanking cells and in a broad L3 prevein region. *ct* is now also detected in the edge cells. (D) Late third instar, stained with anti-Dl and anti-Ct. *Dl* is expressed in the pre-vein regions and cells flanking the margin edge cells. *ct* is expressed in the edge cells, as is *wg-lacZ* (not shown).

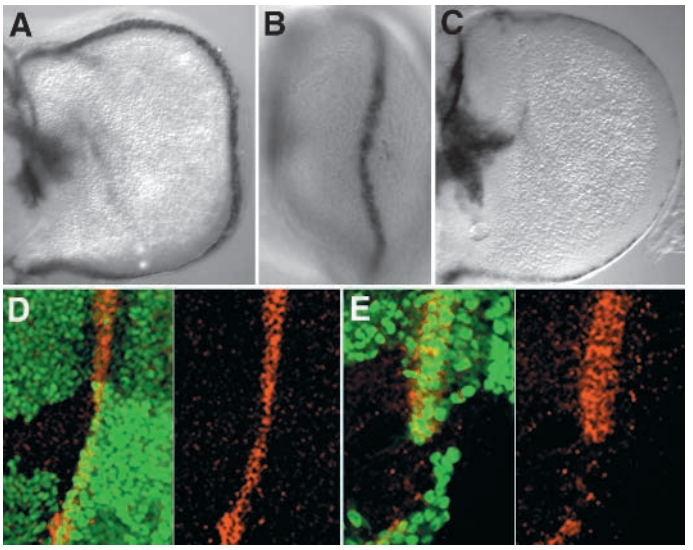

Fig. 2. Regulation of *wg* expression by *ct*. (A-C) In situ hybridization showing levels of *wg* transcript along prospective wing margins. (A) Wild type, WPP stage. (B) ct^{2s}, mid-late third instar; *wg* expression is nearly wild type. (C) ct^{2s}, WPP; *wg* expression is almost totally eliminated. (D,E) anti-Wg staining (red) in ct^{-}clones (indicated by absence of green πM marker). (D)ct^{-}clone limited to dorsal compartment eliminates dorsal but not ventral *wg* expression (note thinning of normal *wg*-expressing stripe). (E) ct^{-}clone that crossed the D/V boundary autonomously eliminates most or all anti-Wg staining.

occasionally displayed traces of Wg protein, clones in older discs did not show any detectable *wg* expression. *ct* clones that crossed the compartment boundary completely lacked *wg* expression on both sides of the margin (Fig. 2E), while those positioned in the interior of the wing blade had no effect upon *wg* expression. Therefore, while the initiation of *wg* expression along the margin during mid third instar is not dependent on *ct*, maintenance of *wg* through late third instar is *ct* dependent. The expression of *wg* in the two rings encircling the presumptive wing and in the notum are not coincident with *ct* expression during third instar and were not sensitive to *ct* loss.

N is required for *cut* expression on the margin

While previous results suggest that N is involved *ct* expression (see Introduction), the autonomy and penetrance of this requirement has not been previously tested. Therefore, we examined *ct* expression in clones lacking *N* (Fig. 3A). N^{-}clones confined to either the dorsal or ventral compartment caused a cell autonomous loss of *ct* expression. Such clones also had domineering non-autonomous effects, such that clones that abutted the D/V boundary on one side without crossing resulted in a loss of *ct* expression on both sides of the D/V boundary. In some cases *ct* expression was also lost a few cell diameters anterior or posterior to the clone; however, this may be due to the sporadic loss of *ct* observed in N^{-}heterozygotes (not shown). Clones in the interior of the wing did not show any detectable phenotype. These N^{-}phenotypes are similar to those previously described in adult wings (de Celis and Garcia-Bellido, 1994a) and in activating *wg* and the *vg* second intron enhancer (Rulifson and Blair, 1995; Diaz-Benjumea and Cohen, 1995). The domineering aspects of the phenotypes are likely due to N's earlier role in reciprocal signaling between dorsal and ventral compartments (see Introduction).

Analysis of the temperature-sensitive *N* genotype, N^{55e11}/N^{ts1}, show that *N* is required for *ct* expression from mid to late third instar. Larvae shifted to the nonpermissive temperature from 24 to 0 hours BP showed a complete loss of *ct* expression along the margin (Fig. 3B,C). Larvae shifted from 12 to 0 hours BP showed thinning and incomplete loss of expression which was most pronounced at the distal tip of the margin (not shown). *wg* expression is also lost from such discs (Rulifson and Blair, 1995); double staining for *wg* and *ct* expression showed that *ct* loss was always more extreme (Fig. 3C).

Reception of the Wg signal is not required for *cut* expression

To test if *wg* is required directly for *ct* expression, clones were generated that were incapable of receiving the Wg signal. *dsh*, a ubiquitously expressed cytoplasmic protein, is required in a cell- autonomous fashion for the reception of the Wg signal (Klingensmith et al., 1994; Noordermeer et al., 1994; Theisen et al., 1994; Couso et al., 1994; Rulifson et al., 1996). We found that dsh^{-}clones that intersected the *ct*-expressing cells on the

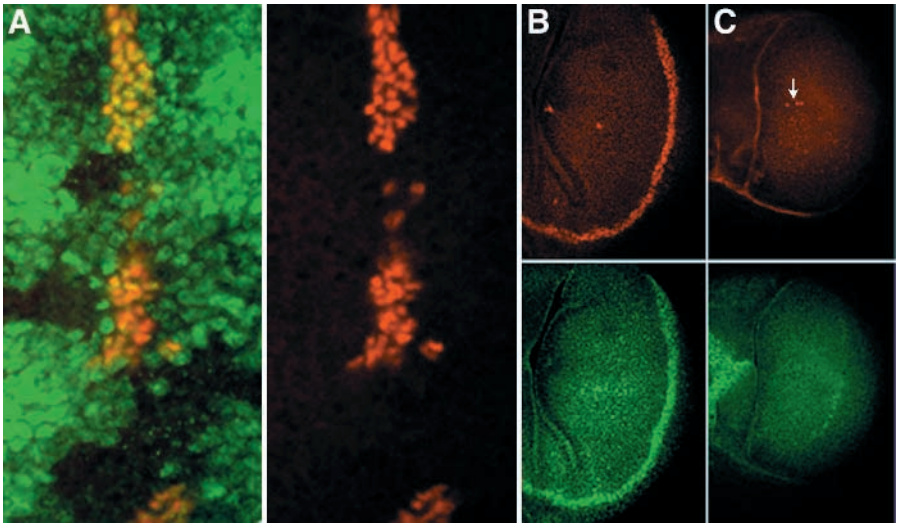

Fig. 3. Regulation of margin *ct* and *wg* by *N*, observed at late third instar. (A) Anti-Ct staining (red) in N^{-}clones (indicated by absence of green πM marker, –). *ct* expression is eliminated both within and adjacent to clones that are in contact with margin (upper, lower clones), but not adjacent to clones not in contact with margin (middle clone). (B,C) Anti-Ct (red) and anti-Wg (green) staining in N^{ts}. (B) Reared at permissive temperature. (C) Reared at non-permissive temperature for previous 24 hours. Reduction of margin anti-Cut staining is more extreme than reduction of anti-Wg. *ct* is still expressed in the precursors of the campaniform sensilla (arrow).

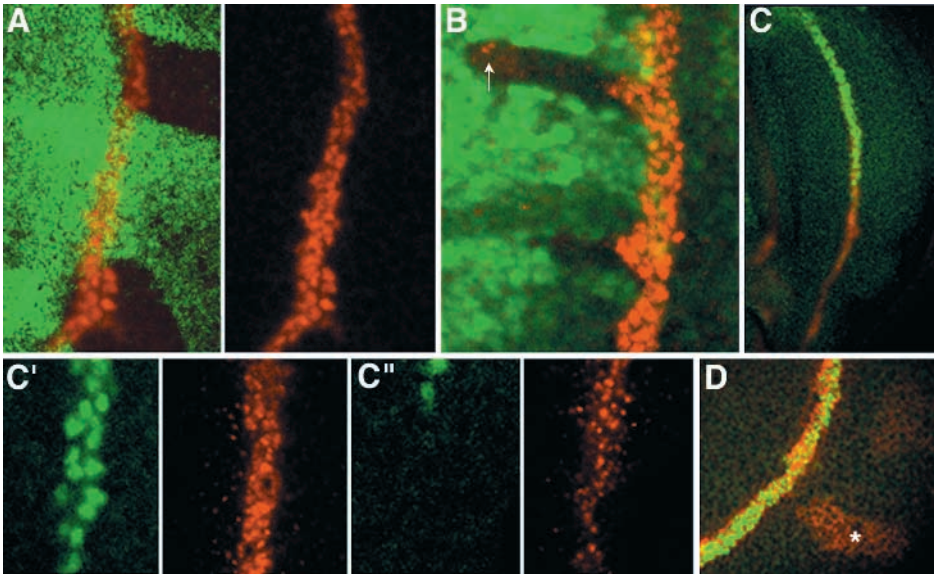

Fig. 4. Regulation of *ct* by Wg pathway. (A,B). *dsh*$^{-}$clones; *ct* is still expressed in these clones. Anti-Ct staining is shown in red. (A) Clones marked by absence of anti-Dsh staining (green). *ct* expression is slightly expanded in posterior clone. (B) Clones marked by absence of green πM marker. Ectopic*ct*-expressing cells are present in the anterior clone, distant from the margin (arrow). (C,C′,C″) *en*-GAL4 UAS-*dsh* disc, stained with anti-Ct (green) and anti-Wg (red). Posterior expression of *dsh*, driven by *en*-GAL4, reduces anti-Wg and eliminates anti-Ct staining. (C′) Detail of anterior. (C″) Detail of posterior. (D) Disc containing *wg*-expressing Ubx>f>*wg*$^{+}$ FLP-out clones, stained with anti-Ct (green) and anti-Wg (red). Sizable FLP-out clone (*) does not express *ct*.

margin still had the capacity to express *ct* (Fig. 4A,B). While occasional loss was observed, especially in large clones that extended outside of the edge cell region, many clones had completely normal expression. Therefore, *ct* expression can be stimulated in the absence of Wg signal reception and the loss of *ct* in *wg*^{ts} wings and *wg*$^{-}$ clones (Couso et al., 1994; Neumann and Cohen, 1996) is an indirect effect.

It was shown previously that *dsh*$^{-}$clones near the normal region of margin *wg* expression ectopically express *wg* at margin-like levels, indicating that Wg signaling represses *wg* expression in the flanking cells (Rulifson et al., 1996). Similarly, *dsh*$^{-}$ clones occasionally contained ectopic *ct*-expressing cells along clone boundaries, in cells up to 10 cell diameters from the margin (Fig. 4B). No ectopic expression was observed outside the clones. Ectopic *ct* expression was less common and usually less extensive than the ectopic expression of *wg* (not shown).

Recent results suggest that Dsh can inhibit N activity by directly binding to the intracellular domain of N (Axelrod et al., 1996). This may account for the ectopic expression phenotype, as loss of *dsh* should derepress *N* activity, leading to increases in *N*-dependent *ct* and *wg* expression. To test the possibility that *ct* expression within clones is *N* dependent, we generated *N*$^{-}$ *dsh*$^{-}$ double mutant clones. In contrast to *dsh*$^{-}$ clones, which often expressed *ct*, *N*$^{-}$*dsh*$^{-}$clones displayed the *N*$^{-}$ phenotype: an autonomous loss of *ct* and domineering nonautonomy on the wing margin (Fig. 5A).

Wingless signaling is not sufficient to stimulate *cut* expression

We further tested whether Wg was able to stimulate *ct* expression using the Ubx>f>*wg*$^{+}$ FLP-out construct (Diaz-Benjumea and Cohen, 1995) to generate clones of *wg*-expressing cells within the wing blade. The levels of *wg* expressed in these clones can elicit other *wg*-dependent events, such as the formation of margin-like bristles (Diaz-Benjumea and Cohen, 1995). However, such clones failed to express detectable levels of *ct* (Fig. 4D). This is consistent with the result of Neumann and Cohen (1996) using GAL4-driven UAS-*wg*.

The overexpression of *dsh* also mimics many aspects of Wg signaling (Axelrod et al., 1996), but failed to elicit ectopic *ct* expression. In fact, overexpressing *dsh* in the posterior compartment of the wing using *en*Gal4/UAS*dsh* resulted in a reduction of normal margin *ct* expression (Fig. 4C,C′,C″), even more dramatic than the reduction of *wg* expression observed previously (Rulifson et al., 1996). Since *dsh* overexpression appears to inhibit N activity (Axelrod et al., 1996), this result is consistent with our proposal that *ct* is a direct target of *N* signaling.

Wingless is necessary and sufficient for high levels of N ligands flanking the margin

If Wg signaling is not directly required for *ct* expression, why is *ct* lost in a *wg*^{ts} wing disc and in *wg*$^{-}$clones, and sporadically lost from *dsh*$^{-}$clones? One possibility is that Wg affects N activity indirectly by regulating the levels of N ligand available near the margin. At mid to late third instar, the period during which *ct* is sensitive to N activity (see above), the N ligands encoded by *Dl* and *Ser* are normally expressed at higher levels immediately flanking the *wg*- and *ct*-expressing edge cells. We will show that Wg signaling is required for these high levels of *Dl* and *Ser*.

wg^{-} clones that intersected the *wg*-expressing edge cells often induced lowered levels of *Dl* and *Ser* (not shown). The *wg*$^{-}$phenotype was not cell autonomous, as expected since Wg from outside the clone can act over a distance of several cell diameters; Dl and Ser appeared at wild-type levels in cells two to three cell diameters away from *wg*-expressing cells at clone boundaries. Loss was seen both within clones that crossed the D/V boundary and those limited to either compartment.

dsh^{-} clones of any size within the flanking cells displayed cell autonomous reduction of *Dl* and *Ser* (Fig. 5B). Identical loss was observed in *N*$^{-}$*dsh*$^{-}$clones (Fig. 5A), indicating that the loss was not due to derepression of N signaling. *Dl* and *Ser* expression along the presumptive wing veins was not reliably lost from *dsh*$^{-}$clones.

Heightened Wg signaling was also sufficient to activate high levels of *Dl* and *Ser* expression. *wg* overexpressing clones, generated using the Ubx>f>*wg*$^{+}$ FLP-out construct (Diaz-Benjumea and Cohen, 1995), expressed high levels of both *Dl*

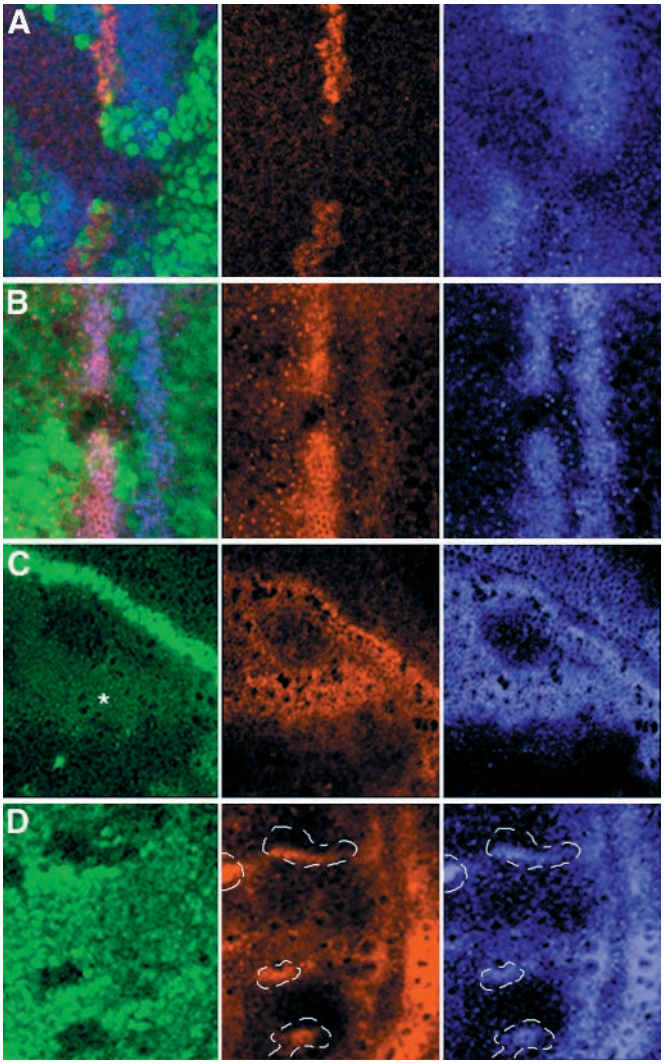

Fig. 5. Regulation of margin *Dl* and *Ser* by Wg pathway. (A) $N^{-}dsh^{-}$ clone, marked by absence of green πM marker. Anti-Ct (red) and anti-Dl (blue) staining is eliminated in the clone. (B) dsh^{-}clone, marked by absence of green πM marker. Anti-Ser (red) and anti-Dl (blue) staining is eliminated in the clone. (C) Disc containing *wg*-expressing Ubx>f>wg^{+} FLP-out clones, stained with anti-Wg (green), anti-Ser (red), and anti-Dl (blue). *wg*-expressing clone (*) raises *Ser* and *Dl* expression to margin-like levels. Phenotypes induced using the Ubx>f>wg^{+} FLP-out are largely cell autonomous, apparently due to the lower levels of *wg* expressed in the clones (Diaz-Benjumea and Cohen, 1995). (D) $sgg\text{-}zw3^{-}$clones, marked by absence of green πM marker (outlined in other panels). Anti-Ser (red) and anti-Dl (blue) staining is raised in clones to margin-like levels.

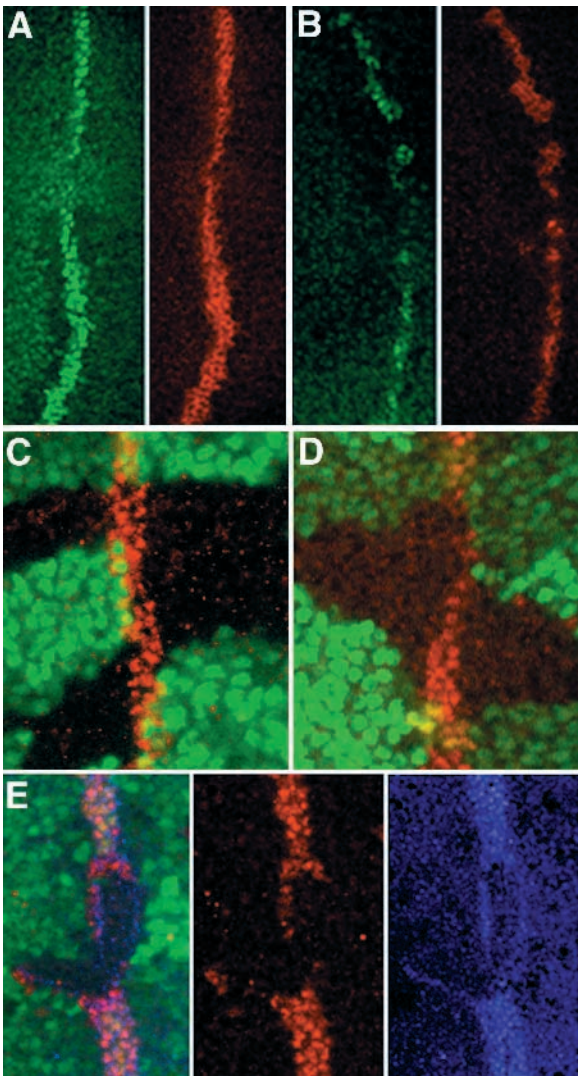

Fig. 6. Regulation of margin *ct* and *wg* by *Dl* and *Ser*. (A,B) Dl^{RF}/Dl^{-} discs, stained with anti-Ct (green) and anti-Wg (red). (A) Disc reared at permissive temperature, showing slight thinning and occasional breaks in *ct* and *wg* expression, especially near the prospective distal tip. (B) Disc reared at non-permissive temperature for the previous 18 hours. Thinning of *ct* and *wg* expression is more extreme, and breaks in expression are more frequent and widespread. (C,D) Large Dl^{-}(C) and Ser^{-}(D) clones, marked by absence of green πM marker, which crossed the D/V boundary. Nearly normal anti-Ct staining (red) is retained in the clones. (E) Large $Dl^{-}Ser^{-}$double mutant clone, marked by absence of green πM marker, which crossed the D/V boundary. Anti-Ct (red) and anti-Wg (blue) staining is disrupted in the clone, except along the clone boundaries.

and *Ser* (Fig. 5C). Cells lacking the Shaggy-zeste white 3 (Sgg-zw3) serine-threonine kinase mimic reception of the Wg signal (Siegfried et al., 1992; Blair, 1994) and, in the adult wing, form margin-like bristles (Simpson et al., 1988). $sgg\text{-}zw3^{-}$ clones throughout the wing pouch contained elevated levels of *Dl* and *Ser* (Fig. 5D). Although the anti-Dl and anti-Ser staining appeared concentrated in the center of some $sgg\text{-}zw3^{-}$ clones, we feel that this apparent non-autonomy is an artifact, caused by the apical concentration of Dl and Ser and the abnormal arrangement of cells within these clones (see Blair, 1994).

N ligands are required for normal *ct* and *wg* expression on the wing margin

As with *N*, *Dl* helps direct normal *ct* and *wg* expression during late third instar. Dl^{RF} is temperature sensitive (Parody and Muskavitch, 1993), although Dl^{RF}/Dl^{-} discs showed partial reduction of *ct* and *wg* expression even when reared at the permissive temperature (Fig. 6A). This reduction became more extreme, however, when larvae were shifted to the non-permissive temperature for the previous 18 hours, and frequent gaps were observed (Fig. 6B).

Clonal loss of either *Dl* or *Ser* alone also occasionally induced loss of margin *ct* or *wg* expression. However, such loss was not reliable. Even in large clones that crossed the D/V boundary, loss or reductions in *ct* or *wg* expression were sporadic and, in many clones, *ct* or *wg* expression was almost normal (Fig. 6C,D). In adults, ventral *Dl*$^{-}$or dorsal *Ser*$^{-}$clones that contact the D/V boundary can induce loss of margin and adjacent tissues (Couso et al., 1995; Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996; de Celis et al., 1996a); although little or no loss is observed in some smaller adult clones, clones that cross the D/V boundary reliably induce extensive notching (de Celis et al., 1996a). Our results in imaginal discs suggest that some of the notching observed in adults may be due to cell loss during early pupal stages, as occurs in *N*^{ts1}/+ and *Ser*^{D}/+ flies (Jack and DeLotto, 1992; Thomas et al., 1995).

Comparison of single mutant clones with double mutant *Dl*$^{-}$ *Ser*$^{-}$ clones show that Dl and Ser functions are partially redundant. While clonal loss of *Dl* or *Ser* alone had only variable effects, all *Dl*$^{-}$*Ser*$^{-}$double mutant clones that crossed the D/V boundary completely lacked normal margin *ct* and *wg* expression, except 1-2 cell diameters inside the clone boundaries, (Fig. 6E). The rescue at clone boundaries was expected, as cell-bound Dl and Ser should be able to signal to cells immediately inside the clone, and there are also indications that normal Ser can be secreted (Couso et al., 1995). Clones observed in pupal wings (24-36 hours AP) showed wing notching phenotypes, associated with clones apparently limited to either the dorsal or ventral compartments (not shown). As expected from the phenotypes of *Dl*$^{-}$and *Ser*$^{-}$single mutant clones (Kim et al., 1995; Couso et al., 1995; Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996; de Celis et al., 1996a), adult wings containing unmarked *Dl*$^{-}$*Ser*$^{-}$clones had regions with lost margin bristles, notched margins and thickened veins.

High levels of Dl and Ser have dominant negative effects on *ct* and *wg* expression

If Dl and Ser drive *ct* and *wg* expression along the margin, why is there no expression in the regions of highest *Dl* and *Ser* expression, that is, in the cells flanking the edge cells? The temperature-sensitive experiments indicate that N and Dl function are required at the same stage when high levels of *Dl* and *Ser* are expressed in the flanking cells; the flanking cells must be relatively insensitive to Dl- and Ser-mediated N signaling. The phenotypes of our *Dl*$^{-}$*Ser*$^{-}$clones show that it is in fact the high levels of Dl and Ser that render these cells insensitive.

In order to rule out effects due to earlier functions of Dl and Ser, small clones were generated during third instar. *Dl*$^{-}$*Ser*$^{-}$ double mutant clones that lay within or adjacent to the normal margin region of high *Dl* and *Ser* expression (approximately 5 cell diameters to either side of the D/V boundary) ectopically expressed *ct* and *wg* (Fig. 7A,B). This was true even in extremely small clones of approximately four cells in either the dorsal or ventral flanking regions. Such expression was limited to *Dl*$^{-}$*Ser*$^{-}$cells at the edges of the clones. Thus, cells expressing high levels of *Dl* and *Ser* can signal to adjacent cells and stimulate *ct* and *wg* expression, but cells expressing high levels of *Dl* and *Ser* are themselves incapable of receiving the signal. These phenotypes differ from those caused by interfering with earlier *apterous*-driven signaling between dorsal and ventral compartments, as dorsal clones lacking *apterous* induce *ct* and *wg* expression both within and outside the clone (Blair, unpublished data).

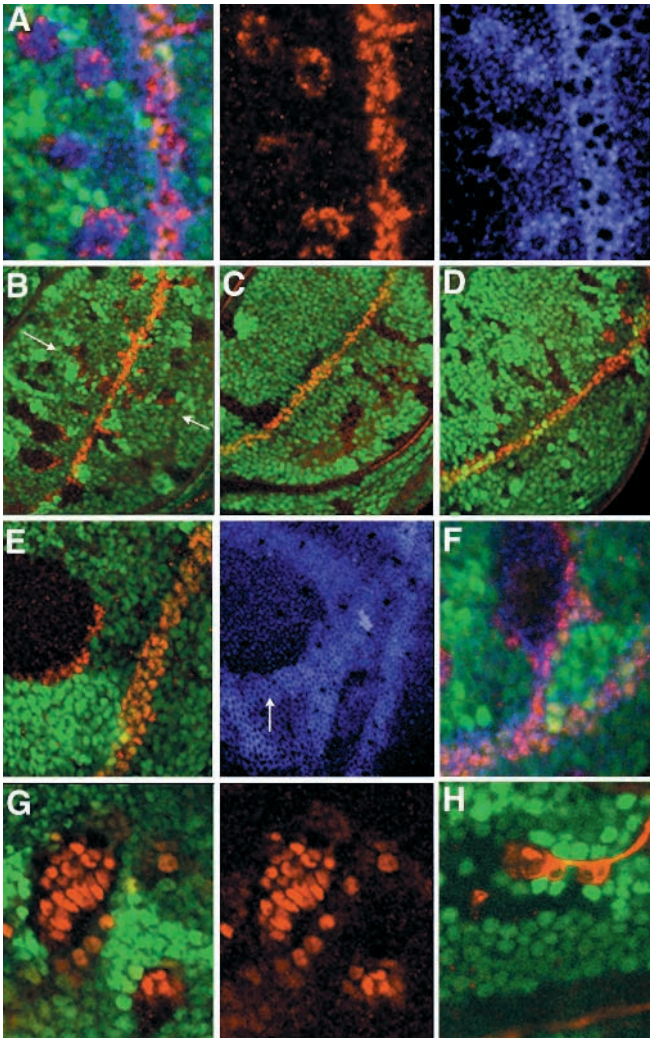

Fig. 7. Regulation of margin *ct* and *wg* by *Dl* and *Ser*. Clones marked by absence of green πM marker, stained with anti-Cut (red) or, in A, F, with anti-Wg (blue), or, in H, with anti-HRP. (A,B) Small *Dl*$^{-}$*Ser*$^{-}$ double mutant clones, generated during third instar. Ectopic *ct* and *wg* expression is observed in clones near the margin, and over slightly wider region in middle of wing in region of vein *Dl* and *Ser* expression (arrow in B, *Dl*/*Ser* expression not shown). (C) Small *Dl*$^{-}$ clones. No ectopic *ct* expression is observed. (D) Small *Ser*$^{-}$clones. Occasional ectopic ct expression is observed; this expression is rarer than in *Dl*$^{-}$*Ser*$^{-}$double mutant clones, and primarily in dorsal clones. (E). *Dl*$^{-}$*Ser*$^{-}$double mutant clone, stained with anti-Ct (red) and anti-Dl (blue). Note expression extends further from the margin on the edge facing the *Dl*$^{+}$ *Ser*$^{+}$/*Dl*$^{+}$ *Ser*$^{+}$ twin spot. *Dl* expression also appears heightened next to the *ct*-expressing cells (arrow). (F) Larger *Dl*$^{-}$*Ser*$^{-}$double mutant clone, generated during second instar. Ectopic expression extends further from the margin than in A. Also, note that expression occurs only along clone boundary, and is biased towards the margin. (G) Ectopic anti-Scute staining (red) within and adjacent to *Dl*$^{-}$*Ser*$^{-}$double mutant clones. (H) Detail of pupal wing (24-36 hours AP), just posterior to anterior margin, containing *Dl*$^{-}$*Ser*$^{-}$ clones. Clones contain scattered neurons, visualized with anti-HRP (red).

Little ectopic expression was observed in either dorsal or

ventral Dl^-clones (Fig. 7C), underscoring the partial redundancy of Dl and Ser in both the dorsal and ventral compartments. Ectopic expression was observed in some Ser^-clones, but this was not as common as in Dl^-Ser^-clones, and was seen primarily in clones on the dorsal side where *Ser* expression is highest (Fig. 7D). Thus, the simultaneous absence of *Ser* uncovers a requirement for *Dl* in both the dorsal and ventral wing; the simultaneous absence of *Dl* strengthens the Ser^- phenotype in the dorsal wing, and uncovers a requirement for *Ser* in the ventral wing.

Rarely, Dl^- Ser^- clones outside the margin region also expressed ectopic *ct* and *wg*. Interestingly, the regions in which such expression appeared correlated with regions of high *Dl* and *Ser* expression: they were more common in the endogenous domain of *Dl* and *Ser* flanking the veins, most notably in the domain between L3 and L4 (Fig. 7B), and along the edge of clones that abutted Dl^+ Ser^+/Dl^+ Ser^+ twin spots (Fig. 7E). In some cases, the ectopic *ct* and *wg* expression was flanked by margin-like levels of *Dl* and *Ser* expression, apparently ectopic, in the cells outside the clone (Fig. 7E). Our results above suggest that the Wg secreted by the clone is inducing these high levels of *Dl* and *Ser* expression in adjacent cells.

There was, in most cases, a bias towards ectopic expression in clones and at clone boundaries nearest the margin (Fig. 7B,E,F). Interestingly, when clones were generated earlier in development, *ct*- and *wg*-expressing cells were formed further from the margin (Fig. 7F). It is possible that the distribution of N ligands controls this region of competence, since both *Dl* and *Ser* are more generally expressed in the wing blade at earlier stages (see above). However, other factors, such as Scalloped, may be play a role in the distal bias (see Discussion).

Previous work has shown that ectopic Wg signaling can induce the formation of margin-like regions of proneural gene expression in the anterior of the wing and bristle formation in both anterior and posterior (Simpson et al., 1988; Blair, 1992; Diaz-Benjumea and Cohen, 1995; Axelrod et al., 1996). We have observed that Dl^- Ser^- clones can also induce ectopic anterior proneural gene expression (*scute*) outside the normal proneural regions (Fig. 7G), presumably induced by the ectopic *wg* expressed in such clones. Ectopic proneural gene expression was not limited to the Dl^-Ser^- cells, as would be expected from the long-range action of Wg secreted by Dl^-Ser^- cells. Anti-HRP-labeled neurons are formed in dorsal and ventral Dl^-Ser^-clones near the anterior margin of pupal wings (Fig. 7H), but only within the clones, so the levels of proneural gene expression outside the clone are apparently insufficient to induce neuronal development. Ectopic margin-like bristles were found near the anterior and posterior margins of adult wings containing unmarked clones (not shown).

DISCUSSION

A novel role for Cut on the wing margin

Our study indicates that *ct* acts during late larval and pupal stages to maintain *wg* transcription along the presumptive wing margin, and that this role is cell autonomous. Interestingly, this suggests a basis for the long-range effects of *ct* mutations. During pupal stages, *ct* mutants lose not only *ct*-expressing cells but also adjacent epithelial cells, resulting in a notched wing phenotype; these effects may be explained by the loss of the secreted Wg morphogen. Many aspects of *ct* and *wg* phenotypes appear consistent. *wg* is required during mid to late third instar to properly pattern and maintain the presumptive wing margin (Phillips and Whittle, 1993; Couso et al., 1994; Diaz-Benjumea and Cohen, 1995). Reducing *ct* function during third instar induces similar margin defects (Dorsett, 1993).

However, *ct* loss only affects the maintenance, not the initiation, of *wg* expression, and it is unclear whether such a late loss of Wg expression can induce the same amount of cell loss observed along the margins of pupal *ct* mutant wings. It has also been reported that hypomorphic ct^6 clones that do not cross the D/V boundary can in 21% of the cases induce notching and bristle loss both within the clone and in the adjacent wild-type compartment (Santamaria and Garcia-Bellido, 1975). Our results show that such clones should affect *wg* expression only within the clone. However, when wg^- clones are limited to one compartment, defects have not been observed (Baker, 1988b; Diaz-Benjumea and Cohen, 1995), suggesting that Wg secreted in the other compartment is sufficient to maintain the margin. Thus, while the loss of Wg is undoubtedly a component of the *ct* phenotype, it is possible that *ct* is required for processes on the margin in addition to *wg* maintenance.

Regulation of *cut* on the wing margin

ct expression on the margin depends on both N and Wg activity. However, our evidence indicates that only the N requirement is direct. We demonstrated that *N* is required in a cell autonomous fashion to maintain *ct* expression on the margin. Thus, *ct* appears to be like other margin-specific genes: *N* or *Su(H)* is required autonomously for the margin expression of *wg*, the *vg* second intron enhancer, and certain members of the *E(spl)* complex (Rulifson and Blair, 1995; Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996; Kim et al., 1996; de Celis et al., 1996a; Neumann and Cohen, 1996).

In contrast, the requirement for *wg* is indirect. Clones lacking *dsh*, which are unable to receive the Wg signal (Klingensmith et al., 1994; Noordermeer et al., 1994; Theisen et al., 1994; Couso et al., 1994; Rulifson et al., 1996), can continue to express *ct*. Moreover, ectopic expression of *wg* or *dsh* was not sufficient to induce margin-like *ct* expression (see also Neumann and Cohen, 1996). This is consistent with the previous finding that *ct* is not expressed *sgg-zw3*$^-$ clones; in other respects, *sgg-zw3*$^-$ cells in the wing blade mimic reception of Wg signals (Blair, 1994). Although Wg signaling is neither necessary nor sufficient for *ct* expression, it is possible that direct Wg signaling contributes to *ct* expression. However, the complete loss observed in temperature-sensitive Wg mutations (Couso et al., 1994; Neumann and Cohen, 1996) must be in a large part due to indirect effects.

Regulation of N ligands by Wg provides a plausible mechanism for this indirect effect. We showed that *Dl* and *Ser* were required to activate or maintain *ct* and *wg* expression and, in addition, that reception of the *wg* signal can directly modulate the levels of the N ligands *Dl* and *Ser* expressed in the cells adjacent to the margin. *Dl* and *Ser* expression was lowered or lost in dsh^-clones and was heightened after *wg* overexpression or the loss of *sgg-zw3*. It is likely that universal removal of Wg function using a temperature-sensitive allele would also reduce the levels of N ligands expressed adjacent

to the margin, and thus the levels of N-dependent *ct* expression on the margin. It is interesting to note that the loss of N ligands observed in *dsh*$^-$ clones does not occur because of the heightened N activity thought to be induced by the loss of *dsh* (Axelrod et al., 1996; Rulifson et al., 1996). Heightened N activity is thought by some to downregulate the levels of N ligands, but *Dl* and *Ser* levels were lowered in both *dsh*$^-$ and *N*$^-$ *dsh*$^-$ clones.

Although margin-specific genes are all directly sensitive to N activity, they are not expressed in identical patterns. *wg*, the *vg* second intron enhancer, and various *E(spl)* complex members are all expressed at higher levels near the margin beginning in late second or early third instar, while *ct* is not expressed until mid to late third instar. Each is also expressed in different sized domains that change during development. Some of these differences might be due to different sensitivities to N signaling. For example, *ct* expression might require higher levels or more sustained N activity. This is consistent with our finding that *ct* was more sensitive to N^{ts} than *wg*.

Our *dsh*$^-$ and *dsh*-overexpression phenotypes could also be interpreted in this manner. Dsh can inhibit N activity, perhaps by direct N-Dsh binding (Axelrod et al., 1996). Thus, the N targets *wg* and *ct* should react to changes in Dsh levels. As expected, clones lacking *dsh* expand the domain of *wg* expression, while *dsh* overexpression reduces normal margin *wg* expression (Rulifson et al., 1996). *ct* reacted similarly to such manipulations, but appeared to require higher levels of N activity than *wg*. In *dsh*$^-$ clones, ectopic *ct* expression was observed less frequently and was restricted to a smaller domain within the clone than was expression of *wg*. *ct* expression on the wing margin was also more sensitive to suppression by overexpression of *dsh* than *wg*.

However, it is likely that additional factors regulate margin-specific gene expression. Recent reports suggest that *ct* is directly regulated by Scalloped, a transcription factor that is expressed throughout the wing blade but at higher levels near the margin (Campbell et al., 1992; Williams et al., 1993), as Scalloped binds to the wing margin enhancer of *ct* (Morcillo et al., 1996). Although *ct*-expressing cells autonomously require N and Su(H) activity (Neumann and Cohen, 1996; this study), little or no Su(H) binding is found to the *ct* wing margin enhancer (Morcillo et al., 1996). It is thought that most or all N signaling is mediated by the Su(H) transcription factor (reviewed in Artavanis-Tsakonas et al., 1995). Thus, *ct* may be regulated by some other Su(H) target.

Activation and repression: dual roles for Dl and Ser

At mid to late third instar, the N ligands *Dl* and *Ser* are expressed at high levels in cells flanking the *ct*- and *wg*-expressing edge cells. Temperature-sensitive alleles were used to show that N and Dl activities were required during this period for *ct* and *wg*. Nonetheless, *ct* and *wg* are not expressed in the flanking cells.

Previous analyses have suggested that Dl and Ser can act both as activators and repressors of N. Overexpression of *Dl* or *Ser* induces ectopic margin-like gene expression, but only in cells adjacent to the overexpressing cells, and normal margin gene expression is also inhibited in the overexpressing cells (Speicher et al., 1994; Thomas et al., 1995; Kim et al., 1995; Couso et al., 1995; Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996; de Celis et al., 1996a,b; Jonsson and Knust, 1996). Overexpression of *Dl* or *Ser* can also induce other *N* loss-of-function phenotypes (Sun and Artavanis-Tsakonas, 1996; T. R. Parody and M. A. T. Muskavitch, personal communication), and raising the dosage of Dl^+ can increase the severity of *N*$^-$ defects and decrease hypersensitive $N^{Abruptex}$ defects (de la Concha et al., 1988; de Celis and Garcia-Bellido, 1994b). Our experiments show that even wild-type levels of Dl and Ser can both activate and repress N targets during normal development. When *Ser*$^-$ *Dl*$^-$ double mutant clones were generated in the flanking region, where *Ser* and *Dl* levels were highest, ectopic *wg* and *ct* expression was induced within the clone in cells adjacent to wild-type cells. The phenotype was not due to some earlier function of *Ser* and *Dl* in wing development, as it held even in very small clones generated during the third instar. Rather, cells expressing high levels of Dl and Ser can signal to adjacent cells and induce *ct* and *wg* expression, but are themselves incapable of receiving the signal. Signaling is therefore highest at sharp boundaries of ligand expression.

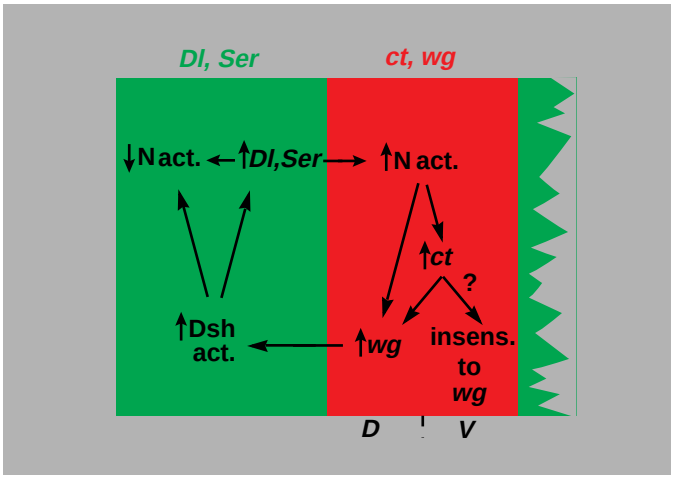

Fig. 8. Model of reciprocal signaling between edge and flanking cells on the wing margin. See text.

We suggest that a similar event occurs along the normal margin at the boundary between the edge and flanking cells (Fig. 8). We suppose that signaling between dorsal and ventral compartments biases cells at the D/V boundary to take on the edge cell fate. This signaling could be mediated by high levels of N activity, or some unknown signal. Cells immediately flanking the D/V boundary would express their edge cell bias by expressing *ct*, higher levels of *wg* and lower levels of N ligand, and would themselves become relatively insensitive to Wg signal. Hypothetically, *ct* expression itself may be responsible for making these cells insensitive to Wg. Once this bias between the edge cells and the flanking cells is established, the signaling between the two regions would sharpen and maintain the boundary between them. Wg secreted by the edge cells would induce high levels of N ligand in the flanking cells, as shown in this study, and also repress *wg* expression in those cells, as shown previously (Rulifson et al., 1996). The insensitivity of the edge cells to Wg would, however, prevent these events from occurring in the edge cells. N activity would be repressed in the flanking cells by the high levels of N ligand, but would be high in the edge cells through the non-

autonomous activity of Dl and Ser in the flanking cells, reinforcing the expression of *wg* and *ct*.

One implication of our results is that high levels of ligand expression alone cannot be taken to indicate high levels of N activity, even in the wild-type wing. Rather, boundaries of ligand expression may be more critical. Apparently, such boundaries must also be fairly sharp. *wg*-FLPout and *sgg-zw3*$^{-}$ clones induce higher N ligand levels, but the boundaries between such clones and the lower but still substantial levels of ligands in surrounding cells do not elicit *ct* or *wg* expression. Moreover, ligand misexpression driven using *ptc*-GAL4 is more effective at the sharp posterior boundary of *ptc* expression than at the fuzzy anterior one (Kim et al., 1995; Couso et al., 1995; Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996; Jonsson and Knust, 1996)

But how could *Dl* and *Ser* autonomously repress N activity? A number of studies have sought to illuminate the nature of the interactions that exist between N and its ligands Dl and Ser, as dimerization or multimerization of receptors or ligands constitutes a potentially rich site for regulating the *N* pathway. In vitro studies using an aggregation assay in S2 cells show that *Dl*-expressing cells can bind in a homotypic fashion, but *N*-expressing cells do not (Fehon et al., 1990). Thus, it is possible that situations that favor homotypic Dl-Dl binding could sequester ligand from the N receptor. Dominant negative phenotypes can also be accentuated by expression of extracellular fragments of N ligand, so the activity of ligands may be modified outside the cell (Sun and Artavanis-Tsakonas, 1996; T. R. Parody and M. A. T. Muskavitch, personal communication). However, our data and previous studies suggest that cells with high levels of ligand expression can signal to adjacent cells; all ligand cannot be sequestered in such cells, nor can such ligand be in a permanently inactivated form. Nor does the evidence as yet support the idea that cells receiving high levels of N activity become desensitized, as hypersensitive $N^{Abruptex}$ mutations, or overexpression of wild-type or activated forms of *N*, does not disrupt development of the normal margin (Doherty et al., 1996; de Celis et al., 1996a,b; Kim et al., 1996). The molecular basis of cell autonomous repression thus remains an interesting puzzle.

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