The *dachsous* gene, a member of the cadherin family, is required for Wg-dependent pattern formation in the *Drosophila* wing disc

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Summary

The *dachsous* (*ds*) gene encodes a member of the cadherin family involved in the non-canonical Wnt signaling pathway that controls the establishment of planar cell polarity (PCP) in *Drosophila*. *ds* is the only known cadherin gene in *Drosophila* with a restricted spatial pattern of expression in imaginal discs from early stages of larval development. In the wing disc, *ds* is first expressed distally, and later is restricted to the hinge and lateral regions of the notum. Flies homozygous for strong *ds* hypomorphic alleles display previously uncharacterized phenotypes consisting of a reduction of the hinge territory and an ectopic notum. These phenotypes resemble those caused by reduction of the canonical Wnt signal Wingless (Wg) during early wing disc development. An increase in Wg activity can rescue these phenotypes, indicating that Ds is required for efficient Wg signaling. This is further supported by genetic interactions between *ds* and several components of the Wg pathway in another developmental context. Ds and Wg show a complementary pattern of expression in early wing discs, suggesting that Ds acts in Wg-receiving cells. These results thus provide the first evidence for a more general role of Ds in Wnt signaling during imaginal development, not only affecting cell polarization but also modulating the response to Wg during the subdivision of the wing disc along its proximodistal (PD) axis.

Key words: *Drosophila melanogaster*, *dachsous*, Wg signaling, Pattern formation, Imaginal disc, PD axis

Introduction

Wnt proteins are a major family of signaling molecules that play central roles in many developmental processes in both vertebrates and invertebrates (Cadigan and Nusse, 1997). In *Drosophila*, two different Wnt pathways have been described: the canonical pathway has been implicated in the control of cell proliferation, cell fate specification and gene expression (Wodarz and Nusse, 1998), whereas the non-canonical PCP pathway regulates cell- and tissue polarization (Adler, 2002). The two pathways initiate their signaling cascade with the activation of the transmembrane Frizzled (Fz) receptor upon ligand binding, and both require the activity of the downstream component *dishevelled* (*dsh*) (Klingensmith et al., 1994; Krasnow et al., 1995; Strutt and Strutt, 2002). Several other signaling components have been identified, but these seem to be specific to one or the other pathway (Wodarz and Nusse, 1998; Adler, 2002).

The Wnt-ligand controlling PCP still remains to be characterized. Nevertheless, it is known that the asymmetrical localization of Fz at the plasma membrane is the response to a polarity signal that is distributed as a gradient within the epithelium. Members of the cadherin family have been implicated at different steps in PCP signaling (Adler, 2002). Cadherins form a family of well-conserved transmembrane molecules with common structural features, such as an extracellular domain containing several copies of a cadherin motif, a transmembrane region, and a cytoplasmatic tail that, in most cases, binds other proteins, such as α- and β-catenin (Vleminckx and Kemler, 1999). They are located at adherens junctions and mediate cell-cell adhesion through homophilic protein-protein interactions via the cadherin repeats (Tepass, 1999). Five cadherins have been isolated in *Drosophila*: *DE-cadherin*/*shotgun* (Tepass et al., 1996; Uemura et al., 1996), *DN-cadherin*/*Cadherin-N* (Iwai et al., 1997), *flamingo/starry night* (*fmi*; *stan* – FlyBase) (Usui et al., 1999; Chae et al., 1999), *fat* (*ft*) (Mahoney et al., 1991) and *dachsous* (*ds*) (Clark et al.), and several more have been predicted (Hill et al., 2001). Cadherins are also involved in cell proliferation (Mahoney et al., 1991; Garoia et al., 2000) and tissue organization (Uemura et al., 1996; Tepass et al., 1996), although the molecular mechanisms underlying their role in these processes are unclear.

Studies of the PCP pathway in the alignment of hairs within the wing (Strutt and Strutt, 2002; Ma et al., 2003) and the abdomen (Lawrence et al., 2002), and in ommatidial rotation during eye development (Yang et al., 2002; Rawls et al., 2002), have shown that Fmi, Ds and Ft, in combination with the transmembrane protein Four-jointed (Fj) (Villano and Katz, 1995), establish a gradient of polarity signal within the epithelium that contributes to the asymmetrical distribution of Fz within the cell membrane (Adler, 2002).

The *Drosophila* Wnt-protein Wingless (Wg) acts as the main ligand in the canonical Wnt pathway. Binding of Wg to Fz and the co-receptor Arrow (Wehrli et al., 2000) prevents the degradation of Armadillo (Arm)/β-catenin by the APC/Axin/Zw3 complex, leading to its stabilization and accumulation within the cytoplasm (Henderson and Fagotto, 2002). Once stabilized, Arm protein moves to the nucleus and

activates target gene expression in complex with the transcription factor Pangolin Pan/dTCF (Brunner et al., 1997; Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996; Korinek et al., 1997; Korinek et al., 1997; Kuhl and Wedlich, 1997), and with the participation of the nuclear factors Legless (Lgs) and Pygopus (Pygo) (Belenkaya et al., 2002; Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002).

The imaginal disc serves as an excellent model system to gain important insight into the role of the Wg pathway in pattern formation (Klein, 2001). During larval development, two initial groups of 30-50 imaginal cells proliferate and differentiate to form the adult wings and thorax. Crucial to the growth and patterning of the wing disc is its subdivision into compartments, a sequential process that starts early and implicates the differential activation of 'selector' genes (Garcia-Bellido et al., 1973).

The expression of *engrailed* (*en*) and *apterous* (*ap*) in cells of the posterior (P) and dorsal (D) compartments, respectively, confers distinct adhesion properties to the cells to prevent them from intermingling with anterior (A) and ventral (V) cells that do not express those genes (Dahmann and Basler, 1999; Blair, 2001). The minimal contact between cells from opposite compartments leads to the formation of a straight border along the AP and DV interfaces. These compartment boundaries serve as sources of the signaling molecules Decapentaplegic (Dpp) and Wg, which coordinate cell proliferation and patterning in the disc (Tabata, 2001). At second larval instar, when the wing disc contains only a few hundred cells, an additional subdivision occurs along its proximodistal (PD) axis, which segregates cells into notum, hinge and wing (Klein, 2001). One of the earliest signals in this process is the expression of Wg in a group of anterior cells located at the distal-most part of the disc (Ng et al., 1996). This Wg expression defines the wing territory by the differential activation of *vestigial* (*vg*) within the domain and *homothorax* (*hth*) in the surrounding cells. Within the wing territory, Wg represses the expression of *teashirt* (*tsh*), which promotes body wall formation (Wu and Cohen, 2002), and the activity of the Epidermal growth factor receptor (Egfr) pathway, which specifies notum fate by activating the expression of the Iroquois complex (*iro-C*) genes within the proximal-most region of the disc (Wang et al., 2000; Zecca and Struhl, 2002). The elimination of this early *wg* function causes a transformation of the wing territory into an ectopic notum (Couso et al., 1993).

Reported data in vertebrates (Polakis, 2000) and invertebrates (Sanson et al., 1996) indicate that cadherins can modulated Wg signal transduction by affecting the balance between the levels of cytoplasmic and membrane anchored Arm/β-catenin (Vleminckx and Kemler, 1999). Here, I describe a new role of the cadherin Ds in pattern formation when territories along the PD axis are specified in the wing disc. This study suggests that localized expression of Ds controls PD subdivision by modulating the response to Wg.

Materials and methods

Fly stocks

All the mutant alleles for *ds*, *wg*, *dsh*, *naked* (*nkd*) and *Df(2L)S2* are described in FlyBase, except ds^{D36}, which was generated by the excision of *P1394* (*ds-lacZ*). This line contains a P(lacZ) insertion in the second intron of *ds*. *Df(2L)S2* partially uncovers *ds* locus and the escapers show a strong *ds* phenotype similar to ds^{38k} at 25°C. The *UAS-*wg^{ts} transgene only produces active Wg protein when larvae are allowed to develop at 18°C (provided by I. Guerrero). The drivers used for misexpression experiments were *omb-Gal4* and dpp^{disk}*-Gal4* (Cavodeassi et al., 2002).

Clonal analysis and ectopic expression experiments

The FRT/FLP technique (Xu and Rubin, 1993) was used to induce clones of *ds* mutant cells in animals of the following genotypes: *hs-FLP122; FRT40A* ds^{D36}*/FRT40A Minute(2L) arm-lacZ* and *hs-FLP122; FRT40A* ds^{38k}*/FRT40A Minute (2L) arm-lacZ.* Larvae were heat shocked at 36±12 and 60±12 hours after egg laying (AEL). Mutant cells were marked by the absence of anti-β-galactosidase antibody staining.

Rescue assays were performed in the following genotypes:
omb-Gal4; ds^{38k}*/SM5-TM6b X* ds^{38k}*; UAS-*wg^{ts}*/SM5-TM6b*
w; ds^{38k}*;* dpp^{disk}*-Gal4/SM5-TM6b X* ds^{38k}*; UAS-*wg^{ts}*/SM5-TM6b*
omb-Gal4; ds^{38k}*/SM5-TM6b X* ds^{38k}*; UAS-dpp/SM5-TM6b*
w; ds^{38k}*;* dpp^{disk}*-Gal4 SM5-TM6b X* ds^{38k}*; UAS-dpp/SM5-TM6b*

Larvae were developed at 25°C except when *UAS-*wg^{ts} was overexpressed; in those cases they were raised at 18°C. At 18°C, the levels of Wg were able to promote ectopic cell proliferation in the hinge without changing the cell fate to wing.

Histochemistry

Imaginal discs were dissected and stained as described previously (Gomez-Skarmeta et al., 1995). The following primary antibodies were used: mouse anti-Nub (Ng et al., 1995), rat anti-Ci (Motzny and Holmgren, 1995), guinea pig anti-Hth (Azpiazu and Morata, 2000), rabbit anti-Vg (Williams et al., 1991), rabbit anti-Tsh (Wu and Cohen, 2002), rat anti-Ds (Yang et al., 2002), rat anti-Iro (Diez del Corral et al., 1999), rat anti-Zfh2 (Whitworth and Russell, 2003), mouse anti-Wg and mouse anti-En (Iowa University Hybridoma Bank), rabbit anti-β-galactosidase (Cappel) and mouse anti-β-galactosidase (Amersham). Fluorescent secondary antibodies were from the Jackson Immunostaining Laboratory.

Adult cuticles

For microscopic examination, the wing and legs were dissected and treated in 10% KOH and mounted in a solution of lactic acid mixed 6:5 with ethanol.

Results

ds mutations are associated with novel adult phenotypes

All known *ds* alleles are homozygous viable to different extents and the flies show a PCP phenotype consisting of disorganized cuticle hairs (Adler et al., 1998). In addition, strong *ds* alleles induce pupal lethality at high frequency, and the adult escapers display defects such as enlarged wings, shortened legs with missing tarsi, and a widened notum with extra bristles at the dorso-central and postalar positions. The sum of these defects will be further referred to as the 'classical' *ds* phenotype (Lindsley and Zimm, 1992; Clark et al., 1995). In a new screen, I have isolated an allele that can be classified as a null allele for two reasons. First, *ds* mRNA is undetectable by in situ hybridization (data not shown) in homozygous individuals. Second, homozygotes die at larval stages with severe morphological alterations of the imaginal discs not found in other allelic combinations.

From an allelic series that includes most of the *ds* alleles, ds^{38k} represents the strongest hypomorphic condition. In addition to

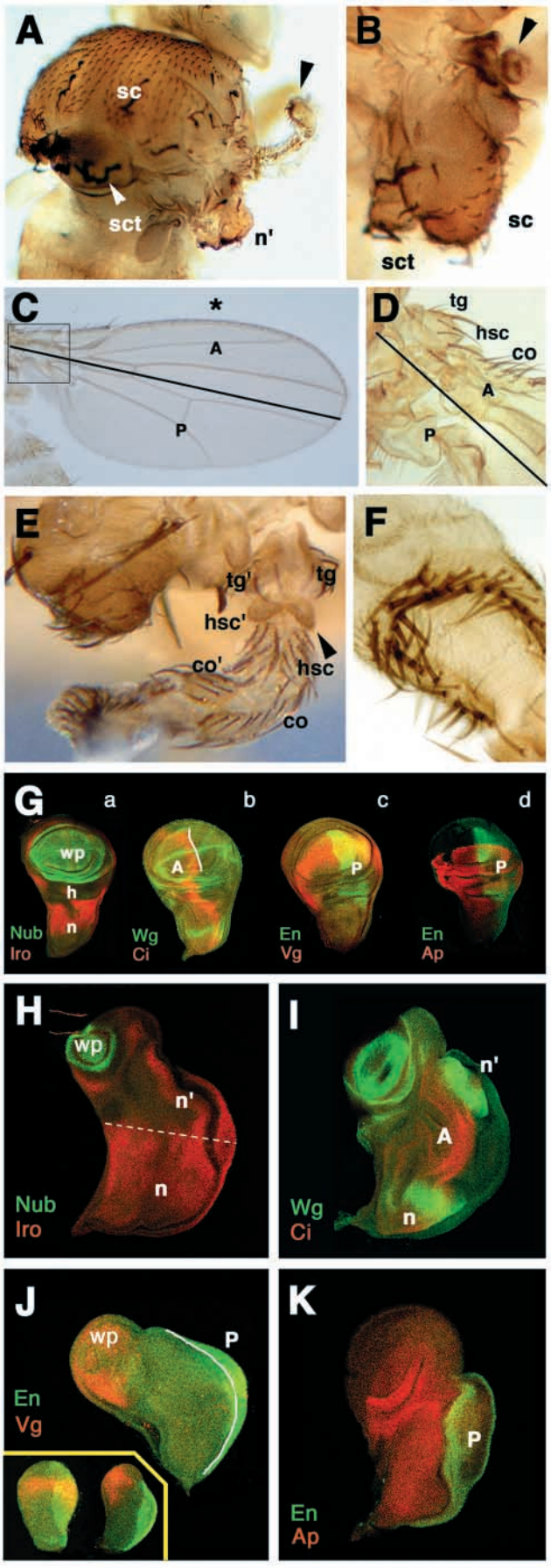


Fig. 1. DNW phenotype is caused by abnormal subdivision along the PD and AP axis. Adult structures (A-F) and wing imaginal discs (G-K) of wild-type (C,D,G) and ds^{38k} mutant (A,B,E,F,H,I,J,K) specimens are shown. (A) Reduced wing (black arrowhead) associated with an ectopic notum (n′). (B) Magnification of an ectopic notum shows the scutum (sc) and scutelum (sct) structures next to a minimal winglet that is exclusively formed by a duplicated tegula (black arrowhead). (C) Wild-type hinge (square) and the wing blade regions divided by the AP boundary (black line). The asterisk marks the position of the anterior wing margin. (D) High magnification view of the hinge in C, showing anterior proximal structures such as the tegula (tg), humeral sclerite (hsc) and costa (co). (E) Detail of a winglet composed of anterior proximal structures arranged in a mirror-image duplication. (F) Detail of a rudimentary wing blade in which only anterior wing margin bristles (normally formed at the position marked by the asterisk in C) are present. (G) Third instar wild-type discs stained for (a) Nub (green) and Iro (red) to delimit the territories along the PD axis. wp, wing pouch; n, notum. The AP and DV compartments are marked by (b) Ci (red) and Wg (green); (c) En (green) and Vg (red); and (d) En (green) and Ap (red). The white line highlights the border between the A and P compartments. (H-K) Late DNW wing discs show (H) a reduced wing pouch (Nub; green) and an expansion of the notum territory (n and n′) (Iro; red). (I) The notal duplication, visualised by a double notal band of Wg (green), is expanded into the hinge territory and is formed by A (Ci; red) and P cells. (J) P cells (En; green) are only present in the notum territory. The wing pouch (Vg; red) cells are confined in the A compartment. The inset in J represent early discs of wild-type (left) and ds^{38k} (right) larvae. The comparison shows that the DNW phenotype occurs very early. The dorsal (red) and ventral compartments are apparently normal (K).

the ‘classical’ phenotype, a percentage of ds^{38k} escapers (approximately 5%) show striking phenotypes that were not described previously. These consist of the presence of a lateral protuberance similar to an ectopic scutum (sc) and scutellum (sct), indicating a possible notum duplication (Fig. 1A,B), and the replacement of the normal wing (Fig. 1C) by a winglet (arrowhead in Fig. 1A,B). The ectopic notum and the winglet are always associated. A comparison with the wild-type wing (Fig. 1C,D) shows that the winglet is composed of proximal anterior structures that are arranged in a mirror-image duplication (Fig. 1E). The smallest winglet is exclusively formed by a duplication of the tegula and humeral sclerite structures (Fig. 1B, arrowhead), whereas the largest one also has a rudimentary wing blade composed of a small costa and anterior wing margin (Fig. 1E,F). I shall refer to these newly described anomalies as the ‘double-notum-winglet’ (DNW) phenotype. This phenotype is also found in *Df(2L)S2* homozygous flies (1 out of 100 heminota), and in individuals from heterozygous combinations of ds^{38k} and other strong alleles, such as ds^{33k} (3 out of 72 heminota), the P insertion *ds-lacZ* (approximately 2% of heminota) and ds^{UA071} (Adler et al., 1998).

DNW wing discs show abnormal subdivisions along PD and AP axis

To explore the origin of the DNW phenotype, I examined the expression of several markers in wild-type and homozygous ds^{38k} wing discs from early to late larval stages (Fig. 1G-K). Approximately 22% of the ds^{38k} mutant wing discs that reach the third larval instar (n=60) have a dramatically altered size and morphology, and thus could correspond to discs that will give rise

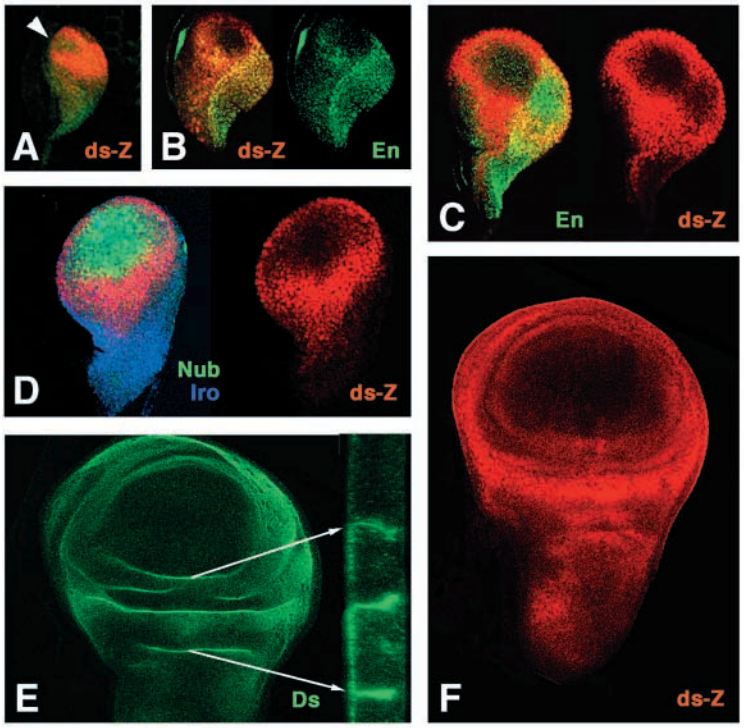


Fig. 2. *ds* is expressed in the wing disc from early stages. (A-E) Wild-type imaginal wing discs. (A,B) In early- (A) and mid-second (B) instar discs, *ds-lacZ* expression (red) is confined to the distal part of the wing disc, except in those anterior cells that express Wg (arrowhead). (C) At late second instar, *ds-lacZ* expression fades away from the P cells adjacent to the AP border (En; green). (D) At early third instar, the *ds-lacZ* domain forms a ring around the wing pouch that spans the whole hinge territory delimited by Nub (green) and Iro (blue) domains. (E) Spatial distribution of Ds protein is similar to *ds-lacZ* expression. A cross-section shows that Ds protein is accumulated apically at the plasma membrane. (F) At late third instar, *ds-lacZ* is expanded into the lateral regions of the notum territory.

to the DNW phenotype (Fig. 1A). First, I analyzed the expression of markers outlining the subdivision along the PD axis. Indeed, mutant discs show a reduced wing pouch, as revealed by the wing-specific marker Nubbin (Nub) (Fig. 1G, part a; H, green). Moreover, they show an expansion of the notum territory into the prospective hinge territory as assessed by Iro expression (Fig. 1G, part a; H, red), and the presence of an additional stripe of notal-specific Wg expression (Fig. 1G, part b; I, green).

Two other striking features of *ds* mutant discs are the relative position of the prospective wing pouch with respect to the AP border, and the differences in size between the A and P compartments, as revealed by the expression of Cubitus interruptus (Ci) (Fig. 1G, part b; I, red) and En (Fig. 1G, part c; J, green), respectively. In the wild type, the wing pouch is subdivided in two compartments of a similar size (Fig. 1G, parts b,c). By contrast, the reduced wing pouch in DNW discs is located entirely within the A compartment (Fig. 1G, part c; J,K). These observations are in agreement with the exclusive presence of anterior structures in the ds^{38k} winglet (Fig. 1E,F). Nevertheless, the A and P compartments are both present within the notum, where they are of a normal size (Fig. 1G, part b; I). Note that the extant and the ectopic nota [Fig. 1H,I (n,n′)] are arranged in a mirror-image disposition, and that the Iro domains are kept in contact (Fig. 1H, dotted line), in contrast to *wg* mutant discs in which they are separated by a wide stripe of hinge cells (Cavodeassi et al., 2002). The absence of P cells in the wing territory was observed as early as the mid second instar (Fig. 1J, inset). However, the size of the D and V compartments do not seem to be altered, as revealed by the expression of Ap in dorsal cells (Fig. 1G, part d; K, red).

ds is expressed in the wing disc from early stages of development

To gain insight into the role of *ds* during early wing development, I examined the expression of *ds* in the wing disc of second and early third instar larvae. *ds* expression was monitored by the *ds-lacZ* reporter gene, which reflects the spatial pattern of *ds* mRNA (Clark et al., 1995). In second instar larvae, *ds-lacZ* expression is essentially confined to the distal part of the wing disc (Fig. 2A,B), but is absent in those distal A cells in which Wg strongly accumulates (Fig. 3A, green). This Wg expression constitutes the earliest marker for the nascent wing pouch (Couso et al., 1993; Ng et al., 1996). Soon thereafter, when Wg expression is expanded to the adjacent P cells, *ds-lacZ* expression fades away (Fig. 2C) and becomes confined to a ring of cells around the prospective wing pouch (Fig. 2D). At this stage, most of the hinge cells located between the prospective notum and wing pouch express *ds-lacZ* at high levels, as revealed by the Iro and Nub markers (Fig. 2D). A weak expression of *ds-lacZ* overlaps with the periphery of the Nub domain (Fig. 2D) and marks the region that will become the proximal wing. At third instar, *ds-lacZ* expression is also observed within the lateral regions of the prospective notum (Fig. 2F). An antibody directed against the cytoplasmic region of Ds protein (Yang et al., 2002) reveals a Ds protein distribution similar to the *ds-lacZ* expression pattern and an apical location at the plasma membrane (Fig. 2E). From these results, I conclude that *ds-lacZ* expression is one of the earliest and most specific markers of the prospective hinge during the second and early third larval instar.

ds function is required to specify the proximal wing and hinge territory

The DNW phenotype and the *ds-lacZ* spatial pattern strongly suggest an involvement of *ds* during the specification of the proximal wing and hinge territories. To better define the role of *ds*, I analyzed the expression of genes involved in this process, such as *hth*, *wg*, *tsh* and *zfh2* in wild-type and *ds* mutant backgrounds. *hth*, *zfh2* and *tsh* are regulated by *wg*, and they are expressed in the distal region of early second instar discs (Azpiazu and Morata, 2000; Casares and Mann, 2000; Whitworth and Russell, 2003; Wu and Cohen, 2002). The specification of the hinge structures requires the activation of *hth* and *zfh2*, and the repression of *tsh* expression within the putative hinge territory (Wu and Cohen, 2002). Overexpression of *tsh* and *hth* in the prospective wing pouch represses *nub*, indicating an antagonism between Hth and/or Tsh expression and the specification of the wing cell fate. Moreover, the loss

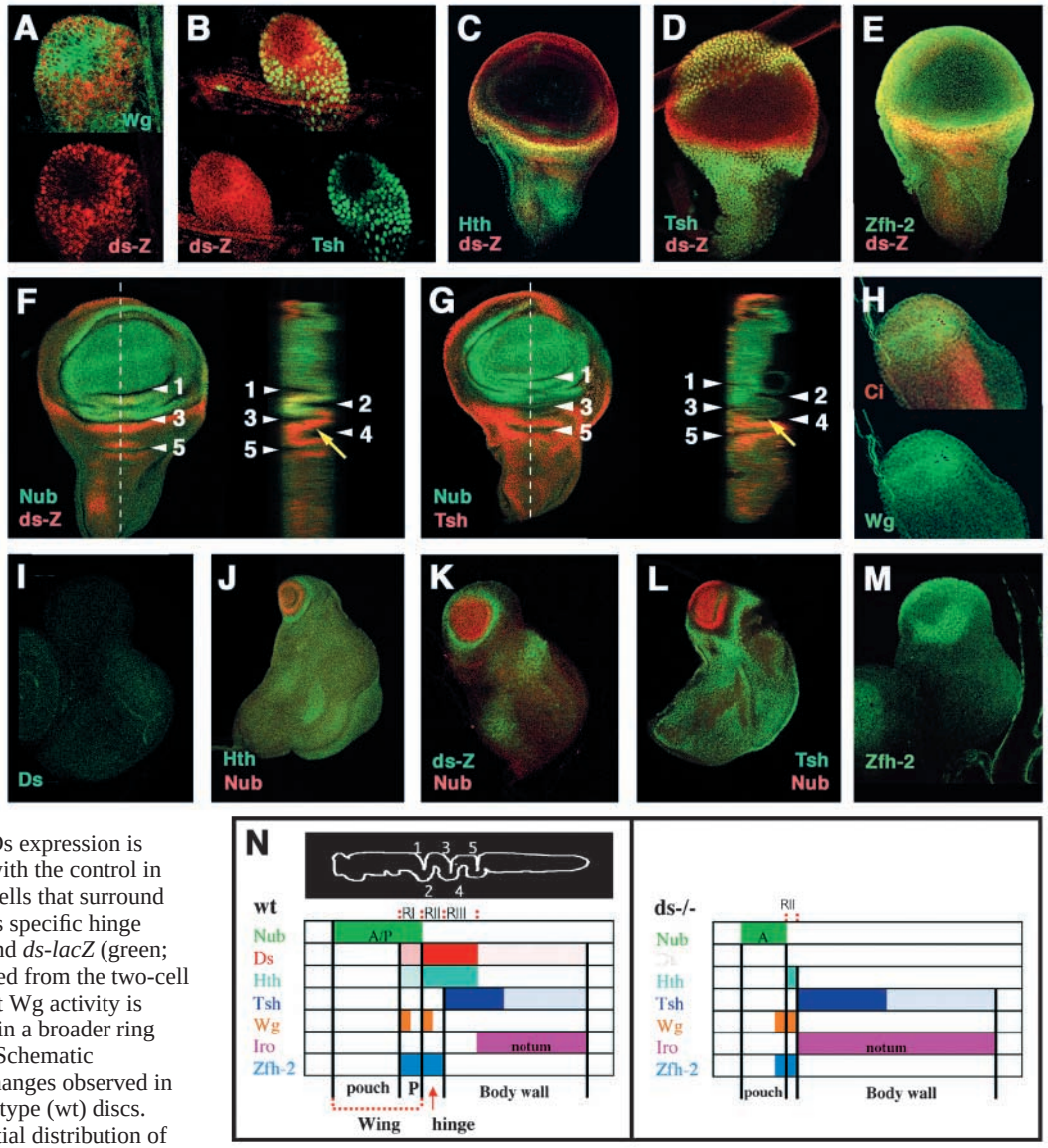


Fig. 3. The hinge territory is not specified in DNW discs. Wild-type (A-G) and DNW mutant (H-M) wing discs are shown. (A) In early wild-type discs, the spatial pattern of expression of *ds-lacZ* (red) and Wg (green) is complementary. At the periphery of the Wg domain, cells express *ds-lacZ* (red) at low levels, and Tsh expression (green; B) is excluded from those cells. At early third instar, *ds-lacZ* (red; C,D,E), Hth (green; C) and Zfh2 (green; E) are highly expressed in a ring of cells around the wing pouch where Tsh expression (green; D) is excluded. (F) In late third instar, a cross-section along the AP border (dotted line) shows a ring of cells abutting the Nub domain (green) that express *ds-lacZ* (red) at high levels (yellow arrow); (G) Tsh expression (red) is absent (yellow arrow). In DNW discs, early Wg expression is apparently normal but restricted to the A cells (H), and Ds expression is almost undetectable (I, compare with the control in 2E). (J,K) Only a narrow ring of cells that surround the wing pouch (Nub, red) express specific hinge markers, such as Hth (green; J), and *ds-lacZ* (green; K). By contrast, Tsh (L) is excluded from the two-cell wide ring (as in G), indicating that Wg activity is decreased. (M) Zfh2 is expressed in a broader ring around the wing pouch cells. (N) Schematic representation summarising the changes observed in DNW ($ds^{-/-}$) with respect to wild-type (wt) discs. Different stripes represent the spatial distribution of genes involved in the specification of territories along the PD axis. Low (light) and high (dark) levels of expression are indicated for each gene in different colours. A/P, anterior/posterior; P, proximal wing. Numbers 1 to 5 indicate the folds of a mature wing imaginal disc. RI, RII and RIII correspond to the three concentric rings around Nub domain.

of *zfh2* activity eliminates proximal wing and hinge territories (Whitworth and Russell, 2003).

In the wild type, the initial wing territory is specified around second instar by the expression of Wg in distal A cells. *ds-lacZ* is almost eliminated within the nascent wing primordium, except in a few cells at the periphery, which express both *wg* and *ds-lacZ* at low levels (Fig. 3A). A similar expression pattern was also observed for *hth* and *zfh2* (Casares and Mann, 2000; Whitworth and Russell, 2003). By contrast, *tsh* expression is turned off within the Wg domain, and in a ring of surrounding cells, but remains uniformly expressed in the rest of the wing disc (Fig. 3B) (Wu and Cohen, 2002). The pattern of *ds-lacZ* expression with respect to Hth, Tsh and Zfh2 is maintained until the third instar (Fig. 3C,D,E). To visualize the distal border of each expression domain in more detail, I also examined optical cross-sections of late third instar wing discs. *ds-lacZ*, *zfh2* and *hth* are co-expressed at high levels in a ring of cells abutting the Nub domain (Fig. 3C,E,F; arrow in F). Tsh is repressed within these cells (Fig. 3D) by the activity of Wg (Fig. 3D,G; arrow in G) (Wu and Cohen, 2002). Based on these expression patterns, I can define three concentric domains with respect to Nub expression (Fig. 3N). The cells of the innermost ring (ring I) express *nub* and low levels of *ds-lacZ* and *hth* (Fig. 3F, folds 1 to 3), and will give rise to the proximal wing structures eliminated in $wg^{spd\text{-}fg}$ (Neumann and Cohen, 1996) and $zfh\text{-}2^{MS209}$ (Whitworth and Russell, 2003) mutants. The middle ring (ring II) spans the region in which *tsh* is repressed (Fig. 3G, folds 2 to 4), and in which *ds-lacZ* and *hth* are expressed at high levels (Fig. 3F, folds 2 to 4). These cells will develop the hinge structures eliminated in ds^{38k} flies. Finally, the outermost ring (ring III) consists of cells that co-express low levels of *ds-lacZ* and *hth* within the Tsh domain (Fig. 3G, folds 4 to 5). Those cells will form the body wall structures excluded from the notum territory, such as the thoracic pleura.

DNW discs from early larval stages suggest altered specification of the proximal wing and hinge territories (Fig. 1J, inset). Two main differences are observed in DNW discs with respect to the wild-type discs. First, *hth* and *ds-lacZ* expression is eliminated from rings I and III. Only a few cells in the ring II are still expressing both genes (Figs 3J,K). Second, cells within ring III maintain Tsh expression (Fig. 3L), but now express notum-specific genes such as *iro-C* (Fig. 1H). Note that the *zfh2* domain is wider than the domains of *hth* and *ds-lacZ* expression (Fig. 3M). Thus, reduction of *hth* and *zfh2* expression might be the cause of the loss of proximal wing and hinge territories in DNW discs. The ectopic notum observed in DNW flies is most likely to derive from the outermost ring of cells that missexpress the *iro-C* genes.

Ds regulates Wg signaling

The notum duplication observed in DNW flies resembles a similar phenotype described for wg^1 flies (Sharma and Chopra, 1976). In general, a decrease of Wg signaling during early wing disc development transforms the wing into an ectopic notum (Brunner et al., 1997; Kramps, 2002). However, DNW flies retain a rudimentary wing, suggesting that the ectopic notum in these mutants is formed predominantly at the expense of proximal wing and hinge structures.

Wg signaling has been shown to control both *hth* and *zfh2* expression during the specification of these structures (Casares and Mann, 2000; Whitworth and Russell, 2003). The analysis of DNW discs described above indicates that these same genes are affected by the reduction of Ds activity, suggesting a role of Ds in Wg signaling. In order to investigate this possibility, I tested whether an increase of Wg protein levels could rescue the DNW phenotype of *ds* mutant discs. An ectopic dose of Wg was provided to ds^{38k}/ds^{38k} discs using the *UAS-wg* transgene in combination with either the *dpp-Gal4* or *omb-Gal4* drivers. Under these conditions, the DNW discs of ds^{38k} larvae were completely rescued in size and pattern (Fig. 4A) (over 130 larvae were analyzed). The rescued wing discs consisted of just a single notum and a wing pouch of normal size and AP subdivision. In particular, the specification of the proximal wing and hinge territories was restored, as assessed by *hth* expression (Fig. 4B; although the hinge domain is expanded compared with the wild type), due to the later role of Wg in the induction of cell proliferation within the hinge (Neumann and Cohen, 1996). The ds^{38k} larvae expressing *UAS-wg* under *dpp-Gal4* control also showed enhanced viability, although they died before reaching adulthood. Thus, these data suggest that all aspects of the DNW phenotype are caused by a reduction in Wg signaling.

Next, I investigated whether *wg* expression was affected in DNW discs. In this *ds* mutant background, in which Ds protein is almost undetectable (Fig. 3I, compare with Fig. 2E), the distribution and levels of Wg protein looked similar to those of wild-type discs at either early (Fig. 3H) or late larval stages (Fig. 1I). This result shows that *ds* does not regulate the expression of Wg.

In order to test whether other signaling pathways are affected by the reduction of Ds activity, I ectopically expressed Dpp in *ds* mutant discs using a similar approach to that described for ectopic *wg* expression. Dpp secreted by a thin stripe of cells along the AP boundary acts directly, and at long range, on all cells within the developing wing pouch to organize pattern formation and growth (Capdevila et al., 1994; Zecca et al., 1995; Burke and Basler, 1996; Lecuit et al., 1996; Nellen et al., 1996). In addition, Dpp also functions outside of the wing pouch to confine Iro-C expression to the notum (Cavodeassi et al., 2002). In wild-type discs, the Dpp source (AP border) is located at the center of the prospective wing pouch (Fig. 1G, part a; white line). As the AP border is far removed from the wing pouch in DNW discs (Fig. 1J,K), a reduced level of Dpp within the wing pouch cells could be responsible for the winglet phenotype. To address this issue, *UAS-dpp* was expressed under *dpp-Gal4* control in ds^{38k} larvae. Under these experimental conditions, the DNW phenotype was retained. By contrast, ds^{38k} larvae bearing the *UAS-dpp* and *omb-Gal4* transgenes produced wing discs with a remarkable expansion of the wing pouch (Fig. 4C,D). The rescued wing pouch is formed of A and P cells, indicating that Dpp also contributes to the recruitment of P cells into the wing fate (Fig. 4D). However, the notum duplication was still present in these discs

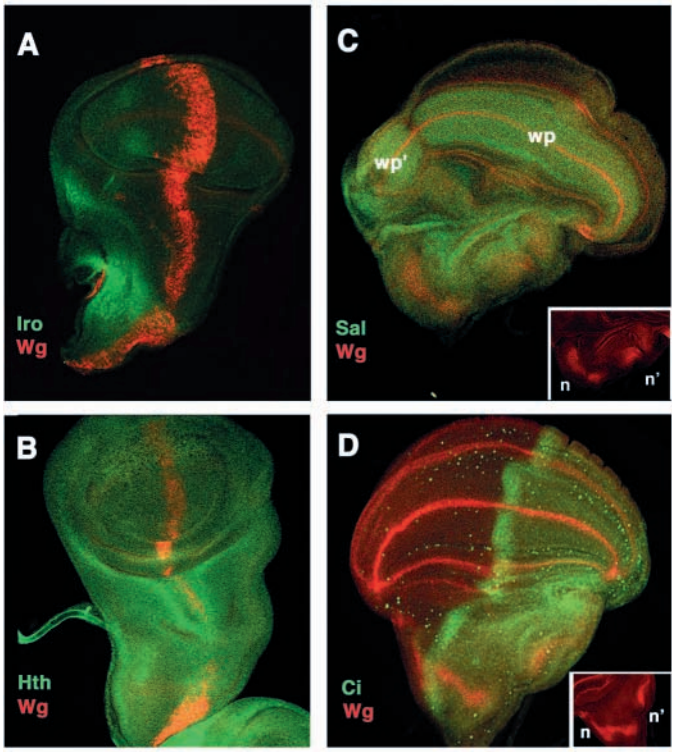


Fig. 4. An increase of Wg activity rescues the DNW phenotype. (A-D) Third instar ds^{38k} mutant wing discs. (A,B) Expression of *UAS-wg* (red) under the control of *dpp-Gal4* driver rescues the DNW morphology to wild type. (B) The hinge territory is specified and the expression pattern of Hth (green) is restored. The hinge is enlarged in respect to wild type due to an increase in cell proliferation caused by ectopic Wg. (C,D) Ectopic expression of Dpp driven by *omb-Gal4* increases the proliferation in the wing pouch but fails to rescue the notal duplication. (C) The size of wing pouch is increased. In some cases wing duplications can be recovered (wp and wp′); however, the double notal Wg (red) stripe indicates that the notum duplication is still retained. Dpp activity was monitored by Sal (green), a target gene of the Dpp pathway. (D) The symmetrical location of the AP border into the wing pouch territory is restored in ds^{38k} wing discs, as visualised by Ci (green) expression in A cells. Red channels (insets) show the notal Wg stripes.

(Fig. 4C,D; insets). In summary, I conclude that a disruption of Wg signaling is mainly responsible for the hinge to notum transformation in DNW flies. However, the winglet phenotype due to the absence of P cells recruited into the wing fate is caused, not only by the lack of Wg, but also by Dpp expression, although the DNW phenotype retained in the *UAS-dpp/dpp-Gal4* combination suggests that only cells specified into the wing fate by Wg expression can respond to Dpp in this process. Finally, the data indicate that Ds is required for efficient Wg and/or Dpp signaling for the recruitment of the P cells into the wing fate during early stages of wing development.

PD patterning requires Ds in Wg-receiving cells during early stages of disc development

The analysis of *ds* mutant discs reveals early patterning defects (Fig. 1J, inset). To assess the temporal requirement of Ds in Wg-mediated PD patterning, I analysed *zfh2* expression (Fig. 5A, inset) in *ds* mutant clones. *zfh2* is activated by Wg in second instar wing discs to become independent soon thereafter (Whitworth and Russell, 2003). Using the Minute technique (Morata and Ripoll, 1975), I generated large clones of ds^{D36}cells at different stages of larval development. Clones induced at early second instar, eliminate *zfh2* expression (Fig. 5A,B). Moreover, an overgrowth was observed within the mutant territory, similar to that described for mutations in other cadherins, such as *ft* (Mahoney et al., 1991). By contrast, clones of ds^{D36} cells induced later do not eliminate *zfh2* expression, although expression levels were reduced in the hinge cells (Fig. 5C,D; asterisk). Thus, it seems that Ds, similar to Wg signaling, is essential for the initiation, but not the maintenance, of *zfh2* expression at later developmental stages. Together, these findings support a role for Ds in Wg-mediated initiation of *zfh2* expression during early stages of wing development. I have previously shown that *wg* and *ds* are expressed in complementary domains at these stages (Fig. 3A), thus I conclude that Ds must act in Wg-receiving cells to achieve PD patterning.

Ds affects the distribution of Wg protein in the Wg-producing cells

During patterning and growth of the wing blade, Wg distribution has been proposed to signal to distant cells in a concentration-dependent manner (Zecca et al., 1996; Neumann and Cohen, 1996; Strigini and Cohen, 1996). Several mechanisms, such as the interaction of Wg with heparin sulfate-containing proteoglycans, as well as regulated endo- and exocytosis, are involved in shaping the gradient and delimiting the range of signaling (reviewed by Seto et al., 2002). Wg protein is predominantly located at the apical surface in the producing cells (Strigini and Cohen, 2000), and in the embryo it has been demonstrated that this sub-cellular location is essential for its signaling activity (Simmonds et al., 2001). When the hinge territory is already specified at early third instar, *wg* is activated in these cells and acts as a cell proliferation signal necessary for the development of most structures (Neumann and Cohen, 1996; del Alamo et al., 2002). Wg expression within the hinge can be described as two rings, the ‘inner ring’ (IR) and the ‘outer ring’ (OR), which overlap with the areas of low (ring I) and high expression of *ds-lacZ* (ring II), respectively (data not shown). In this context, as Ds is also apically located (Fig. 2E), I wished to determine whether Ds has a role in Wg-producing cells. To test this, I examined the distribution of Wg in large clones of *ds* mutant cells. Two results were obtained from these experiments: first, the level of Wg in the producing cells was slightly increased with respect to neighbouring wild-type cells (Fig. 6A, arrowheads); and second, the Wg gradient within mutant tissue appears to be broader. These results should imply a higher signaling capacity of Wg in *ds* mutant cells (Giraldez et al., 2002); however, that was not the case (Fig. 1I,J, and data not shown). In *ds* clones, Wg accumulation was less marked apically and was relatively more abundant in the baso-lateral region than in the wild-type cells (Fig. 6B). Interestingly, this phenomenon seems not to be strictly cell autonomous, as adjacent wild-type cells also displayed a similar abnormal sub-cellular localization of Wg protein (Fig. 6B, bracket). This effect could be due to basal Wg protein diffusing more rapidly to adjacent cells than apical protein does, as has been observed in the embryo (Simmonds et al., 2001).

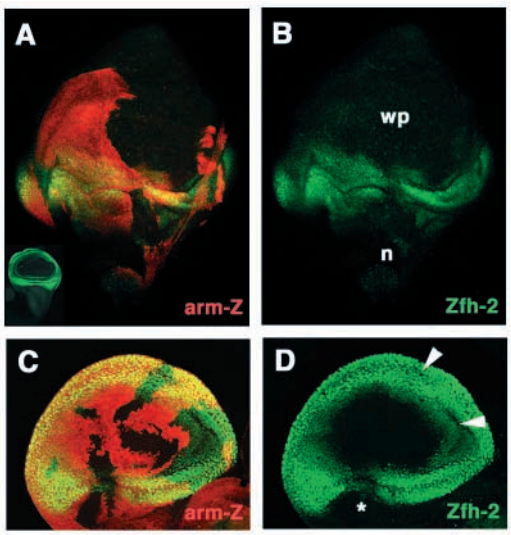


Fig. 5. Ds function is required for the early activation of *zfh2*, a target gene of Wg pathway. (A-D) Third instar wing discs containing ds^{D36} clones marked by absence of *lacZ* expression (red). (A,B) Early-induced ds^{D36} clones (36±12 hours AEL) eliminate *zfh2* expression in the hinge region. Observe that the *ds* mutant territory overproliferates with respect to the wild type. Inset shows Zfh2 expression in wild-type wing disc. (C,D) Late-induced ds^{D36} clones (60±12 hours AEL) only downregulate *zfh2* expression at the proximal hinge region (asterisk in D), whereas in the pleura region its expression looks unaffected (arrowheads in D). (B,D) Green channels show the expression of Zfh2.

Taken together, these observations suggest that Ds protein contributes to the apical localization of Wg protein at the plasma membrane. It is though unlikely that this function of Ds is responsible for the early PD patterning defects in DNW discs, as *ds* and *wg* are expressed in complementary domains during early larval development.

ds regulates Wg signaling in other developmental contexts

I also investigated whether Ds is required for Wg-mediated patterning in imaginal discs other than the wing disc by analyzing the genetic interactions between *ds* and several

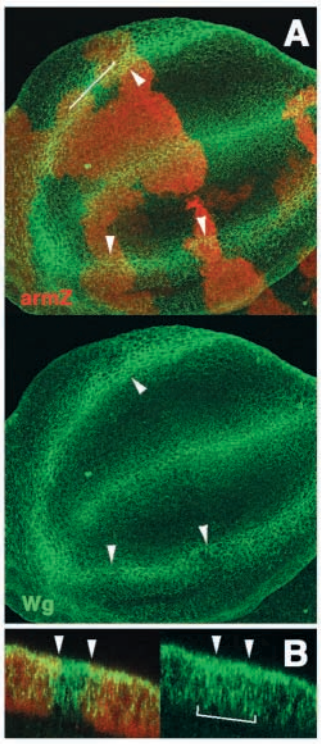
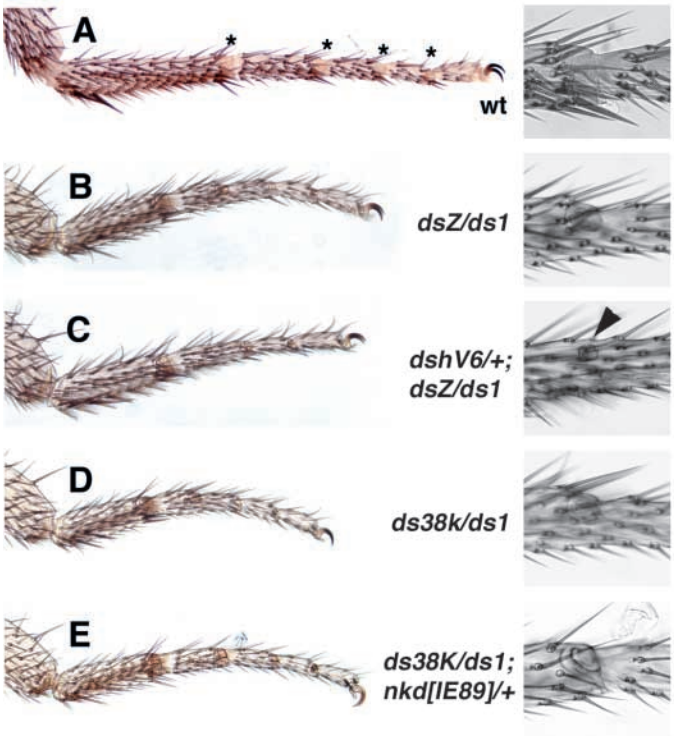


Fig. 6. The sub-cellular distribution of Wg protein in the Wg-producing cells is altered in *ds* mutant cells. (A,B) Third instar discs containing ds^{38k} clones generated by the *Minute* technique. Clones were induced during second instar and are marked by the absence of *armZ* (red). (A) In ds^{38k} cells, the gradient of extracellular Wg (green) is expanded with respect to the adjacent wild-type cells (arrowheads). (B) A cross-section from the region marked by the white line in A, showing a higher accumulation of Wg protein at the apical surface of *ds* mutant cells (compare with wild-type cells, red marker). Wg is uniformly distributed along the apical-basal axis. Note that the mislocalization of Wg also affects the wild-type cells adjacent to ds^{38k} mutant cells (bracket). Wg expression is shown in the green channel.

components of the Wg pathway during leg development. Homo- and hetero-allelic combinations of *ds* cause a reduction of the segment size and fusion of the tarsal segments, with partial elimination of the tarsal joints (Fig. 7B,D) (Clark et al., 1995). This phenotype resembles some defects associated with the loss of function of *pangolin/dTCF*, (Brunner et al., 1997) and *legless/BCL9* (Kramps et al., 2002). Therefore, the levels of Wg signaling were manipulated in mid-strength heteroallelic combinations of *ds*. The loss of one wild-type copy of *dsh* enhanced the fusion of leg tarsi and shortened the leg segments (Fig. 7B,C). By contrast, the leg phenotype of *ds* showed a complete recovery of the tarsal joints and an increase in the length of the segments when one dose of the *nkd* gene, an antagonist of the Wg pathway, was eliminated (Zeng et al., 2000; Rousset et al., 2001) (Fig. 7D,E). Taken together, these findings support a more general role for *ds* in Wg-mediated patterning processes.



Discussion

The present work describes a new role of *ds* in the specification of the proximodistal axis in the early wing imaginal disc, which is independent of its previously characterised role in PCP. At present, *ds* is the only known cadherin in *Drosophila* that shows a spatially restricted pattern of expression in wing imaginal discs from early stages onwards, and it can be considered one of the earliest specific markers for the hinge territory.

ds is required for early specification of the proximal wing and hinge

The wing primordium is specified as a few anterior cells that express *wg* at the distal-most part of the wing imaginal disc at second larval instar. Slightly later, *wg* is also expressed in P cells and these cells are recruited into the wing fate (Ng et al., 1996). In DNW discs, the level of Ds protein is highly reduced and only the initial anterior group of Wg-expressing cells becomes specified into the wing fate (Fig. 1H,J). The levels of this initial Wg expression seems not to be affected in DNW discs. However, neither the P cells abutting the initial anterior Wg domain nor the surrounding cells of this early wing primordium are able to respond to Wg, leading to the formation of a wing pouch composed exclusively by A cells (Fig. 1I,J). Moreover, the activation of Wg target genes, such as *hth*, required for the specification of hinge cells fails in DNW discs (Fig. 3J), and, consequently, the proximal wing and hinge

Fig. 7. Ds regulates Wg signaling during leg development. Removal of one dose of several genes that participate in the Wg pathway modifies the *ds* phenotype in the leg. Proximal leg of a wild-type (A) and *ds* mutant (B,C,D,E) specimens. Right panels show a higher magnification of the third tarsal joint for each genotype. In all panels, proximal is to the right and distal to the left. (A) The wild-type tarsus is divided into five segments connected by four tarsal joints (asterisks). (B) In dsZ/ds^{1} mutants, tarsal segments are shortened and some tarsal joints are incomplete. (C) Insufficiency of the Wg pathway, caused by removal of one dose of *dsh*, increases the severity of the dsZ/ds^{1} phenotype. The length of the segments is reduced and most of the tarsal joints are almost eliminated (arrowhead, right). In several cases the tarsal joints are completely absent (not shown). Reduction of *nkd*, by one dose, produces an increase in Wg pathway signalling. (D) The leg phenotype of ds^{38k}/ds^{1} is similar that shown in B. (E) Elimination of one dose of *nkd* in ds^{38k}/ds^{1} background completely rescues the tarsal joints and the size of the tarsal segments is recovered almost to that of wild type. dsZ/ds^{1} and ds^{38k}/ds^{1} are considered mild allelic *ds* combinations with respect to the leg phenotype. A representative phenotype for each genotype was illustrated. dsh^{V6} or nkd^{1E89} are null alleles.

structures do not develop (Fig. 1B,E) (Casares and Mann, 2000; Whitworth and Russell, 2003). The significantly reduced rings of *ds-lacZ*, *hth* and *zfh2* expression (Fig. 3J,K,M) in DNW discs most likely reflect the residual Ds activity retained in the ds^{38k} mutant. Cells close to the Wg source might thus still be able to respond to high Wg levels during early stages of wing development. However, under null conditions for *ds* (ds^{D36}) the expression of *zfh2* is eliminated (Fig. 5A,B).

Thus, in addition to its function in PCP, *ds* plays a role in early patterning when the specification of the different territories along the PD axis takes place in response to Wg. Initially, *ds* facilitates the recruitment of P cells into the wing fate in response to Wg. Subsequently, Ds promotes the activation of Wg target genes in the surrounding cells to specify the hinge. Note that once the hinge cells have been specified in response to Wg signaling, *ds* seems to be dispensable for global wing disc patterning, as the 'classical' ds^{38k} phenotype shows (Clark et al., 1995). In this case, only mild defects such as slight tissue overgrowth or polarity defects were observed, suggesting additional functions of *ds* related to cell adhesion.

As shown above, ectopic expression of Dpp (Fig. 4D) in wing cells of DNW discs restores both the formation of the AP border and cell proliferation within the wing pouch, indicating that both Wg and Dpp orchestrate these events. Only cells previously committed to the wing fate by Wg are able to proliferate in response to Dpp, as the *UAS-dpp/dpp-Gal4* and *UAS-dpp/omb-Gal4* experiments suggest. In the *ds* mutant background, *omb* is expressed in anterior wing cells, albeit in the absence of the AP border/Dpp source within the wing pouch, suggesting that this initial *omb* expression might not be Dpp dependent. Similar results were observed for *spalt* (*sal*), another known target gene of *dpp*. I propose that Ds primarily regulates Wg signaling in the initial recruitment of P cells into putative wing territory. Once this initial recruitment has occurred, Dpp expression is established and Dpp signaling can contribute to the further recruitment of P cells. Expression of *UAS-dpp* in anterior wing pouch cells of *ds* mutant discs using *omb-Gal4* can bypass the initial requirements for Wg in P cell recruitment, leading to the observed wing pouch rescue (Fig. 4C,D).

Ds contributes to the maintenance of the hinge/notum boundary

In vertebrates, during telencephalon formation, the organization into different structures requires the expression of different cadherins in adjacent regions to maintain a compartment boundary based on differential cell affinity features. It has been suggested that the expression pattern of each of these cadherins is under the control of specific signaling cascades (Inoue et al., 2001).

In *Drosophila*, during imaginal disc development, indirect evidence has suggested that cell adhesion might be under the control of the same signaling pathways that control cell proliferation and patterning. The smooth borders of clones mutant for *thick vein* (*tkv*), the receptor of Dpp (Burke and Basler, 1996), or *smoothened* (*smo*) (Blair and Ralston, 1997; Rodriguez and Basler, 1997), a downstream component of the Hedgehog (Hh) signaling pathway, indicate that mutant cells change their affinity properties and therefore try to minimise the contact with surrounding wild-type cells. Nevertheless, little is known about the molecules involved in these adhesiveness differences. Recent work has proposed that both *tartan* and *capricious* (*caps*), two transmembrane proteins regulated by *ap*, are putative candidates to maintain the affinity boundary between dorsal and ventral cells (Milan et al., 2001). However, whereas clones ectopically expressing *tartan* and *caps* in V cells tend to contact D cells, the elimination of *tartan* and *caps* in clones from D cells had no effect on DV boundary formation.

In the DNW phenotype, the ectopic notum develops from cells of the hinge territory (Fig. 1A,B). According to the proposed subdivision into concentric rings (I to III), cells from the outermost ring III expressing Tsh and Ds will give rise to that part of the body wall that is excluded from the notum region (Fig. 3N, wt). In DNW discs, the absence of Ds produces an expansion of notal-specific *iro-C* expression to more distal positions to fill up the Tsh domain (compare Fig. 1H with Fig. 3L,N; $ds^{-/-}$). These distal cells acquire a notum fate (Fig. 3N), generating an ectopic notum similar to wg^{1} mutant flies (Sharma and Chopra, 1976).

Thus, Ds protein contributes to hinge/notum boundary formation by means of an affinity border. This process would occur at early second instar when Iro-C expression is capable of specifying the notum fate. This finding provides the first evidence that a cadherin is able to maintain the cell boundary between two adjacent territories in *Drosophila*.

How does *ds* participate in Wg signaling?

Several findings point out a specific role of Ds in the modulation of Wg signaling: (1) the elimination of *zfh2* expression in *ds* mutant clones (Fig. 5A,B); (2) the genetic interactions of *ds* alleles with several components of the Wg signaling pathway; and (3) the rescue of the DNW phenotype by increasing Wg levels. It has been shown that Ds is associated with adherens junctions at the apical surface of the imaginal cells (Fig. 2E) (Ma et al., 2003), to mediate cell-cell adhesion. A major step of the cell adhesion mechanism requires interaction of the cytoplasmic tail with Arm/β-catenin to connect the cadherin-catenin complex to the actin cytoskeleton (Vleminckx and Kemler, 1999). Thus, the phenotype could reflect changes in the balance between cytoplasmic Arm versus Arm anchored to the plasma membrane. If this were the case, then a reduction of *ds* function would increase Wg signaling; however, the results presented above indicate that loss of *ds* decreases Wg signaling. Moreover, sequence analysis has shown that the β-catenin binding motifs in the Ds protein, which have to be in tandem to be functional, are separated by a stretch of amino acids, further discarding the possibility that Ds binds directly to Arm to modulate its cytoplasmic levels (Clark et al., 1995).

Alternatively, the apical plasma membrane acts as a structural centre that contains crucial components that modulate the Wg pathway, such as Dsh (Cliffe et al., 2003; Axelrod, 2001), E-APC (Yu et al., 1999) and Axin (Cliffe et al., 2003). Axin and E-APC, promote the degradation of cytoplasmic Arm, the main effector of the Wg cascade (Ikeda et al., 1998; Yu et al., 1999). Previous work has shown that, upon binding of Wg in the receiving cells, the Axin/E-APC complex becomes anchored to the plasma membrane to prevent Arm degradation (Kishida et al., 1998; Cliffe et al., 2003). In this context, Ds protein, as part of the adherens junctions, could be the cadherin required to anchor this degradation complex to

the plasma membrane. In *ds* mutant cells, the cytoplasmic levels of the Axin/E-APC complex would be higher and, therefore, Wg signaling would decrease. In agreement with this hypothesis, I have observed that mild *ds* phenotypes are enhanced when a copy of *dsh* gene is eliminated (Fig. 7C). Still, Ds could act at the level of Wg reception, by increasing the Fz/Wg-binding affinity or by recruiting Fz molecules to the apical plasma membrane, as has been demonstrated for the cadherin Fmi in the PCP processes (Strutt, 2001).

Early anterior Wg activity initiates specification of the PD axis in the wing disc

To date, the current model explaining the specification of the territories along the PD axis assumes that the initial anterior Wg expression at second instar is required only for cells to acquire the wing fate. It is only later, when *wg* is expressed in two concentric rings that its function is required to specify the hinge territory.

Wg has been shown to be required for the development of the hinge. On the one hand, Wg activates downstream genes such as *hth* (Casares and Mann, 2000) or *zfh2* (Whitworth and Russell, 2003) to specify the hinge fate. On the other hand, Wg controls cell proliferation when it is expressed from early third instar into the IR and OR rings (Neumann and Cohen, 1996; Del Alamo et al., 2002). It has been established that the specification of the hinge takes place later than the wing; however, my data show that an early and timely limited depletion of Wg activity causes a failure in hinge specification. This is mainly based on the observation that only early-induced *ds* clones abolish *zfh2* expression required for hinge formation. In *ds* mutant clones induced later, hinge development is unaffected, although a perdurance of *ds* activity in these clones cannot be excluded. Still, the rescue of hinge development in DNW discs that ectopically express Wg under *dpp-Gal4* further support an early specification of the hinge. In these discs, ectopic Wg expression stays confined to the AP border. At early stages, the AP border must be located close enough to the nascent wing primordial to allow the spreading of Wg into regions destined to become hinge territory. At late stages, the narrow stripe of ectopic Wg expression can no longer account for the maintenance of the whole hinge territory. It is rather the Wg within the IR and OR that maintains *hth* expression and, with it, the specification of the hinge fate. At this stage, either Wg works independently of *ds* or its requirements for *ds* are lower. Thus, if hinge specification is not initiated early upon *ds* and *wg* activities, wg expression cannot be established and the development of the hinge is aborted.

The present results provide insights that help us to understand how the PD axis is established in the wing disc. The initial event in this process would be the early activity of Wg. When Wg is expressed at the distal part of the wing disc in a small group of anterior cells, it not only promotes the activation of target genes like *vg*, *nub* or *scalloped (sd)* in the wing cells, but also the expression of *hth* and *zfh2* to specify the hinge. At the same time, Wg would repress *tsh* or *vein* (*vn*) at the distal part of the wing disc to separate the proximal wing and hinge regions from the body wall where Egfr signaling activates notum-specific genes like *iro-C*. Thus, in cooperation with *dpp*, *wg* establishes the AP and PD axis in the prospective wing and hinge regions.

In DNW discs, even though the Dpp source is distantly and asymmetrically located with respect to the wing pouch (Fig. 1G, part c; J), anterior wing cells differentiate into distinct cell types (Fig. 1E,F) in a mirror image disposition. This result suggests that specific positional information might be provided independently of *dpp*. Ap in combination with Wg might contribute to this initial AP positional information. Once P cells are recruited into the wing fate, Dpp takes over and promotes pattern formation along the AP axis, as well as proliferation within the wing pouch.

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