A Directed Mutagenesis Screen in Drosophila melanogaster Reveals New Mutants That Influence hedgehog Signaling

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ABSTRACT

The Hedgehog signaling pathway has been recognized as essential for patterning processes in development of metazoan animal species. The signaling pathway is, however, not entirely understood. To start to address this problem, we set out to isolate new mutations that influence Hedgehog signaling. We performed a mutagenesis screen for mutations that dominantly suppress Hedgehog overexpression phenotypes in the Drosophila melanogaster wing. We isolated four mutations that influence Hedgehog signaling. These were analyzed in the amenable wing system using genetic and molecular techniques. One of these four mutations affects the stability of the Hedgehog expression domain boundary, also known as the organizer in the developing wing. Another mutation affects a possible Hedgehog autoregulation mechanism, which stabilizes the same boundary.

MEMBERS of the Hedgehog (Hh) family of proteins are secreted intercellular signaling molecules that provide vital patterning information in a wide range of developmental contexts. Signaling by Hh proteins has been identified in patterning of the vertebrate neural tube, the somites, specification of different neuronal cells, and myotome and sclerotome differentiation. The determination of left-right asymmetry, hair follicle development, and limb morphogenesis have all been found to involve Hh signaling (for review, see Hammerschmidt et al. 1997). The hh mutation was first identified in a mutagenesis screen for embryonic lethals in Drosophila melanogaster that disrupt the segmental organization of the embryo (Nüsslein-Volhard and Wieschaus 1980). Strong hh alleles cause deletion of the posterior naked portion of each embryonic segment and loss of cell polarity in the remaining (dentine-covered) part, resulting in a “spiky” embryo with deranged denticles everywhere. This phenotype led to the hh mutant being classed as one of the segment polarity mutants. This group of mutants is now well characterized and the underlying genes are known to be required for correct patterning and polarity of each embryonic trunk segment (Martinez-Arias et al. 1988). The hh gene was cloned (Mohler and Vani 1992) and its amino acid sequence suggested that the protein would be secreted. Within the segment polarity group of mutants, several lead to phenotypes similar or identical to the hh mutant phenotype. It is now clear that some of these genes are required downstream of the Hh signal (Ingham 1998).

Hh genes were cloned from vertebrate species on the basis of homology to the Drosophila hh gene (see Hammerschmidt et al. 1997). In addition, for most of the genes identified in Drosophila as functioning in the Hh pathway, homologous vertebrate genes have been isolated. At least for some of these, a direct role downstream of vertebrate Hh proteins has been defined similar to what is seen in flies. Loss of hh function in mice is embryonic lethal and midline defects are seen in humans in whom the hh gene is affected (Chiang et al. 1996; Roessler et al. 1996). A driving role for Hh signaling in the generation of oncogenic transformation has been described on the basis of mutations in Hh signaling genes (see Ruiz i Altala 1999). The best-known example of this is the role the patched (ptc) gene plays in the noninvasive skin tumors of the basal cell carcinoma type (Johnson et al. 1996). The ptc gene normally functions to inhibit Hh signaling. This becomes obvious as the ptc loss-of-function phenotype in Drosophila embryos is identical to the phenotype caused by overexpression of hh (Ingham 1993). In fact, ptc encodes the only protein yet known to bind the Hh protein directly (Stone et al. 1996).

As in vertebrates where Hh proteins play important roles in the development and differentiation of many tissues, the single hh gene in flies (based on latest genomic database searches) plays multiple roles. Its expression in the embryonic segment is maintained in the tissues that will form most of the adult fly. In these imaginal discs, hh plays an essential role in patterning (Basler and Struhl 1994).

Although several proteins are known to function downstream of Hh, either to transduce or to inhibit the signal (Ingham 1998), their interactions are poorly understood. To identify possible new components in or
modifiers of Hh signaling, we made use of the role Hh signaling plays in the patterning of the imaginal wing disc in Drosophila. An existing dominant hh allele, hh\textsuperscript{hominat}, induces overgrowth and repatterning in the wing (Tabata and Kornberg 1994). The hh\textsuperscript{hominat} phenotype is mild and thus can be used as a background to isolate suppressors. Several mutants were isolated that reduce the phenotype and these are presented here with their genetic analysis.

**MATERIALS AND METHODS**

**Fly strains:** A second chromosome isogenized stock was generated (cn bu sp) for the mutagenesis. All GALA lines (30A, 34B, and 1348) have been described (Brand and Perrimon 1993). The following deficiency stocks were used: Df(2L)J39, Df(2R) PCA, Df(2R)or-Br-I, and Df(3R)rin7; some of these form part of the Bloomington deficiency kits. UAS hh, UAS sonic hh, UAS dpp, UAS ci, and dpplacZ have been described (Ingham and Fietz 1995; Alexandre et al. 1996). The stocks used for mosaic recombination mapping were net dp cn bu, and st p e.

**Mutagenesis:** A total of 10,000 male flies of an isogenized cn bu sp stock were treated with 4000 rad using a Co source. After 24 hr they were mated to hh\textsuperscript{hominat}/TM3 females and the F\textsubscript{1} non-TM3 progeny were screened for reduction of the hh\textsuperscript{hominat} phenotype. The suppressing loci were rescued, tested again over hh\textsuperscript{hominat}, and balanced using the suppression phenotype to determine the chromosomal location. All stocks were then kept balanced or as homozygous (if viable). Regularly, the suppression phenotype was tested against either hh\textsuperscript{hominat} or 30-AGAL4 UAS shh.

**B-Galactosidase stainings of imaginal discs:** Larval heads were cut off the larvae in 1× PBS. They were inverted and fixed for 6 min in 1× PBS, 0.1% glutaraldehyde. The inverted heads were washed five times with 1× PBS and incubated at 37°C for 1 hr in 10 mm phosphate buffer, 3.1 mm K\textsubscript{4}(Fe[II]CN\textsubscript{6}), 3.1 mm K\textsubscript{3}(Fe[III]CN\textsubscript{6}), 150 mm NaCl, 1.0 mm MgCl\textsubscript{2} with 0.32% X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside). The heads were washed with 1× PBS, 0.1% Tween 20, and the discs were dissected in the same buffer. The wing imaginal discs were mounted in 1× PBS, 70% glycerol on glass slides. They were photographed with a Zeiss Axioscop microscope using differential interference contrast optics.

**Antibody stainings of imaginal discs:** Larval heads were cut off the larvae in 1× PBS on ice. They were inverted and fixed for 20 min in 1× PBS, 4% paraformaldehyde. The inverted heads were washed five times with 1× PBS and again washed five times for 5 min each with 1× PBS, 5% normal serum (usually donkey in case secondary antibodies were derived from donkey), and 0.5% saponin (PBT). They were incubated overnight at 4°C with the primary antibodies in PBT and washed again five times with PBT. Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). They were used at the manufacturer’s recommended concentrations for 2 hr in PBT at room temperature, after which the heads were washed again five times with PBT. Discs were dissected from the heads in PBT and mounted in VectaShield (Vector Laboratories, Burlingame, CA) on glass slides. They were viewed using a Leica confocal laser scanning microscope.

The antibody against the Cubitus interruptus (G) protein originated from Motzny and Holmgren (1995), while the antibody against Engrailed (En) protein came from Patel et al. (1989).

**RESULTS**

**Suppression of hh\textsuperscript{hominat} phenotype:** The hh\textsuperscript{hominat} allele leads to ectopic expression of the hh gene in the anterior wing compartment, where normally hh is not expressed (Tabata and Kornberg 1994; Felsenfeld and Kennison 1995). The resulting phenotype, shown in Figure 1B, varies between clear overgrowth of the (usually distal) part of the wing to slight disorganization of the wing margin and the addition of extra vein material. This phenotype can be used to screen for suppressing mutations; however, its variability can raise difficulties.

Male flies were exposed to 4000 rad of radiation. These males were mated to hh\textsuperscript{hominat} females and the resulting F\textsubscript{1} progeny were studied. In total, 25,000 chromosomes were screened and 12 mutants were identified in which the hh\textsuperscript{hominat} phenotype was suppressed. Seven of the mutations were stabilized and balanced to second and third chromosomes [labeled Su(hh) I–VII]. These consistently suppressed the hh\textsuperscript{hominat} wing phenotype. To compensate for the isolation of spurious suppressors due to the variability of the hh\textsuperscript{hominat} phenotype, all isolated mutants were screened three times against the hh\textsuperscript{hominat} stock; average suppressed wings are shown in Figure 1, C–I.

**Figure 1.—** *D. melanogaster* wings showing the suppression of hedgehog\textsuperscript{hominat} phenotypes; distal to the left and anterior upward. Wings were derived from adult flies of the following genotypes: (A) +/++; (B) hh\textsuperscript{hominat}/+; (C) Su(hh) 1/++; hh\textsuperscript{hominat}/++; (D) Su(hh) II/++; hh\textsuperscript{hominat}/++; (E) Su(hh) III/++; hh\textsuperscript{hominat}/++; (F) Su(hh) IV/hh\textsuperscript{hominat}, (G) Su(hh) V/++; hh\textsuperscript{hominat}/++; (H) Su(hh) VI/hh\textsuperscript{hominat}, (I) Su(hh) VII/hh\textsuperscript{hominat}. Note suppression of the hh\textsuperscript{hominat} phenotype in all wings (C–I); wings are representatives of at least three individual crosses and several hundred flies.
Suppression of ectopic hh phenotypes: In the hh\textsuperscript{me} wing imaginal discs, hh is ectopically expressed in the anterior region of the disc. To verify that our mutants did not suppress Hh overexpression specifically in this region of the wing, all isolated mutants were crossed into a background where Hh is expressed in the disc at this and other sites. However, ectopic expression of Drosophila hh in the wing imaginal disc using transgenes leads to severe duplications and often flies do not emerge. Vertebrate Hh genes induce similar but milder duplications when overexpressed (Figure 2B). In addition, by using a vertebrate Hh gene, we reassert that the observed suppression is specific to Hh signaling.

The seven mutants were tested using a phenotype generated by UAS-GAL4-driven expression of zebrafish sonic hh in the presumptive proximal areas of the wing (Brand and Perrimon 1993; Krauss et al. 1993). Using 30A-GAL4, four of the seven mutants clearly suppressed the strong wing duplication phenotype caused by overexpression of shh [Figure 2, C–F; Su(hh) I–IV]. Three mutants did not suppress this phenotype [Figure 2, G–I; Su(hh) V–VII]. Using a second GAL4 driver (34B-GAL4) that drives expression in cells that will form only the most proximal parts of the wing, the duplication is milder (Figure 2J). Su(hh) I–IV that suppress the strong duplication phenotype driven by 30A-GAL4 also suppress the 34B-GAL4 phenotype (Figure 2, K–N).

Suppression of ectopic decapentaplegic phenotypes: A transcriptional target of Hh signaling in the wing imaginal disc is the decapentaplegic (dpp) gene, a member of the tgfβ/BMP superfamily (Zecca et al. 1995). The Dpp protein is expressed in a narrow band of cells adjacent to the hh expression domain (see Figure 5A). The Dpp protein is considered responsible for some of the growth and patterning in the wing imaginal disc (Nellen et al. 1996).

The suppression of the overgrowth and repatterning phenotypes seen above could be caused by suppression of Dpp signaling rather than of Hh signaling itself. To distinguish between these possibilities we introduced the Su(hh) I–VII mutants into a dpp overexpression background. In this experiment, the dpp gene is ectopically expressed using the same GAL4 driver as above (30A), leading to a mirror image duplication of tissue at the anterior and overgrowth at the posterior (Figure 3B). Suppression of the phenotype caused by dpp overexpression was observed in four mutants [Su(hh) I, II, IV, and VII; Figure 3, C, D, F, and I]. The strong suppression observed in Su(hh) VII was very consistent, while in Su(hh) I, II, and IV only mild suppression was observed and sometimes it was impossible to distinguish suppressed from nonsuppressed. Three mutants did not suppress the dpp-induced phenotype [Su(hh) III, V, and VI; Figure 3, E, G, and H].

On the basis of these and the above results, we excluded Su(hh) V and Su(hh) VI (shown in Figures 2 and 3, G and H) from further study; these did not suppress ectopic shh nor ectopic dpp. Su(hh) VII clearly reduces the phenotype caused by ectopic dpp but the phenotypes caused by ectopic hh expression are not affected (Figures 2 and 3I). As we were interested in Hh signaling, we did not pursue this mutant either. Of the four remaining mutants, Su(hh) III (Figure 2, E, and M; Figure 3E) suppresses only ectopic hh expression. The other three [Su(hh) I, II, and IV; Figures 2 and 3, C, D, and F; Figure
The variable suppression is due to the fact that ectopic dpp drives developmental changes in the regions also suppressed by Shh signaling, possibly by cooperation, deficiencies covering the indicated regions were depicted as regions within chromosome arms; shown are left and right arm of second and left arm of third chromosome. Also shown are deficiencies that take out (parts of the) regions identified in the meiotic mapping. Sut(hh) I maps to position 31–33 on the left arm of the second chromosome (hatched bar). Recombination frequency with net, 46%; with dp, 18%. A deficiency (Df(2L)J39) within this region (31C–31D) suppressed the GAL4-derived phenotype (gray hatched bar). Sut(hh) II maps to the region 53A–57A on the right arm of the second chromosome (vertically hatched bar). Recombination frequency with cn, 19%; with bw, 12%. Again a deficiency (Df(2R)PC4) within this region suppresses the ectopic shh phenotype (gray vertically hatched bar). Sut(hh) III is embryonic lethal. The mutant was mapped to the region 59–60 using the suppression of the ectopic shh phenotype. Recombination frequency with bw, 0%. This mutant is lethal over a deficiency uncovering 59F–60A (Df(2R)br11); this deficiency also suppresses the test phenotypes. Sut(hh) IV was mapped to the left arm of the third chromosome to 68–69 using meiotic mapping based on suppression. Recombination frequency with st, 9.5%; with p, 10%. The mutant is lethal over a deficiency with breakpoints at 68C and 69B (Df(3L)win7); again this deficiency also suppresses the test phenotypes.

Sut(hh) I was mapped to map position 31–33 and Sut(hh) II to 53–57. Deficiencies that fall within these regions also suppressed the 34BGAL4 UAS shh phenotype. Neither of these regions contains mutants that are thought to influence Hh signaling. Sut(hh) III was mapped to the region 59–60 on the right arm of the second chromosome. A deficiency covering this region (59F–60A) suppresses the 34BGAL4 UAS shh phenotype. Sut(hh) III is homozygous embryonic lethal and lethality is also seen in trans over the deficiency. Sut(hh) III mutant embryos display a normal cuticle phenotype. In the 59–60 region on the second chromosome, several interesting genes are known. One of these is the glass bottom boat (gbb) gene that encodes a dpp homologue in flies. Mutations in this gene have been shown to influence Dpp-driven wing patterning, possibly by cooperating with Dpp signaling (Haerry et al. 1998). Our locus, however, complements gbb.

Sut(hh) IV was mapped to map position 68–69 on the
left arm of the third chromosome. A deficiency in this region (68C–69B) also suppresses the 34BGAL4 UAS shh phenotype. Like Su(hh) III, Su(hh) IV is homozygous lethal and no embryonic phenotype is discernible. Su(hh) IV is also lethal in trans over the 68C–69B deficiency. There are no mutants known in this region that influence Hh signaling.

**Influence on target gene expression:** Ectopic Hh signaling leads to induction of ectopic dpp transcription. If our suppressor mutants function in the Hh pathway, they should reduce the amount of ectopic dpp expression. Figure 5A visualizes the expression domain of the GAL4 driver we used in these experiments (30A GAL4). If 30AGAL4 is used to express the shh gene, dpp expression is induced in the areas where the driver is active in the anterior area of the wing imaginal disc, in addition to the normal expression domain in the stripe (Figure 5, B–E; left vs. middle). In the posterior part of the disc (where endogenous hh is expressed), ectopic dpp is suppressed by the action of the transcriptional repressor En, coexpressed there with hh (Sanicola et al. 1995).

We introduced Su(hh) I–IV into the 30A GAL4 UAS shh genetic background and examined the expression of dpp using a dpplacZ transgene. Both Su(hh) I and IV reduced the amounts of ectopic dpp expression compared to the transgenic background, as expected (Figure 5, B and E). In Su(hh) III discs, the ectopic expression of dpp is reduced but also the normal endogenous stripe of dpp expression through the middle of the disc is reduced in intensity (Figure 5D). In Su(hh) II, the levels of ectopic dpp were increased especially in areas where 30AGAL4 expression is highest (Figure 5C).

**Suppression of ectopic cubitus interruptus phenotypes:** Transcription of target genes downstream of hh signaling is elaborated through the activation of a large cytoplasmic complex (Kalderon 1997; Aza-Blanc and Kornberg 1999). This complex consists of at least three proteins: Costal-2, a kinesin-like molecule; Fused, a serine-threonine kinase; and Cubitus interruptus (ci), a zinc-finger transcription factor. The Ci protein is believed to enter the nucleus, to bind upstream to the DNA of Hh transcriptional targets, and to activate transcription. This has been shown in assays in yeast (Alexandre et al. 1996). The cytoplasmic complex is thought to mediate Ci activation, perhaps through release to allow entrance into the nucleus but also through proteolytic control.

Overexpression of the full-length ci gene leads to extreme duplication phenotypes and no flies emerge when the 30AGAL4 driver is used. We used another GAL4 driver to express ci that allows flies to emerge with a mild

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**Figure 5.** Dissected wing imaginal discs stained for β-galactosidase activity, showing the expression of the 30AGAL4 transgene (A) and decapentaplegic-lacZ transgene (B–E); anterior to the left; wing pouch upward. The imaginal discs were derived from third instar larvae of the following genotypes: (A) 30AGAL4 UAS lacZ; (B–E) left, dpplacZ/+; middle, dpplacZ/30AGAL4; UAS shh/+; Right (B) 30AGAL4 dpplacZ/Su(hh) I; UAS shh/+; (C) 30AGAL4 dpplacZ/Su(hh) II; UAS shh/+; (D) 30AGAL4 dpplacZ/Su(hh) III; UAS shh/+; (E) 30AGAL4 dpplacZ/+; UAS shh/Su(hh) IV. Note the increase in ectopic dpplacZ staining in the disc derived from Su(hh) II larvae (arrowheads) and the reduction of the endogenous stripe of dpp expression in the Su(hh) III disc (arrow). The control discs (left and middle in B–E) were derived from the same staining dish as the suppressed examples shown.
Figure 6.—Drosophila wings showing the suppression of phenotypes generated by overexpression of *cubitus interruptus* (A–E); distal to the left and anterior upward. Wings were derived from adults of the following genotypes: (A) 1348GAL4/+; UAS ci/1; (B) 1348GAL4/Su(hh) I; UAS ci/+; (C) 1348GAL4/Su(hh) II; UAS ci/+; (D) 1348GAL4/Su(hh) III; UAS ci/+; (E) 1348GAL4/+; UAS ci/Su(hh) IV. Note the differential suppression of the ci overexpression phenotype in the two compartments in Su(hh) IV (compartment boundary indicated by dashed line).

vein phenotype (1348GAL4; Figure 6A). We introduced the suppressor mutants into this background and analyzed the suppression of the UAS-ci phenotype. Su(hh) I did not suppress the phenotype caused by overexpression of ci (Figure 6B). Su(hh) II and III reverted the phenotype to close to wild-type vein patterning (Figure 6, C and D), while Su(hh) IV effected differential rescue of the phenotype with anterior compartment vein patterning being close to normal (Figure 6E).

**Further analysis of Su(hh) II and III:** Su(hh) III was analyzed further because this locus seems to suppress only Hh signaling and not Dpp signaling. In Su(hh) III/+ discs in a *shh* overexpression background, reduced levels of Dpp expression at the anterior-posterior compartment boundary are observed (Figure 5D). In wild-type wing imaginal discs, the expression of Dpp is confined to a precise stripe of cells anterior to the expression domain of hh, creating a sharp boundary between hh- and dpp-expressing cells (Figure 7A). The Hh signaling pathway is required for the expression of Dpp across this boundary but is also thought to play a crucial role in the maintenance of the boundary. We were interested to see if markers of this boundary were disrupted in Su(hh) III heterozygous mutant wing imaginal discs. The expression of Dpp along this boundary in anterior cells is abnormal; the normal sharp stripe is diffuse and not as much of a “straight” line down the disc as in wild type (Figure 7A). However, we could only analyze this using the *dpplacZ* transgenic line since antibodies to Dpp protein are no longer available. As alternative markers for the boundary, we analyzed the expression of the Ci and En proteins in discs using antibodies directed against the proteins. The levels of the Ci protein are elevated in cells where the Hh pathway induces its release from the cytoplasmic complex, but the protein is absent from cells expressing hh. Lower levels of Ci protein are seen throughout the rest of the disc where hh is not expressed (Aza-Blanc et al. 1997). The En protein is coexpressed...
with the cells expressing \( hh \) (Tabata and Kornberg 1994). As shown in Figure 7, B and C, Ci and En distributions are both disrupted. The stripe of high protein levels of Ci normally running very sharply along the boundary now forms a diffuse variable line down the middle of the disc. Similarly, the expression domain of the En protein, which in wild type exactly complements the Ci stripe, is diffuse and disorganized. The area most affected in the discs is in most cases the more distal part of the presumptive wing blade area (arrows in Figure 7).

In three of the suppressor mutants analyzed, we detected a reduction in the levels of target gene expression induced by the overexpression of Hh. However, in \( Su(hh) \) II, although it clearly suppressed the overexpression phenotypes (Figure 2D), higher levels of ectopic \( dpp \) were found especially in the areas of the disc where the GALA expression is highest (Figure 5C). Hh signaling has been shown to lead to expression of En in anterior cells, but only in cells that are closest to the \( hh \) expression domain (de Celis and Ruiz-Gomez 1995; Guillen et al. 1995; Tabata et al. 1995; Gomez-Skarmeta and Modolell 1996; Sanchez et al. 1996; Blair and Ralston 1997; Mullor et al. 1997; Strigini and Cohen 1997). It has been proposed therefore that it is the high levels of Hh that these cells are exposed to that induce the cells to express En. Possibly the high levels of ectopic \( dpp \) in our experimental setup could induce En expression in the anterior part of the disc. En activity represses \( shh \) in our experimental setup could thus lead to absence of \( dpp \) expression. We first investigated if the expression of \( shh \) in the anterior compartment induces ectopic En expression. In the \( GALA UAS shh \) background, ectopic En is seen in the anterior compartment in the areas where the ectopic \( shh \) expression is highest (Figure 8B). Second, we investigated whether in \( Su(hh) \) II discs the induction of En expression was lost. In \( Su(hh) \) II \( 30AGAL4 UAS shh \) discs, En expression in the part of the imaginal disc was not observed (Figure 8C). This indirectly would lead to the gain of \( dpp \) expression.

**DISCUSSION**

We present here four mutations that influence Hh signaling. These have not been previously isolated as suppressing Hh-induced patterning and they thus represent new genes that might play a role in Hh signaling. Alternatively, some of these could be acting in pathways downstream of Hh patterning activity (\( i.e. \), the Dpp signaling pathway).

We utilized a known dominant allele of \( hh \) (\( hh^{oe} \)) in an extensive radiation mutagenesis screen. The combination of this \( hh \) allele and transgenes to overexpress vertebrate Hh genes increased the specificity with which the mutants could be selected for further analysis. Additional combinations of the mutants with transgenic backgrounds for genes in the Hh signaling pathway allowed a placement in the genetic hierarchy. In addition, by using vertebrate homologues for Hh overexpression, the possibility of finding evolutionarily conserved components in the Hh pathway will have increased.

We isolated one mutant, \( Su(hh) \) I, that suppressed the phenotypes caused by overexpression of \( hh \) (or \( shh \)), and this mutant suppresses the induction of ectopic target gene expression (\( dpp \)) by Hh signaling. It maps to the left arm of the second chromosome at map position 31–33. No mutants that affect Hh signaling have been characterized in this region. This mutant could thus
represent a new gene that functions in the Hh pathway. Interestingly, a homologue of the ptc gene can be found here in the annotated genome database (accession no. CG5722). This ptc homologue shows significant homology to the human NPC 1 gene; this gene was isolated in humans as the cause of the Nieman-Pick C1 disease (Carstea et al. 1997).

In contrast, in Su(hh) II discs, we found that the expression domain of the Hh target gene dpp expands. This locus was isolated as the strongest suppressor of Hh overexpression phenotypes; it also suppresses albeit weakly ci and dpp overexpression phenotypes. Upon further analysis of the induction of dpp in this mutant background, we found that the gain of dpp in the discs is due to a loss of ectopically induced En. We show that ectopic shh induces endogenous En expression in the anterior compartment (for references see results). In Su(hh) II discs, En expression is lost in the areas where high levels of shh ectopically induced it. En suppresses dpp expression as a transcriptional repressor (Sanicola et al. 1995), thus loss of En will lead to gain of dpp expression, as seen in our mutant. However, in the 30AGAL4 UAS shh wing imaginal discs, in addition to En expression in the cells exposed to high levels of shh, endogenous hh expression is also seen in these cells (data not shown). We do not know if this ectopic hh is directly induced by ectopic shh or dependent on the ectopic En. The expression of endogenous hh in these cells does, however, point to a possible change of cell identity of these cells, from anterior (non-hh-expressing cells) to posterior (hh-expressing cells). The interpretation of the loss of dpp expression in these cells might thus be due to the fact that these cells are now “true” posterior cells. Our results indicate, though, that the Su(hh) II acts in a pathway that leads to concomitant En and hh expression in the anterior compartment induced by Hh signaling. It is thought that the signaling leading to En expression in the anterior compartment proceeds through the normal Hh pathway (Blair and Ralston 1997; Strigini and Cohen 1997; Ohlmeier and Kalderon 1998). Since Su(hh) II also suppresses ectopic ci phenotypes, we would favor a position for Su(hh) II downstream of ci function but upstream of the induction of hh and En expression. It would thus function in a Hh autoregulatory pathway, upstream of hh and En expression but downstream of the Hh signaling pathway. Su(hh) II maps to the right arm of the second chromosome at map position 55–57 and no genes previously characterized as interacting with the Hh signal transduction pathway are known to map to this position.

Both Su(hh) III and IV are embryonic lethal but neither shows any distinct abnormal cuticle phenotype. If the lethality of these mutants is associated with the suppression and if these mutants are absolutely required for Hh signaling, perhaps embryonic segmentation phenotypes would be expected. The apparent lack of these might indicate a maternal contribution for the gene. This would be consistent with the fact that these loci have not been found in mutagenesis screens for zygotic embryonic phenotypes that resemble the hh phenotype. We have not generated any germline clones to remove maternal contribution since the use of radiation on a mutagen might have caused mutations in more than one gene in our mutants. A role in embryonic patterning for these mutants is thus possible but one cannot exclude an exclusive role for these genes in wing/imaginal Hh patterning.

Su(hh) III maps to a region where several loci of interest to wing patterning driven by Hh or Dpp signaling have been placed (map position 59–60). We show that our mutant is not allelic to gbb, a dpp homologue in this region that has been shown to cooperate with Dpp signaling in disc patterning. Another gene situated close by is the G-protein a-subunit encoding gene (Gos). Since the Hh signaling pathway contains a putative G-protein-coupled receptor, smoothened (van den Heuvel and Ingham 1996), a G-protein might influence Hh signaling. However Su(hh) III does not appear to map to Gos either, since a combination of a deficiency covering Su(hh) III and Gos combined with a translocation containing Gos to the X chromosome still suppresses the test phenotypes. Upon further analysis of the expression of dpp in discs heterozygous mutant for Su(hh) III, we observed differences in the normal expression domain. The normally sharp boundary between the posterior and anterior compartments was less defined, leading up to (in extreme cases) a very disrupted and unclear compartment boundary. Indeed, we confirmed this observation using other markers for the boundary, Ci and En. It is known that Hh signaling is required for proper establishment of the boundary but little is known of the cell biology underlying the clonal and affinity restrictions at the boundary (Blair and Ralston 1997; Rodriguez and Basler 1997; Dahmann and Basler 2000). It is possible that Su(hh) III is in some way involved downstream of Hh in establishing the boundary. Consistent with its being downstream of Hh but upstream of Dpp signaling is that Su(hh) III suppresses ectopic ci but not ectopic dpp. There are many loci in this region of the chromosome and further analysis will be needed to determine which of these genes (or more) are affected in Su(hh) III.

Su (hh) IV is localized on the third chromosome to map position 68–69. It suppresses well the phenotypes generated by overexpression of shh. It also weakly suppresses those generated by dpp ectopic expression. One observation indicates a particular role for this locus. When ci is overexpressed to a low level overlying the boundary between hh-expressing and nonexpressing cells, rescue of the resulting phenotype is observed but only in the domain of the wing that did not express hh. This result indicates that this locus plays a role in Hh signaling at the level of Ci or directly downstream but
only in anterior compartment cells since here the ci-driven phenotypes are rescued. There are no genes in this area that show homology to genes known to act in the Hh signaling pathway.

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