Development 129, 843-851 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV5978

rasp, a putative transmembrane acyltransferase, is required for Hedgehog signaling

Craig A. Micchelli[1,2,*], Inge The[1,†], Erica Selva[1], Vladic Mogila[1] and Norbert Perrimon[1,2]

[1]Department of Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115, USA
[2]Howard Hughes Medical Institute, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115, USA
[†]Present address: Department of Molecular Medicine, NRB Floor 6, Office 621, 364 Plantation Street, Worcester, MA 01615, USA
*Author for correspondence (e-mail: cmicchel@genetics.med.harvard.edu)

Accepted 6 November 2001

SUMMARY

Members of the Hedgehog (Hh) family encode secreted molecules that act as potent organizers during vertebrate and invertebrate development. Post-translational modification regulates both the range and efficacy of Hh protein. One such modification is the acylation of the N-terminal cysteine of Hh. In a screen for zygotic lethal mutations associated with maternal effects, we have identified *rasp*, a novel *Drosophila* segment polarity gene. Analysis of the *rasp* mutant phenotype, in both the embryo and wing imaginal disc demonstrates that *rasp* does not disrupt Wnt/Wingless signaling but is specifically required for Hh signaling. The requirement of *rasp* is restricted only to those cells that produce Hh; *hh* transcription, protein levels and distribution are not affected by the loss of *rasp*. Molecular analysis reveals that *rasp* encodes a multipass transmembrane protein that has homology to a family of membrane bound *O*-acyl transferases. Our results suggest that Rasp-dependent acylation is necessary to generate a fully active Hh protein.

Key words: *rasp*, *hedgehog*, Acyltransferase, Wing imaginal disc, *Drosophila*

INTRODUCTION

Hh proteins make up a conserved family of secreted signaling molecules required during both vertebrate and invertebrate development. These potent signals play a role in specifying cell fate and establishing pattern in many distinct developmental contexts (Hammerschmidt et al., 1997; Chuang and Kornberg, 2000). The most extensively characterized mammalian ortholog is Sonic Hedgehog (Shh), which is required for patterning the neural tube, somites and for left-right asymmetry. *Drosophila* has a single *hh* gene that is also required in numerous developmental processes, including embryonic segmentation, imaginal disc development and abdominal patterning. The potency of Hh as a global determinant of pattern is illustrated by considering the consequences of Hh misexpression in the *Drosophila* wing imaginal disc (Basler and Struhl, 1994). Many of the basic parameters of the epithelium, such as cell fate, cell number; wing size and morphology are all affected by ectopic Hh expression. Thus, stringent regulation of Hh signaling during development is essential for proper patterning to occur.

One emerging generalization is that Hh patterns a tissue through the establishment of discrete organizing centers (Lawrence and Struhl, 1996). Hh can act both as a short-range inducer and as a long-range morphogen in patterning. For example, in the *Drosophila* wing imaginal disc epithelium Hh is produced in presumptive posterior cells under the control of *engrailed* (*en*) (Zecca et al., 1995), a transcription factor, that functions as a 'selector' gene for posterior identity. En also prevents posterior cells from responding to the Hh signal (Sanicola et al., 1995; Tabata et al., 1995; Zecca et al., 1995). Consequently, only anterior cells respond to Hh by activating target genes in a narrow stripe adjacent to the anteroposterior (AP) boundary. One such target is *decapentapalegic* (*dpp*), which encodes a morphogen that can signal over a long range to specify cell fates in both the anterior and posterior of the wing (Lecuit et al., 1996; Nellen et al., 1996). However, in other contexts, Hh itself can act as a long-range morphogen, as shown in the adult abdomen (Struhl et al., 1997a; Struhl et al., 1997b). Specific regulation of both the range of Hh movement and Hh activity at organizing centers will therefore determine many aspects of a tissues overall pattern.

The post-translational modification of Hh appears to be crucial in regulating both the range of Hh signaling and its activity. Biochemical studies suggest that two distinct activities reside within the full-length precursor form of Hh. The N-terminal fragment is required for signaling, and is liberated from the C terminus by an intramolecular cleavage event (Lee et al., 1994; Porter et al., 1995; Porter et al., 1996a). The C terminus possesses a cholesterol transferase activity, and is sufficient to catalyze the covalent addition of a cholesterol moiety to the N-terminal fragment of Hh (Porter et al., 1996b). Cholesterol modification of Hh is not, however, a pre-condition for generating an active Hh species, as expression of mutant

forms of Hh that cannot be modified retain the ability to signal (Porter et al., 1996a; Burke et al., 1999). Moreover, the unmodified N-terminal fragment of *Drosophila* Hh is capable of signaling over an even longer range than its cholesterol modified counterpart (Porter et al., 1996a; Burke et al., 1999). By contrast, long-range signaling of Shh appears to require cholesterol modification (Lewis et al., 2001). Thus, addition of cholesterol appears to be a key factor in regulating the range of Hh signaling.

There is also evidence that Hh proteins bear at least one additional lipid modification. Mass analysis of full-length human Shh expressed in insect cells showed that the total mass of purified Shh could not be accounted for solely by the addition of cholesterol (Pepinsky et al., 1998). This observation suggested that Shh might be fatty acylated. Palmitoylated proteins contain a 16-carbon saturated fatty acyl group that is almost always attached to a cysteine residue by a thioester bond. Pepinsky et al. have observed that the mass of a palmitoyl moity is sufficient to account for the difference between the calculated and measured mass of Shh (Pepinsky et al., 1998). Direct evidence for palmitic acid modification has come from monitoring the level of radiolabeled palmitic acid incorporation into Shh. While Shh was readily labeled, a variant in which Cys-24 was mutated to Ser-24 showed no palmitic acid incorporation (Pepinsky et al., 1998). Hence, Cys-24, the N-terminal amino acid of Shh, is specifically required for palmitoylation.

Recent experiments have now begun to address the function of fatty-acylated Hh proteins (Kohtz et al., 2001; Lee et al., 2001). These studies sought to compare the activity of wild-type Hh with mutant forms of Hh that lacked the N-terminal Cys, in vivo. In one assay, the ability of Shh to induce rat telencephalic neurons was dependent upon the presence of an N-terminal Cys (Kohtz et al., 2001). Similarly, the activity of Hh in patterning the wing of *Drosophila* was shown to depend on the presence of an N-terminal Cys (Lee et al., 2001). Together, these experiments suggest that fatty-acylation is critical for regulating Hh activity in vivo.

We describe the molecular cloning and characterization of *rasp*, a novel *Drosophila* segment polarity gene. We demonstrate that the function of *rasp* is specific for Hh signaling. Furthermore, we show that the requirement for *rasp* is spatially restricted and limited only to *hh*-expressing cells. Loss of *rasp* activity does not disrupt *hh* transcription, nor does it affect Hh protein levels or distribution. Molecular analysis reveals that *rasp* encodes a putative multipass transmembrane protein with homology to a conserved family of membrane bound *O*-acyl transferases (MBOAT) (Hofmann, 2000). We propose that Rasp, like the other members of the MBOAT family, acts as an acyltransferase and is required to fatty-acylate Hh, a modification required for Hh signaling.

MATERIALS AND METHODS

Drosophila stocks

ovo^{D1} FRT^{2A} (Chou and Perrimon, 1996). *y w*; *rasp*^{7F21}FRT^{2A} FRT^{82B}, *y w*; *rasp*^{9B15}FRT^{2A} FRT^{82B}; and *y w*; *trh*, *rasp*^{7F21}FRT^{2A} FRT^{82B} (this study). *y w* hsflp; πMyc FRT^{80} (kindly provided by S. Blair). *y w* hsflp; *M*(3)^{i55} hsGFP FRT^{2A} (kindly provided by G. Struhl). *en* Gal4, UAS-GFP. *ptc* Gal4. UAS-GFP. UAS-*hh* F^{HA} (Burke et al., 1999) (kindly provided by K. Basler) is a transgene that generates a full-length Hh with an HA tag. UAS-*hh* and UAS-*hh* N (kindly provided by P. Beachy). *hh*^{P30} (*lacZ*) (Lee et al., 1992). *ptc-lacZ* and *dpp*^{10638} (*lacZ*) (kindly provided by S. Cohen). *ci-D*^{plac} (Eaton and Kornberg, 1990). *hh*^{Gal4} (Tannimoto et al., 2000) (kindly provided by Tetsuya Tabata). FRT^{G13} mCD8 GFP (Lee and Luo, 1999).

In a genetic screen for maternal/zygotic genes (N. P., C. Arnold and A. Lanjuin, unpublished) we have identified two *rasp* alleles, *rasp*^{7F21} and *rasp*^{9B15}. These mutations were induced on a *y w;* FRT^{2A} FRT^{82B} chromosome (Chou and Perrimon, 1996). An additional allele, l(3)63Bg, was subsequently identified in the Bloomington stock center collection by complementation testing. The phenotypes of each or these three mutations were tested over a small deficiency in the region and found to be similar to the homozygous phenotype of each allele alone. Furthermore, the molecular lesion associated with *rasp*^{7F21} is consistent with this allele being a protein null, as it introduces a premature stop codon into the predicted protein (see Results). The *rasp*^{7F21} and *rasp*^{9B15} phenotypes we observe are similar to those previously described for l(3)63Bg (Wohlwill and Bronner, 1991). In the present analysis, we used *rasp*^{7F21} in the generation of marked clones and *rasp*^{9B15} to generate discs homozygous for *rasp*.

Clonal analysis

Germline clones (GLC) were induced using the FLP-FRT dominant female sterile method as previously described (Chou and Perrimon, 1996). Recombinant somatic clones were generated by collecting eggs for 3 days and then administering a 37°C heat shock for 1-2 hours (depending on the intended rate of clone induction). To obtain the cohort of larvae that received a heat shock at the beginning of second instar, white prepupae (wpp) were picked from the bottle 72 hours after clone induction. The wpp were transferred to a moist plastic petri dish and heat shocked at 37°C to induce marker gene expression (1 hour for Myc, 2 hours for GFP). Wpp were immediately dissected and prepared for staining.

Histology and immunohistochemistry

For most antibody stains, discs were dissected in *Drosophila* ringers and fixed in 'Browers' buffer + 2% EM grade formaldehyde overnight at 4°C (for details, see Blair, 2000). For anti-Hh staining we found that the protocol described by Burke et al. (Burke et al., 1999) produced the most reliable results. For in situ hybridization to RNA probes, discs were fixed in PBS + 8% formaldehyde and processed according to Hauptmann (Hauptmann, 2001).

Primary antisera used were: Anti-Hh (gift from Ingham), 1:1000; Anti-Hh (gift from Kornberg), 1:10,000; anti-Ptc (gift from I. Guerrero) 1:1000; anti-Sc (gift from S. Carroll) 1:1000; anti-Dll (gift from S. Cohen), 1:1000; anti-Wg (gift from S. Cohen), 1:10; anti-Myc (gift from S. Blair), 1:4; anti-En/Inv (gift from E. Spana), 1:4; anti-En/Inv (gift from N. Patel), 1:10; anti-β-gal (Cappell) 1:2000; anti-HA (Roche), 1:1000; anti-Crumbs (gift from E. Knust), 1:50; and anti-Dig-alkaline phosphatase (Roche), 1:2000. Fluorescent secondary antibodies (low cross reactivity from Jackson ImmunoResearch Labs) were used at 1:200 and incubated at 4°C overnight. In some cases, discs were stained using a nickel-intensified Vector ABC-DAB protocol as described by Blair (Blair, 2000). Discs were mounted in Vectashield mounting media and inspected using a Leica TCS-NT confocal microscope. Single images were merged using Adobe Photoshop.

Molecular biology

The ORF corresponding to CG11495 was amplified from genomic DNA by PCR using specific oligonucleotide primers. PCR products were cloned using the Promega pGemTeasy kit. Genomic DNA was prepared according to a modified form of the single fly PCR protocol described by Gloor et al. (Gloor et al., 1993). To identify mutations, DeepVent DNA polymerase (New England Biolabs) was used to

amplify CG11495 fragments from both *y w*; *rasp*^{7F21}FRT^{2A} FRT^{82B} and *y w*; FRT^{2A}FRT^{82B} genomic DNA. *rasp*^{7F21} homozygous embryos were selected from a *y w*; *rasp*^{7F21}FRT^{2A} FRT^{82B}/TM6, GFP stock under a fluorescent dissecting microscope. For each genotype, the PCR products from each of three independent, but identical, reactions were sequenced and compared. DNA star software suite was used for compiling sequences, generating alignments and analyzing predicted protein structure. RNA probes were generated according to Hauptmann (Hauptmann, 2001).

RESULTS AND DISCUSSION

rasp mutant embryos display a segment polarity phenotype

In a large genetic screen for EMS-induced, zygotic lethal mutations with maternal effects (N. P., C. Arnold and A. Lanjuin, unpublished), we identified two allelic mutations on the third chromosome: 7F21 and 9B15. Normally, the ventral cuticle of a wild-type embryo displays a segmentally repeating pattern of denticle belts and naked cuticle (Fig. 1A). However, 7F21/7F21 and 9B15/9B15 embryos derived from germline clone (GLC) females displayed a lawn of denticles and had little or no naked cuticle – the segment polarity phenotype (Fig. 1B). Based on the resemblance these mutant embryos have with a coarse file, we have named this mutation *rasp* (Mogila and Perrimon, 1998). Subsequently, we will refer to 7F21/7F21 and 9B15/9B15 embryos that lack both the maternal and zygotic gene products as '*rasp* mutant embryos'. By contrast, *rasp*/+ embryos, derived from GLC mothers that received a wild-type paternal chromosome often displayed a cuticle that was indistinguishable from wild type (Fig. 1C). Finally, *rasp/rasp* mutant animals derived from heterozygous females survived embryogenesis but died later during larval or pupal stages.

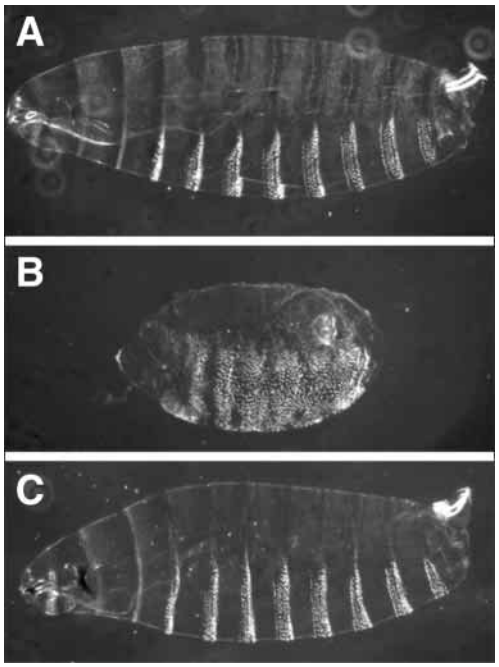

Fig. 1. Identification of a novel gene with a segment polarity phenotype. (A-C) Cuticle preparations were visualized under dark field optics; anterior is towards the left, dorsal is upwards. (A) Wild-type embryos display a ventral cuticle with a segmentally repeated pattern of denticle belts and naked cuticle. (B) Segment polarity phenotype of a *rasp* GLC-derived embryo lacking both maternal and zygotic contributions. Note the reduction in naked cuticle and the lawn of denticles. To identify unambiguously the genotype of the embryos that have received a paternal wild-type copy of *rasp*, we recombined the *rasp* chromosome with a mutation in *trachealess* (*trh*). *trh* results in embryos with a mutant Filtzkorper as described previously (Haecker et al., 1997). Note the Filtzkorper in *rasp, trh/rasp, trh* mutant embryos. (C) Cuticle preparation of *rasp, trh*/+ GLC-derived embryos that carry a wild-type paternal chromosome. The paternal rescue was often complete and cuticles were indistinguishable from those of wild-type embryos (compare A with C).

To characterize the *rasp* segment polarity phenotype further, we examined Wg and En protein distribution in *rasp* mutant embryos. In the ventral embryonic epidermis, Wg signaling is required for maintenance of *en* transcription during stage 10 (DiNardo et al., 1988; Yoffe et al., 1995). En promotes the expression of *hh* (Ingham et al., 1991; Lee et al., 1992), which is subsequently required to maintain *wg* transcription (Martinez-Ariaz et al., 1988; Bejsovec and Martinez-Ariaz, 1991). In *rasp* mutant embryos, both the levels of Wg and En protein failed to be maintained and faded prematurely (data not shown). The observed segment polarity phenotype and the failure to maintain Wg and En protein in *rasp* mutant embryos is consistent with a role for Rasp in Wg signaling, Hh signaling, or both.

rasp is not required for Wg signaling

To test the requirement of *rasp* in Wg and Hh signaling, we analyzed the *rasp* mutant phenotype in developmental contexts where Wg and Hh signaling are known to be independent. One such context is the development of the embryonic stomatogastric nervous system (SNS). By stage 11, three invaginations of the foregut form in the dorsal epithelium and are easily visualized using the epithelial marker Crumbs. Loss of Wg signaling leads to the formation of only a single invagination, while SNS development is not disrupted in *hh* mutants (González-Gaitán and Jäckle, 1995). *rasp* mutant embryos stained with anti-Crumbs showed no defects in SNS formation (data not shown). Thus, Rasp is not required for the Wg-dependent patterning of the SNS.

We next extended our analysis to the wing imaginal disc, where Wg and Hh signaling pathways also have distinct patterning requirements. In the wing, Wg acts over a long range to pattern the dorsoventral (DV) axis of the wing (Zecca et al., 1996; Neumann and Cohen, 1997), while Hh patterns the AP axis (Basler and Struhl, 1994; Strigini and Cohen, 1997). By late third instar, *wg* is expressed in a narrow stripe three to five cells wide along the presumptive wing margin (Baker, 1988a). Wg is necessary and sufficient to activate both proneural gene expression (Blair, 1992b; Phillips and Whittle, 1993) and *Distaless* (*Dll*) expression (Zecca et al., 1996; Neumann and Cohen, 1996) within the wing pouch. We used a GFP marked *Minute* chromosome to generate large *rasp*$^{-}$ clones and examined protein levels of both the proneural gene *scute* (*sc*) and *Dll* (Fig. 2A-F). None of the clones examined resulted in a discernable disruption of either the level or pattern of Sc (Fig. 2A-C) and Dll (Fig. 2D-F) proteins. This was true even for early clones that straddled the DV boundary. Taken together, our data show that *rasp* is not required for Wg signaling.

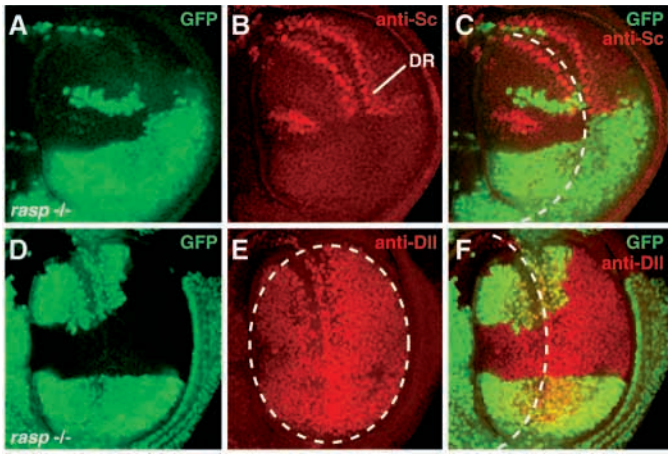

Fig. 2. Loss of *rasp* activity does not affect Wg signaling. (A-F) Confocal micrographs of late third instar wing imaginal discs containing *rasp*^{7F21} (*rasp*$^{-}$) clones. In this and all subsequent micrographs, anterior is upwards and dorsal is on the left. Large *rasp*$^{-}$ clones that cross the DV boundary were generated in a *Minute* heterozygous ($M^{-/+}$) background and are marked by the absence of green fluorescent protein (GFP). (C,F) Broken line marks the approximate position of the DV boundary. (A) The absence of GFP in green marks the tissue homozygous for *rasp*. (B) The double row (DR) of anti-Sc staining shown in red marks the anterior proneural region along the presumptive wing margin. Activation of proneural gene expression along the anterior margin depends on Wg signaling. (C) Overlay of A and B. Note that anti-Sc levels and pattern within the clone are not affected by loss of *rasp*. (D) Absence of GFP in green marks tissue homozygous for *rasp*. (E) Dll is normally expressed throughout the majority of the presumptive wing pouch and is delimited by a broken line. Activation of Dll expression in the wing pouch depends on Wg signaling. (F) Overlay of D,E. Note that anti-Dll levels within the clone are not affected by loss of *rasp*.

rasp is required for activation of Hh target genes

We next examined the requirement of *rasp* for Hh target gene activation. In the wing disc *patched* (*ptc*) (Phillips et al., 1990) and *dpp* (Masucci et al., 1990) are expressed in a narrow anterior stripe along the AP boundary (Fig. 3B,C). Of these, *ptc* expression requires higher levels of Hh signaling (Capdevila et al., 1994; Strigini and Cohen, 1997). In wing discs homozygous for *rasp*, the level of *ptc* expression was reduced, as shown by both the *ptc-lacZ* (Fig. 4B) and *ptc* Gal4, UAS-GFP reporters (data not shown). A striking feature of the *rasp* wing disc phenotype is their markedly reduced size (compare Fig. 4A with 4B). This suggests that *dpp* expression might also be reduced in this mutant background, as reception of *dpp* is normally required for cell proliferation within the wing disc (Burke and Basler, 1996). In situ hybridization of RNA probes to wing discs homozygous for *rasp* revealed a reduction in *dpp* expression levels (compare Fig. 4C with 4D). Thus, *rasp* activity is necessary for the expression of two direct transcriptional targets of Hh.

Hh signaling is also required to maintain distinct populations of anterior and posterior cells in the wing imaginal disc. Failure of anterior cells to receive Hh results in a loss of target gene expression and a distortion of the AP lineage restriction (Blair and Ralston, 1997; Rodriguez and Basler, 1997); distortion of the AP lineage boundary is thus another measure of Hh signaling loss. We observe that in wing

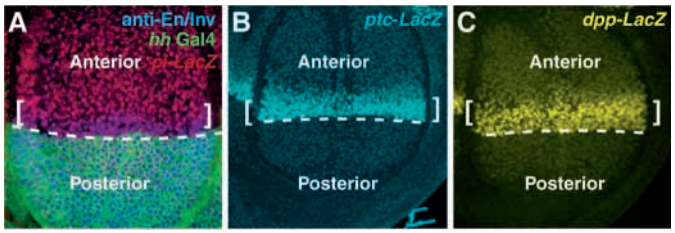

Fig. 3. Hh-dependent gene expression along the AP boundary. (A) Anterior cells are marked by the expression of *ci*, shown in red. Posterior cells are marked by anti-En/Inv staining shown in blue. A broken line marks the approximate position of the AP boundary. *en* is required in posterior cells for *hh* expression; *hh*^{Gal4} UAS-GFP is shown in green. Anterior cells adjacent to the AP boundary respond to Hh by activating target gene expression. A narrow, Hh-dependent stripe of anti-En/Inv staining can be detected in anterior cells by late third instar (purple). The expression of two additional Hh target genes can be detected in anterior cells; (B) *ptc-lacZ*, is shown in blue and (C) *dpp-lacZ*, is shown in yellow. Brackets highlight the domains of anterior Hh target gene expression.

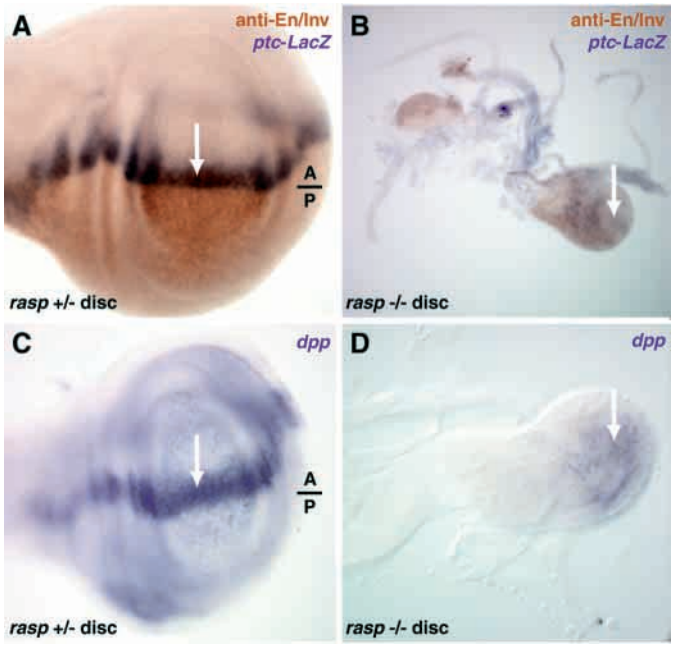

Fig. 4. *rasp* is required for Hh target gene activation. (A-D) Comparison of Hh target gene expression in wing discs heterozygous and homozygous for *rasp*. (A) *ptc-lacZ; rasp*^{9B15}*/TM6* discs stained with anti-β-gal (blue) and anti-En (brown). The domain of elevated *ptc* expression is detectable in a narrow stripe of anterior cells along the AP boundary (arrow). (B) *ptc-lacZ; rasp*^{9B15}*/rasp*^{9B15} discs stained with anti-β-gal (blue) and anti-En (brown). Levels of *ptc-lacZ* are reduced in this mutant background (compare A with B). The anti-β-gal stain was amplified to detect low levels of *ptc-lacZ* reporter signal (see Materials and Methods). We failed to detect *ptc-lacZ* expression using fluorescent secondary antibodies. Note the reduced size of the homozygous wing disc. (C) *ptc-lacZ; rasp*^{9B15}*/TM6* discs hybridized to *dpp* RNA probes (blue). Like *ptc-lacZ*, *dpp* is also expressed in a stripe of anterior cells along the AP boundary (arrow). (D) *ptc-lacZ; rasp*^{9B15}*/rasp*^{9B15} discs hybridized to *dpp* RNA probes (blue). Levels of *dpp* transcripts are reduced in this mutant background (compare C with D).

discs homozygous for *rasp*, the AP boundary, as defined by *en* expression, occasionally appears to be distorted (data not shown). Taken together, we conclude that *rasp* is required for Hh signaling.

rasp is required in cells that send the Hh signal

Reductions in the level of Hh target gene expression can be observed in mutations that disrupt either the process of sending or receiving Hh. Using clonal analysis, we tested the requirement of *rasp* for Hh target gene activation in both sending and receiving cells. Large *rasp*$^-$ clones that were restricted to the anterior compartment and defined the AP boundary, had no detectable effect on Ptc protein levels (Fig. 5A-C). Anterior En protein levels were also examined in this assay; by late third instar, a Hh-dependent stripe of En expression is present along the AP boundary (Blair, 1992a; Blair and Ralston, 1997). The level of anterior En protein was similarly unaffected by large anterior *rasp*$^-$ clones (Fig. 5G-I). Anterior clones distant from the AP boundary also had no effect on either Ptc or En levels. By contrast, large *rasp*$^-$ clones restricted to the posterior compartment resulted in a non-autonomous reduction in the levels of both Ptc and anterior En protein levels (Fig. 5D-F,J-L). These effects were most pronounced in large posterior clones abutting the AP boundary. Posterior *rasp*$^-$ clones that were small or distant from the AP boundary did not result in detectable reductions of either Ptc or En protein. Interestingly, we observed that the presence of wild-type posterior tissue adjacent to the AP boundary could often rescue anterior Hh target gene expression, even when the remaining posterior cells were *rasp* negative (data not shown). Hence, *rasp* is required only in posterior cells, and is both necessary and sufficient for anterior Hh target gene expression along the AP boundary.

Previous observations have suggested that Hh produced in the posterior compartment can travel over a long range to activate target gene expression in cells of the anterior compartment (Chen and Struhl, 1996). The non-autonomous effects we observe in large posterior *rasp* mutant clones are therefore consistent with a role for *rasp* in Hh signaling. One explanation for our observations is that *rasp* is required for *hh* transcription in posterior cells. To test this possibility, we examined *hh* transcription in posterior *rasp* clones using a *hh-lacZ* reporter. No effect on *hh-lacZ* levels was observed (Fig. 6A-C). Thus, *hh* transcription does not require *rasp* activity.

Non-autonomous defects in Hh target gene expression have also been observed in mutations that are required for sending the Hh signal. This is the case for posterior *dispatched* (*disp*) mutant clones where a failure to secrete Hh from sending cells correlates with an autonomous increase in Hh protein levels (Burke et al., 1999). We examined Hh protein in posterior *rasp*$^-$ clones and found that, in general, Hh protein levels were neither elevated nor reduced compared with wild-type cells (Fig. 6D-F). However, we note that in a few cases there did appear to be a detectable increase in Hh protein levels within *rasp* mutant cells (data not shown). We also examined the apical-basal axis of wing disc epithelium and found no discernable effect on the subcellular distribution of Hh (data not shown). Therefore, the primary defect in *rasp* mutants does not appear to be due to a failure of Hh secretion, a reduction in Hh protein stability or mislocalization of Hh protein. We conclude that in the absence of *rasp*, Hh protein is likely to be properly secreted from posterior cells but that it cannot activate the Hh pathway in receiving cells.

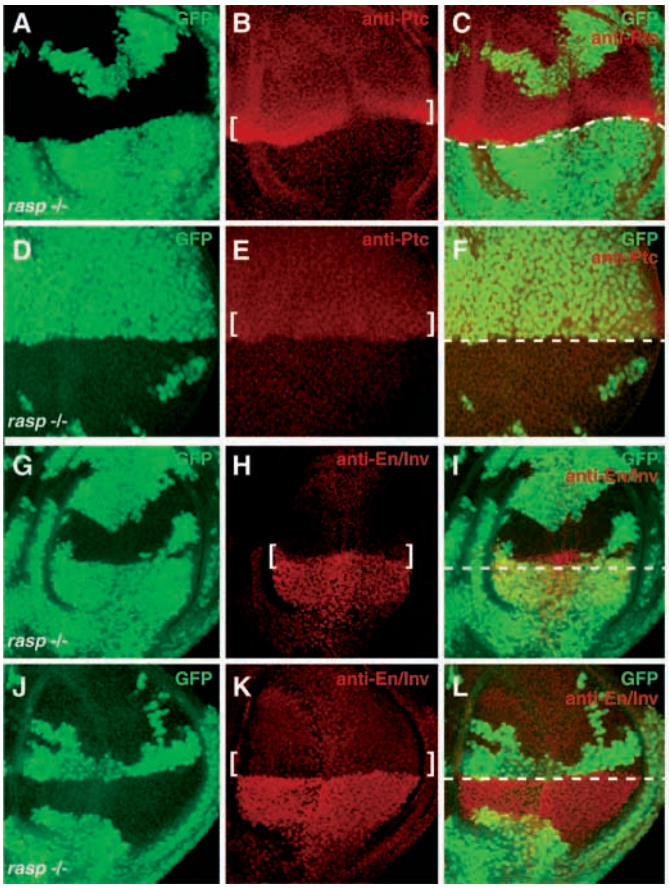

Fig. 5. *rasp* is required in posterior cells to activate Hh target gene expression in anterior cells. (A-L) Analysis of *rasp*^{7F21} (*rasp*$^-$) clones in wing imaginal discs. Clones were generated in a *M*$^{-/+}$ background and marked by the absence of GFP shown in green. (B,E,H,K) Brackets highlight the domain of Hh-dependent gene expression in anterior cells. (C,F,I,L) The broken line marks the approximate position of the AP boundary. (A) A large anterior *rasp*$^-$ clone is marked by the absence of GFP. This clone defines the position of the AP boundary from the anterior side. (B) The pattern of Ptc protein is shown in red and is indistinguishable from wild type. (C) Overlay of A and B. (D) A large posterior *rasp*$^-$ clone is marked by the absence of GFP. This clone defines the position of the AP boundary from the posterior side. (E) The elevated domain of anterior Ptc protein is reduced in this disc (compare with B). (F) Overlay of D and E. (G) A large anterior *rasp*$^-$ clone is marked by the absence of GFP. This clone defines the position of the AP boundary from the anterior side. (H) The domain of both anterior and posterior En protein is shown in red and is indistinguishable from wild type. The brackets mark the approximate position of anterior cells expressing En protein. (I) Overlay of G and H. The domain of anterior En protein is clearly detectable in this micrograph in relation to the AP boundary. Anterior En protein is not detectably altered by the absence of *rasp*$^-$. (J) A large posterior *rasp*$^-$ clone is marked by the absence of GFP. This clone defines the position of the AP boundary from the posterior side. (K) The domain of anterior En protein is reduced in this disc (compare with H). (L) Overlay of J and K.

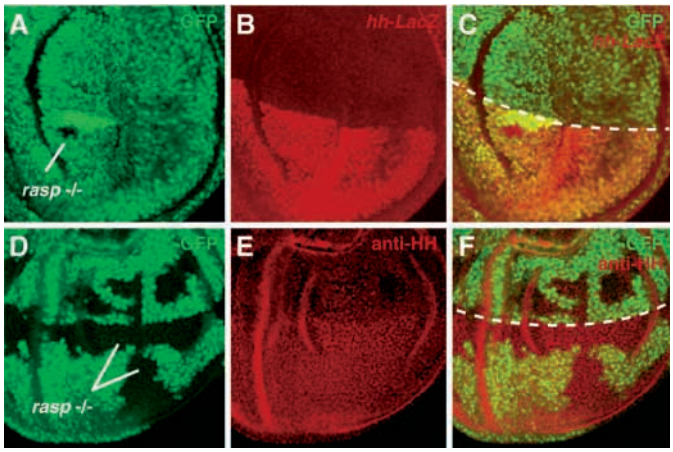

Fig. 6. Hh transcription and protein levels do not require *rasp*. (A-F) *rasp*^{7F21} (*rasp*$^{-}$) clones in wing imaginal discs. (A-C) Analysis of *hh* transcription in *rasp*$^{-}$ clones. (A) Clone is marked by the absence of GFP. (B) Anti-β-gal staining in red. The *hh*^{P30} enhancer trap is a reporter of *hh* transcription. The level of *hh* transcription in posterior cells is not altered by the absence of *rasp*. (C) Overlay of A and B. (D-F) Analysis of Hh protein levels in *rasp*$^{-}$ clones. (D) Clones are marked by the absence of GFP. (E) Anti-Hh protein shown in red. There is no detectable increase or decrease in Hh protein levels in the absence of *rasp*. (F) Overlay of D and F.

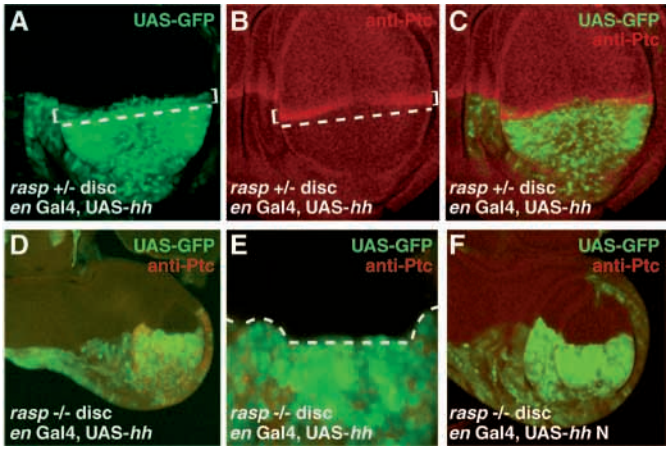

Fig. 7. *rasp* is required to produce active Hh protein. (A-F) A comparison of Hh target gene expression in wing discs that express different Hh transgenes and which are either heterozygous or homozygous for *rasp*^{9B15}. (A,B,E) A broken line marks the approximate position of the AP boundary. (A-C) *en* Gal4, UAS-*GFP*/UAS-*Hh* F ^{HA}*; rasp*^{9B15}*/TM6* discs. (A) The domain of *en* Gal4 expression shown in green. The domain of anterior *en* expression can be detected (brackets). (B) Elevated Ptc protein levels can be detected in a narrow stripe anterior to the AP boundary (brackets). (C) Overlay of A and B. (D,E) *en* Gal4, UAS-*GFP*/UAS-*Hh* F ^{HA}*; rasp*^{9B15}*/rasp*^{9B15} discs stained with anti-Ptc (red); *en* Gal4 expression shown in green. (D) High levels of anterior Ptc protein are not detected (compare C with D). Note that the anterior expression of *en* is also not detectable in these discs. (E) Higher magnification of disc shown in D. (F) UAS-*Hh* N/+*; en* Gal4, UAS-*GFP*/+*; rasp*^{9B15}*/ rasp*^{9B15} discs stained with anti-Ptc (red); *en* Gal4 expression shown in green. High levels of anterior Ptc protein are not detected.

rasp is required to produce a fully active Hh protein

Activation of anterior Hh target gene expression requires that Hh protein produced by posterior cells is both active and that it is transported to anterior receiving cells. The processes of Hh signaling and transport have been experimentally separated. For example, overexpression of Hh in anterior cells results in the activation of Hh target gene at a distance (Strigini and Cohen, 1997; Burke et al., 1999), while activation of a membrane-tethered form of Hh results in target gene activation only in adjacent cells (Strigini and Cohen, 1997; Burke et al., 1999). Furthermore, mutations in *tout-velu* disrupt long range Hh transport, but not the responsiveness of anterior cells to Hh (Bellaiche et al., 1998).

If the function of *rasp* is required for Hh activity and not for transport, then overexpression of Hh in a *rasp* mutant background should fail to activate Hh target gene expression. Alternatively, if *rasp* is required for the transport of Hh, then overexpression of Hh in a *rasp* mutant background should lead to target gene activation only in adjacent cells. To address this issue, we expressed a full-length, tagged form of Hh (Burke et al., 1999) in posterior cells that were either heterozygous or homozygous for *rasp*. The heterozygous discs displayed several features that indicated Hh signaling was occurring normally (Fig. 7A-C). First, the discs were the same size and shape as wild-type discs. Second, the AP lineage boundary was straight, as is the case for wild-type disc. Third, anterior domains of *en* expression and Ptc protein were both detectable. By contrast, discs homozygous for *rasp* were reduced in size, often displayed a distorted AP boundary and showed no detectable anterior *en* expression or Ptc protein (Fig. 7D). Careful examination of these discs revealed that anterior Ptc protein was not detectable, even in the cells directly adjacent to Hh-expressing cells (Fig. 7E). We next tested the requirement of *rasp* for the activity of the *hh*-N transgene (Porter et al., 1995). *hh*-N produces a protein that is truncated at the site of autoproteolysis, and therefore is not modified through the addition of cholesterol. Hh-N is not efficiently sequestered and exhibits the ability to signal over an extended range (Porter et al., 1995, Burke et al., 1999). Nevertheless, we find that *rasp* is required for anterior Ptc expression even in the presence of Hh-N (Fig. 7F). Thus, *rasp* is required for the production of fully active Hh protein.

rasp encodes a putative multipass transmembrane protein with homology to a conserved family of acyltransferases

To molecularly characterize *rasp*, we first determined its location on the third chromosome. Deficiency mapping revealed that *rasp* was localized to a region between 63A1 and 63D1, as *rasp*^{7F21} and *rasp*^{9B15} failed to complement *Df(3L)M21* and *Df (3L) HR370*. Two additional deficiencies in the region, *Df(3L)HR 232* and *Df(3L)HR119*, were found to complement *rasp*^{7F21} and *rasp*^{9B15}, further refining the position of *rasp* between 63A1 and 63C1.

To map the location of *rasp* more finely, we performed complementation tests with available mutations in the region. We identified one mutation, l(3)63Bg, that failed to complement *rasp* and had a similar mutant phenotype (Wohlwill and Bronner, 1991). We next measured the recombination rates between *rasp* and three lethal P-elements in the region, l(3)06803, l(3)j5C2 and l(3)01029. No recombinants between *rasp* and l(3)j5C2 were recovered in our experiment, suggesting a map position for *rasp* close to 63B13.

Examination of the information from the Berkeley *Drosophila* Genome Project (BDGP) revealed the presence of a number of putative open reading frames (ORFs) in the region of 63B13. Furthermore, through personal communication with K. Basler, we became aware that his group was also characterizing a segment polarity gene in the region that failed to complement l(3)63Bg and that corresponded to ORF CG11495, located at 63B11. To determine whether *rasp* was a mutation underlying CG11495, we compared the genomic sequence of the parental, unmutagenized chromosome used in our screen with the genomic sequence of *rasp*^{7F21}. We found that *rasp*^{7F21} was associated with a C to T base substitution that would introduce a premature stop codon at amino acid 52 of the predicted protein encoded by CG11495 (Fig. 8, asterisk). DNA amplified from CG11495 was cloned and used to generate RNA probes. As predicted, based on the domain of Hh responsiveness, *rasp* transcripts were detected ubiquitously at low levels throughout the wing imaginal disc (data not shown).

Sequence analysis of the predicted protein revealed that Rasp is quite hydrophobic and contains at least eleven membrane-spanning regions (Fig. 8). We also identified an invariant histidine residue that may mark the position of the active site. Members of a conserved family of membrane bound MBOAT proteins have two characteristics in common. First, these proteins typically contain between eight and ten membrane-spanning regions. Second, they share a region of sequence similarity in common that includes an invariant histidine residue within a long hydrophobic region (Hofmann, 2000). Based on these criteria, Rasp appears to be a bona fide MBOAT protein.

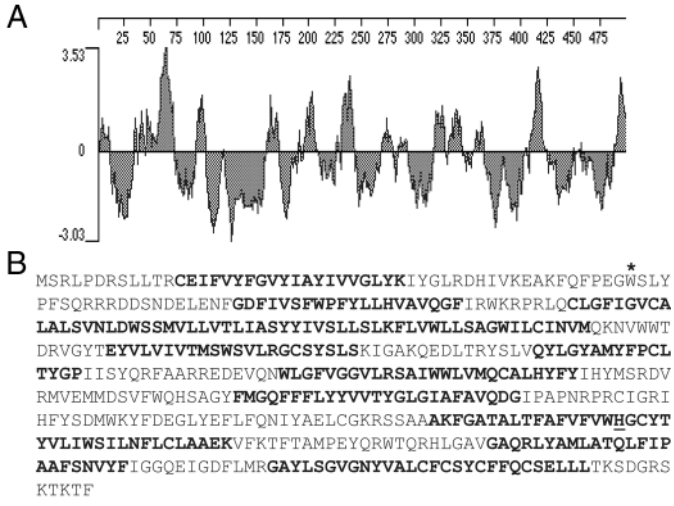

Fig. 8. *rasp* encodes a putative multipass transmembrane protein with homology to acyltransferases. (A) Sequence analysis of *rasp*. Hydrophobicity plot shows that Rasp encodes a predicted protein that is highly hydrophobic. At least eleven transmembrane regions can be detected. (B) Linear sequence of amino acids predicted by the BDGP *rasp* sequence. Hydrophobic regions are indicated in bold. Sequencing genomic DNA from *rasp*^{7F21} homozygous embryos led to the identification of a single base pair change that converts a tryptophan to a premature stop codon at amino acid 52 (asterisk). An invariant histidine residue (underline) conserved among MBOAT family members is a likely candidate for the active site of Rasp.

In this study, we have described the requirement of *rasp* in patterning the *Drosophila* embryo and wing imaginal disc. Our results indicate that *rasp* is required for Hh signaling and that post-translational lipid modification is crucial for long and short range, Hh-dependent patterning. These conclusions are based on the following observations:

(1) We have examined the requirement of *rasp* for both Wg and Hh signal transduction. Our experiments show that loss of *rasp* does not alter Wg target gene expression; however, the expression of direct transcriptional targets of Hh signaling are reduced in the absence of *rasp*.

(2) Using clonal analysis, we have tested the requirement of *rasp* in Hh-sending and -receiving cells. We observe that loss of *rasp* in posterior, Hh-sending cells, has a non-autonomous effect on Hh target gene expression, while no requirement for *rasp* in receiving cells was detected.

(3) *hh* transcription, protein levels and distribution do not depend on *rasp* function.

(4) *rasp* is required for the production of an active Hh protein.

(5) *rasp* encodes a protein with homology to acyltransferases.

What, then, is the role of Rasp in modifying the Hh protein? One possibility is that Rasp is required directly for the cholesterol modification of Hh, and that *rasp* mutant cells produce Hh, but that it lacks a cholesterol moity. Elegant studies in *Drosophila* have examined the role of cholesterol modification for Hh activity. These experiments demonstrate that Hh proteins, which are not cholesterol modified, can still activate Hh target genes, even in the absence of endogenous Hh (Porter et al., 1995; Burke et al., 1999). This, however, is not consistent with what we observe, as lack of *rasp* activity leads to a reduction of target gene expression. Therefore, our data do not appear to be consistent with a role for *rasp* in cholesterol modification.

Members of the MBOAT superfamily that have been well-characterized biochemically encode enzymes that transfer fatty acids onto hydroxyl groups of membrane-tethered targets. Palmitoylation, the attachment of saturated 16-carbon fatty acyl chain to a protein by a thioester bond, is the only other lipid modification of Hh that has been described (Pepinsky et al., 1998). A second possibility, then, is that Rasp is directly required for Hh palmitoylation. Studies of mutant variants in which the N-terminal Cys of *Drosophila* Hh was mutated, fail to be palmitoylated, and lead to phenotypes very similar to those reported here for *rasp* (see Introduction). While our study does not directly address this possibility, these observations suggest that Rasp may be required directly for Hh palmitoylation. Interestingly, biochemical analysis suggests that the palmitoyl moiety of Hh may not be attached to the thiol group of the N-terminal Cys, but rather to the α-amino group (Pepinsky et al., 1998). Thus, if Rasp does palmitoylate Hh, this would constitute a difference in the enzymatic specificity between Rasp and other members of the MBOAT superfamily. Finally, it is possible that Rasp is required for additional lipid modifications that are required for Hh activity, but have yet to be characterized.

Among acylated proteins, there are a growing number that regulate developmental signaling events. Our study has highlighted the importance of lipid modification for Hh signal transduction. Such lipid modifications appear to be important

for protein-membrane interactions and for targeting proteins to membrane microdomains (Casey, 1995). Palmitoylation, in particular, has been the subject of increasing interest (Mumby, 1997; Resh, 1999). This may be due to the fact that, unlike other lipid modifications, palmitoylation can be reversible and thus, constitutes a regulatable step in signal transduction. Finally, like r*asp*, *porcupine* (*porc*) also encodes a protein with homology to members of the MBOAT superfamily (Hofmann, 2000); *porc* is required for sending the Wg/Wnt signal (Kadowaki et al., 1996). Thus, some acylating enzymes appear to be highly specific, and may prove to be of general importance in regulating the function of secreted proteins during development.

Note added in proof

As we were preparing this manuscript for publication, the molecular and phenotypic analysis of *sightless* (*sit*) (Lee and Triesman, 2001) and *skinny hedgehog* (*ski*) (Chamoun et al., 2001) were reported. *sit*, *ski* and *rasp* correspond to the same gene and our findings are in agreement.

We thank S. Blair, G. Struhl, S. Cohen, P. Beachy, Tetsuya Tabata and the Bloomington stock center for generously providing fly stocks, and P. Ingham, T. Kornberg, I. Guerrerro, S. Carroll, S. Cohen and N. Patel for sharing aliquots of precious antibodies. We especially thank K. Basler for sharing data prior to publication. C. A. M. thanks Sara Cherry, Gyeong-Hun Baeg, Stephane Vincent and Lutz Kockel for sharing reagents, and for many helpful discussions. This work was supported by NIH grant GM61110. I. T. was supported by the Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation (DR 1461). N. P. is an investigator for the Howard Hughes Medical Institute.

REFERENCES

Baker, N. E. (1988a). Transcription of the segment polarity gene *wingless* in the imaginal discs of *Drosophila*, and the phenotype of a pupal-lethal *wg* mutant. *Development* **102**, 489-497.

Basler, K. and Struhl, G. (1994). Compartment boundaries and the control of *Drosophila* limb pattern by *hedgehog* protein. *Nature* **368**, 208-214.

Blair, S. S. (1992a). *engrailed* expression in the anterior lineage compartment of the developing wing blade of *Drosophila*. *Development* **115**, 21-34.

Blair, S. S. (1992b). *shaggy* (*zeste-white 3*) and the formation of supernumerary bristle precursors in the developing wing blade of *Drosophila*. *Dev. Biol.* **152**, 263-278.

Blair, S. S. (2000). Imaginal discs. In Drosophila *Protocols* (ed. W. Sullivan, M. Ashburner and R. S. Hawley), p. 159-173. New York: Cold Spring Harbor Laboratory Press.

Blair, S. S. and Ralston, A. (1997). Smoothened-mediated Hedgehog signaling is required for the maintenance of the anterior-posterior lineage restriction in the developing wing of *Drosophila*. *Development* **124**, 4053-4063.

Bejsovec, A. and Martinez-Arias, A. (1991). Roles of *wingless* in patterning the larval epidermis of *Drosophila*. *Development* **113**, 471-485.

Bellaiche, Y., The, I. and Perrimon, N. (1998). *Tout-velu* is a *Drosophila* homologue of the putative tumour suppressor EXT-1 and is needed for Hh diffusion. *Nature* **394**, 85-88.

Burke, R. and Basler, K. (1996). Dpp receptors are autonomously required for cell proliferation in the entire developing *Drosophila* wing. *Development* **122**, 2261-2269.

Burke, R., Nellen, D., Bellotto, M., Hafen, E., Senti, K.-A., Dickson, B. J. and Basler, K. (1999). Dispatched, a novel sterol-sensing domain protein dedicated to the release of cholesterol-modified hedgehog from signaling cells. *Cell* **99**, 803-815.

Capdevila, J., Estrada, M. P., Sanchez-Herrero, E. and Guerrero, I. (1994). The Drosophila segment polarity gene *patched* interacts with *decapentaplegic* in wing development. *EMBO J.* **13**, 71-82.

Casey, P. J. (1995). Protein lipidation in cell signaling. *Science* **268**, 221-225.

Chamoun, Z., Mann, R. K., Nellen, D., von Kessler, D. P., Bellotto, M., Beachy, P. A. and Basler, K. (2001). Skinny hedgehog, an acyltransferase for palmitoylation and activity of the hedgehog signal. *Science* **293**, 2080-2084.

Chen, Y. and Struhl, G. (1996). Dual roles for patched in sequestering and transducing hedgehog. *Cell* **87**, 553-563.

Chou, T.-b. and Perrimon, N. (1996). The autosomal FLP-DFS technique for generating germline mosaics in *Drosophila melanogaster*. *Genetics* **144**, 1673-1679.

Chuang, P.-T. and Kornberg, T. B. (2000). On the range of Hedgehog signaling. *Curr. Opin. Genet. Dev.* **10**, 515-522.

DiNardo, S., Sher, E., Heemskerk-Jongens, J., Kassis, J.A. and O'Farrell, P. H. (1988). Two-tiered regulation of spatially patterned *engrailed* gene expression during *Drosophila* embryogenesis. *Nature* **332**, 604-609.

Eaton, S. and Kornberg, T. B. (1990). Repression of *ci-D* in the posterior compartment of *Drosophila* by *engrailed*. *Genes Dev.* **4**, 1068-1077.

Gonzalez-Gaitán, M. and Jäckle, H. (1995). Invagination centers within the *Drosophila* stomatogastric nervous system anlage are positioned by Notch-nediated signaling which is spatially controlled through *wingless*. *Development* **121**, 2313-2325.

Gloor, G. B., Preston, C. R., Johnson-Schlitz, D. M., Nassif, N. A., Phillis, R. W., Benz, W. K., Robertson, H. M. and Engels, W. R. (1993). Type 1 repressors of P element mobility. *Genetics* **135**, 81-95.

Haecker, U., Lin, X. and Perrimon, N. (1997). The Drosophila sugarless gene modulates Wingless signaling and encodes an enzyme involved in polysaccharide biosynthesis. *Development* **124**, 3565-3573.

Hammerschmidt, M., Brook, A. and McMahon, A. (1997). The world according to *hedgehog*. *Trends Genet.* **13**, 14-21.

Hauptmann. G. (2001). One-, two-, and three-color whole-mount in situ hybridization to *Drosophila* embryos. *Methods* **23**, 359-372.

Hofmann, K. (2000). A superfamily of membrane-bound O-acyltransferases with implications for Wnt signaling. *Trends Biochem. Sci.* **25**, 111-112.

Ingham, P., Taylor, A. and Nakano, Y. (1991). Role of *Drosophila patched* gene in positional signaling. *Nature* **353**, 184-187.

Kadowaki, T., Wilder, E., Klingensmith, J., Zachary, K. and Perrimon, N. (1996). The segment polarity gene *procupine* encodes a putative multitransmembrane protein involved in Wingless processing. *Genes Dev.* **10**, 3116-3128.

Kohtz, J., Lee, H. Y., Galano, N., Segal, J., Ng, E., Larson, T., Baker, D. P., Garber, E. A., Williams, K. P. and Fishell, G. (2001). N-terminal fatty-acylation of sonic hedgehog enhances the induction of rodent ventral forebrain neurons. *Development* **128**, 2351-2363.

Lawrence, P. A. and Struhl, G. (1996). Morphogens, compartments, and pattern:Lessons from Drosophila? *Cell* **85**, 951-961.

Lecuit, T., Brook, W. J., Ng, M., Calleja, M., Sun, H. and Cohen, S. M. (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the Drosophila wing. *Nature* **381**, 387-393.

Lee, J. D. and Treisman, J. E. (2001). Sightless has homology to transmembrane acyltransferases and is required to generate active Hedgehog protein. *Curr. Biol.* **11**, 1147-1152.

Lee, J. D., Kraus, P., Gaiano, N., Nery, S., Kohtz, J., Fishell, G., Loomis, C. A. and Treisman, J. E. (2001). An acylatable residue of Hedgehog is differentially required in *Drosophila* and mouse limb development. *Dev. Biol.* **233**, 122-136.

Lee, J. J., von Kessler, D. P., Parks, S. and Beachy, P. A. (1992). Secretion and localized transcription suggests a role in positional signaling for products of the segmentation gene hedghog. *Cell* **71**, 33-50.

Lee, J. J., Ekker, S. C., von Kessler, D. P., Porter, J. A., Sun, B. I. and Beachy, P. (1994). Autoproteolysis in hedgehog protein biogenesis. *Science* **266**, 1528-1537.

Lee, T. and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22**, 451-461.

Lewis, P. M., Dunn, M. P., McMahon, J. A., Logan, M., Martin, J. F., St-Jacques, B. and McMahon, A. P. (2001). Cholesterol modification of sonic hedgehog is required for long-range signaling activity and effective modulation of signaling by Ptc1. *Cell* **105**, 599-612.

Martinez-Arias, A., Baker, N. and Ingham, P. W. (1988). Role of segment polarity genes in the definition and maintenance of cell states in the *Drosophila* embryo. *Development* **103**, 153-170.

Masucci, J. D., Miltenberger, R. J. and Hoffmann, F. M. (1990).

Pattern specific expression of the *Drosophila decapentapalegic* gene in imaginal disks is regulated by 3′ cis-regulatory elements. *Genes Dev.* **4**, 2011-2023.

Mogila, C. and Perrimon, N. (1998). Maternal products of two new genes, *rasp* and *grater* are required for the segmentation of the *Drosophila* embryo. A. *Dros. Res. Conf.* **39**, 236B.

Mumby, S. M. (1997). Reversible palmitoylation of signaling proteins. *Curr. Opin. Cell Biol.* **9**, 148-154.

Nellen, D., Burke, R., Struhl, G. and Basler, K. (1996). Direct and long-range action of a DPP morphogen gradient. *Cell* **85**, 357-368.

Neumann, C. J. and Cohen, S. M. (1996). A hierarchy of cross-regulation involving *Notch*, *wingless*, *vestigial* and *cut* organizes the dorsal/ventral axis of the *Drosophila* wing. *Development* **122**, 3477-3485.

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

Pepinsky, R. B., Zeng, C., Wen, D., Rayhorn, P., Baker, D. P., Williams, K. P., Bixler, S. A., Ambrose, C. M., Garber, E. A., Miatkowski, K. et al. (1998). Identificarion of a palmitic acid-modified form of human sonic *hedgehog*. *J. Biol. Chem.* **273**, 14037-14045.

Phillips, R. G., Roberts, I. G. H., Ingham, P. W. and Whittle, R. J. S. (1990). The *Drosophila* segment polarity gene *patched* is involved in a positional signaling mechanism in imaginal discs. *Development* **110**, 105-114.

Phillips, R. G. and Whittle, J. R. S. (1993). *wingless* expressing mediates determination of peripheral nervous system elements in late stages of *Drosophila* wing disc development. *Development* **118**, 427-438.

Porter, J. A., von Kessler, D. P., Ekker, S. C., Young, K. E., Lee, J. J., Moses, K. and Beachy, P. A. (1995). The product of hedgehog autoproteolytic cleavage active in local and long-range signalling. *Nature* **374**, 363-366.

Porter, J. A., Ekker, S. C., Park, W.-J., von Kessler, D. P., Young, K. E., Chen, C.-H., Ma, Y., Woods, A. S., Cotter, R. J., Koonin, E. V. and Beachy, P. A. (1996a). Hedgehog patterning activity: role of a lipophillic modification medicated by the carboxy-terminal autoprocessing domain. *Cell* **88**, 21-34.

Porter, J. A., Young, K. E. and Beachy, P. A. (1996b). Cholesterol modification of hedgehog signaling proteins in animal development. *Science* **274**, 255-259.

Resh, M. D. (1999). Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochim. Biophys. Acta* **1451**, 1-16.

Rodriguez, I. and Basler, K. (1997). Control of compartmental affinity boundaries by Hedgehog. *Nature* **389**, 614-618.

Sanicola, M., Sekelsky, J., Elson, S. and Gelbart, W. M. (1995). Drawing a stripe in *Drosophila* imaginal disks: negative regulation of *decapentaplegic* and *patched* expression by *engrailed*. *Genetics* **139**, 745-756.

Strigini, M. and Cohen, S. M. (1997). A Hedgehog activity gradient contributes to AP axial patterning of the *Drosophila* wing. *Development* **124**, 4697-4705.

Struhl, G., Barbash, D. A. and Lawrence, P. A. (1997a). Hedgehog organises the pattern and polarity of epidermal cells in the *Drosophila* abdomen. *Development* **124**, 2143-2154.

Struhl, G., Barbash, D. A. and Lawrence, P. A. (1997b). Hedgehog acts by distinct gradint and signal relay mechanisms to organise cell type and cell polarity in the *Drosophila* abdomen. *Development* **124**, 2155-2165.

Tabata, T., Schwarts, C., Gustavson, E., Ali, Z. and Kornberg, T. B. (1995). Creating a *Drosophila* wing de novo, the role of *engrailed* and the compartment border hypothesis. *Development* **121**, 3359-3369.

Tannimoto, H., Itoh, S., ten Dijke, P. and Tabata, T. (2000). Hedgehog creates a gradient of DPP activity in *Drosophila* wing imaginal discs. *Mol. Cell* **5**, 59-71.

Wohlwill, A. D. and Bonner, J. J. (1991). Genetic analysis of chomosome region 63 of *Drosophila melanogaster*. *Genetics* **128**, 763-775.

Yoffe, K., Manoukian, A., Wilder, E., Brand, A. H. and Perrimon, N. (1995). Evidence for *engrailed*-independent *wingless* autoregulation in *Drosophila*. *Dev Biol.* **170**, 636-650.

Zecca, M., Basler, K. and Struhl, G. (1995). Sequential organizing activities of engrailed, hedgehog and decapentaplegic in the *Drosophila* wing. *Development* **121**, 2265-2278.

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