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Distinct roles of Central missing and Dispatched in sending the Hedgehog signal

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SUMMARY

Secreted Hedgehog (Hh) proteins control many aspects of growth and patterning in animal development. The mechanism by which the Hh signal is sent and transduced is still not well understood. We describe a genetic screen aimed at identifying positive regulators in the *hh* pathway. We recovered multiple new alleles of *hh* and *dispatched* (*disp*). In addition, we identified a novel component in the *hh* pathway, which we name *central missing* (*cmn*). Loss-of-function mutations in *cmn* cause similar patterning defects to those caused by *hh* or *dispatched* (*disp*) mutations. Moreover, *cmn* affects the expression of *hh* responsive genes but not of *hh* itself. Like *disp*, *cmn* acts upstream of *patched* (*ptc*) and its activity is required only in the Hh secreting cells. However, unlike *disp*, which is required for the release of the cholesterol-modified form of Hh, *cmn* regulates the activity of Hh in a manner that is independent of cholesterol modification. Finally, we show that *cmn* mutations bear molecular lesions in CG11495, which encodes a putative membrane bound acyltransferase related to Porcupine, a protein implicated in regulating the secretion of Wingless (Wg) signal.

Key words: Hedgehog, Central missing, Dispatched, Acetyltransferase, *Drosophila melanogaster*

INTRODUCTION

The Hedgehog family of secreted proteins control cell growth and patterning in many key developmental processes in both vertebrates and invertebrates (Ingham, 1994). In *Drosophila* wing development, posterior (P) compartment cells express and secrete Hh proteins that act upon neighboring anterior (A) compartment cells to induce the expression of *decapentaplegic* (*dpp*), which encodes a member of the TGFβ/BMP family of secreted proteins (Basler and Struhl, 1994; Tabata and Kornberg, 1994). Dpp then diffuses bidirectionally into both compartments and functions as a long-range morphogen to control the growth and patterning of cells in the entire wing (Lecuit et al., 1996; Nellen et al., 1996). In addition, Hh activates other genes including *patched* (*ptc*), *collier* (*col*) and *engrailed* (*en*), which are required for proper patterning of the region near the AP compartment boundary (Chen and Struhl, 1996; Strigini and Cohen, 1997; Vervoort et al., 1999).

Hh is synthesized as a full-length protein that undergoes an auto-processing event to generate a cholesterol-modified N-terminal signaling ligand (Porter et al., 1996a; Porter et al., 1996b). The N-terminal fragment of Hh binds to Ptc, resulting in alleviation of Ptc inhibition of Smoothened (Smo) (Chen and Struhl, 1996; Stone et al., 1996). Smo then signals downstream to regulate the Zinc finger transcription factor, Cubitus interruptus (Ci), which transduces the Hh signal into the nuclei (Alexandre et al., 1996; Methot and Basler, 1999; Orenic et al., 1990).

Many components in the *hh* pathway have been identified through genetic screens for embryonic and adult phenotypes. Almost all of the identified components are required in cells that receive the Hh signal to control either the movement or transduction of the Hh signal. The only exception is *dispatched* (*disp*), which encodes a multi-span transmembrane protein with a cholesterol-sensing domain similar to Ptc (Burke et al., 1999). *disp* is required in the Hh sending cells and it appears to control the release of cholesterol-modified Hh (Burke et al., 1999). We describe a genetic screen for positive regulators in the *hh* pathway and present genetic and molecular characterizations of a novel gene required in the Hh sending cells for proper Hh activity.

MATERIALS AND METHODS

Genetic screen using the *eyFLP* system

In the primary screen, isogenic *FRT*-containing males (*FRT80B* or *FRT82B*) were treated with 25 mM EMS overnight. After 24 hours recovery, the males were mated with virgin females with corresponding *eyFLP*/*FRT* (*y w eyFLP2 glass-lacZ; cl3L5 w+FRT80B/TM6B, y+* for 3L screen; *y w eyFLP2 glass-lacZ; FRT82B w+ cl3R3/TM6B*, y+ for 3R screen). Mosaic flies with small-eye phenotypes similar to those caused by *hh* mutation were selected in the F_1 generation and singly backcrossed with several *eyFLP*/*FRT* males or females. F_2 mosaic males with the same phenotypes as the F_1 mosaic flies were selected and mated with female balancers to establish stocks. About one third of fertile F_1 flies

transmitted mutations to the F_2 generation. In the secondary screen, males from individual mutant stocks isolated from the primary screen were mated with virgin females with corresponding *hsFLP* [*y w hsFLP122; Dp(1;3) sc^{J4} y+ M(3) FRT80B/TM2* for 3L screen; *y w hsFLP122; FRT82B hsCD2 y+ M(3)/TM2* for 3R screen]. First or second instar larva were heat shocked to induce FRT/FLP mediated mitotic clones. The F_1 flies with mutant clones on the wings (marked by *y*) were examined for *hh* like phenotypes. From the 3R screen, we recovered 5 new alleles of *hh* (*SH2*, *SH3*, *SH7*, *SH9*, and *SH17*) and 2 new alleles of *disp* (*SH21* and *SH23*) based on the eye and wing phenotypes. The identities of these mutants were assigned by complementation test with previously identified *hh* and *disp* alleles. From the 3L screen, we recovered 9 *cmn* alleles: *M12*, *M82*, *MS1*, *MS6*, *MS7*, *MS13*, *MS16*, *MS18*, and *MS19*. All *cmn* alleles except *M12* are pupal lethal; *M12* may harbor a second lethal hit that causes embryonic lethality.

Molecular characterization of *cmn*

cmn was mapped by complementation tests using the 3L deficiency kit. *cmn* failed to complement *Df(3L)M21* (62F-63D), *Df(3L)HR370* (63A-63D1) and *Df(3L)HR218* (63B6-63D1), but did complement *Df(3L)HR232* (63C1-63D1), thus localizing *cmn* to the 63B6-63C1. Complementation tests with several previously identified lethal mutations within this region revealed that *cmn* failed to complement *l(3)63Bg* (Wohlwill and Bonner, 1991). To generate P-element tagged *cmn* alleles, *l(3)S103012*, which has a P-element insertion at 63B, was mobilized using Δ2-3 as a source of transposase. 3500 new insertion lines were screened by complementation test with *cmn^{M82}* and 3 hopper lines were recovered that are lethal over *cmn^{M82}*. The lethality over *cmn^{M82}* caused by P630 was reversed by precise excision. Both plasmid rescue and inverse PCR were used to determine the new insertion site in *cmn* P-element alleles. Double-stranded RNA interference experiments were carried out as described previously (Kennerdell and Carthew, 1998). To sequence EMS induced *cmn* alleles, late third instar *cmn* mutant larvae were collected and their genomic DNA was extracted using the standard protocol. The genomic DNA of candidate genes was amplified by PCR using primers flanking the coding sequence and was subject to direct sequencing using internal primers.

Transgenes and other mutant stocks

Other mutant stocks used in this study were: *smo^{3}* (Chen and Struhl, 1998); *ttv$^{l(2)00681}$* (Bellaiche et al., 1998); *disp$^{l(3)S037707}$* (Burke et al., 1999); *ptc^{S2}* (Chen and Struhl, 1996). Gal4 driver lines were: *MS1096* (Wang et al., 1999); *actin>CD2>Gal4* (Pignoni et al., 1990). UAS transgenes were: *UAS-Hh* (Wang et al., 2000); *UAS-HhN* (Porter et al., 1996a) and *UAS-CiU* (Methot and Basler, 1999).

Generation of clones of mutant cells

Clones of mutant cells were generated using either the *eyFLP* system or *hs-FLP* system as previously described (Jiang and Struhl, 1995; Newsome et al., 2000). Genotypes for generating clones were as follows.

Eye-specific mutant clones for *smo*: *eyFLP/y w or Y; smo3 stc FRT39E/Dp(1,2)sc19, y+ M(2) FRT39E*.

Eye-specific mutant clones for *ttv*: *eyFLP/ y w or Y; FRT42D ttv$^{l(2)00681}$ /FRT42D w+ cl2R11/Cyo, y+*.

ptc clone in *cmn* mutant background: *y w hs-FLP122; dpp-lacZ FRT42D ptc^{S2}/FRT42D hs-GFP-Myc; cmn^{M82}/cmn^{M82}*.

Immunostaining

Standard protocols for immunofluorescent staining of imaginal discs were used (Jiang and Struhl, 1998). Primary antibodies used were: mouse anti-Ptc (Capdevila and Guerrero, 1994); rabbit anti-Hh (Tabata and Kornberg, 1994); rabbit anti-Col (Vervoort et al., 1999); rat anti-Smo (Denef et al., 2000); rabbit anti-β-gal (Cappel); mouse anti-Myc (Santa Cruz); rabbit anti-GFP (Clone Tech).

RESULTS

Tissue-specific mosaic screen for positive components in the *hh* pathway

We previously identified several novel components in the *hh* pathway including *PKA* (also known as *DCO*) and *supernumary limbs* (*slimb*) in a genetic mosaic screen for mutations that affect patterning of adult structures (Jiang and Struhl, 1995; Jiang and Struhl, 1998). In the previous screen, we utilized a heat inducible FLP/FRT system to randomly induce mutant clones and examined pattern abnormalities in adult structures such as wings and legs (Xu and Rubin, 1993). This screen efficiently identified inhibitory components in the *hh* pathway because clones of mutant cells ectopically activating the *hh* pathway induced pattern duplications if they were situated in the anterior compartment (Jiang and Struhl, 1995; Jiang and Struhl, 1998). In addition to *PKA* and *slimb*, we also identified new alleles of *ptc* and *costal2* (*cos2*), two previously identified inhibitory components in the *hh* pathway (Grau and Simpson, 1987; Hooper and Scott, 1989; Sisson et al., 1997; Wang et al., 2000). However, we were unable to isolate positive components in the *hh* pathway such as *disp* or *tout-velu* (*ttv*), which are involved in either sending or moving the Hh signal (Bellaiche et al., 1998; Burke et al., 1999; The et al., 1999). A likely reason is that Hh signaling only occurs near the AP compartment boundary. Moreover, Hh as well as its major downstream effectors Dpp and Wg acts cell non-autonomously (Basler and Struhl, 1994; Lecuit et al., 1996; Nellen et al., 1996; Zecca et al., 1996). Thus, mutant clones that are defective in Hh signaling may cause significant phenotypes only when they are large enough and are situated near the AP compartment boundary, which are infrequent.

To identify additional positive components that regulate either sending or receiving of the Hh signal, several factors led us to explore the *Drosophila* compound eye. First, Hh signaling activity is required for the initiation and progression of the morphogenic furrow (Dominguez and Hafen, 1997; Greenwood and Struhl, 1999; Heberlein et al., 1993; Ma et al., 1993). Conditional loss of Hh signaling in eyes prevents furrow progression, resulting in a small-eye phenotype (Ma et al., 1993). Second, it is possible to generate mosaic flies that have mutant eyes but wild-type bodies, using the *eyFLP* system (Newsome et al., 2000). Third, eyes are dispensable for viability and fertility; thus, mutant flies with eye defects can be recovered in the F_1 generation. To test the potential of the *eyFLP* system for identifying positive components in the *hh* pathway, we generated mosaic flies with eyes mutant for *smo* or *ttv* using *eyFLP*. As shown in Fig. 2, *smo* or *ttv* mutant eyes exhibit similar small-eye phenotypes to *hh* mutant eyes. We then conducted a two-step screen as illustrated in Fig. 1. In the primary screen, we used *eyFLP* to generate mosaic flies and screened in the F_1 generation for eye phenotypes similar to those caused by the *smo* or *hh* mutation. Once the mutants were bred true in the F_2 generation, we conducted a secondary screen in which we generated mosaic flies carrying large mutant clones in the wing and screened for wing phenotypes similar to those caused by loss or reduction of Hh signaling activity (Basler and Struhl, 1994; Burke et al., 1999). We conducted an extensive screen of randomly introduced mutations on the third chromosome and recovered multiple new alleles of *hh* and *disp* (see Materials and Methods). In

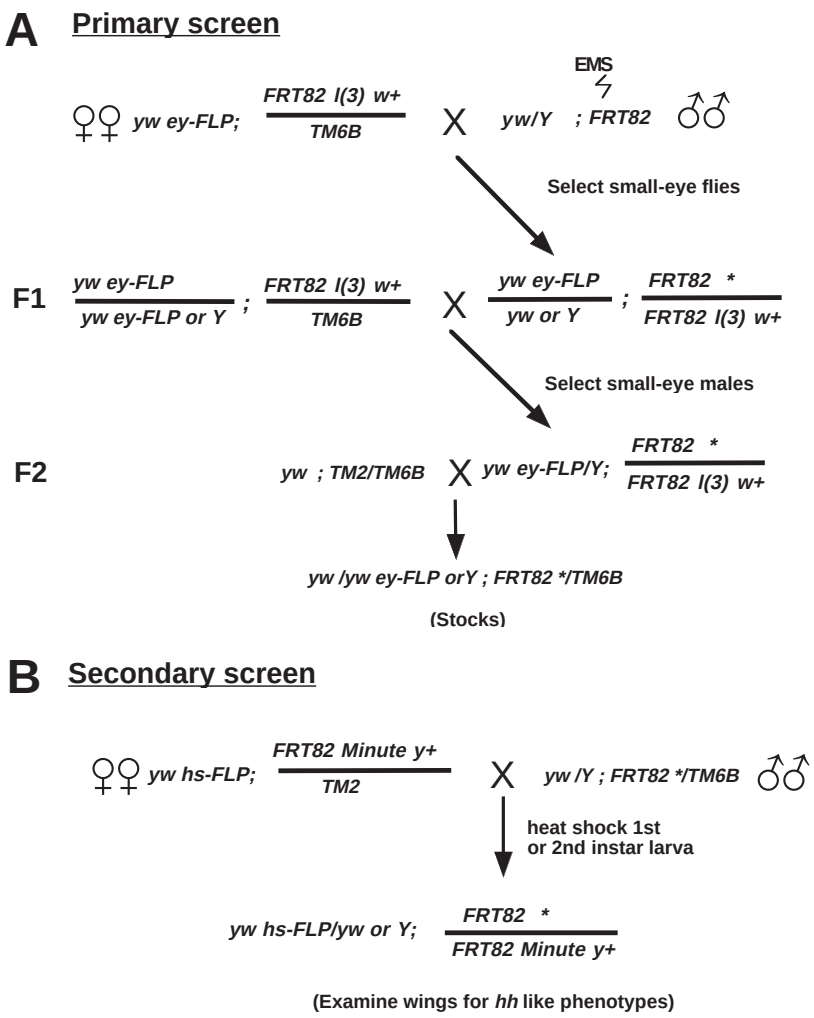

Fig. 1. Tissue-specific mosaic screen for mutations in the *hh* pathway. (A) A two-step screen was designed to identify genes that positively regulate the *hh* pathway. In the primary screen, mosaic flies were generated using the *eyFLP* system and mutations that caused *hh*-like small-eye phenotypes were isolated in the F_1 generation. (B) In the secondary screen, mosaic flies bearing large mutant clones in the wing were generated using *hsFLP* in conjunction with the Minute technique, and were screened for *hh*-like wing phenotypes. For details, see Results and Materials and Methods. The asterisk indicates a newly induced recessive mutation.

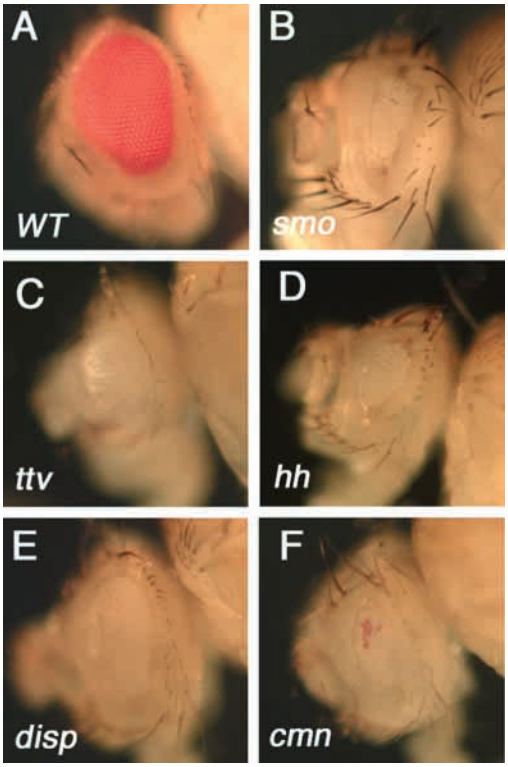

Fig. 2. Eye phenotypes for mutations in the *hh* pathway. (A)Wild-type eye. (B-F) Eyes carrying mutant clones for smo^3 (B), $ttv^{l(2)00681}$ (C), hh^{SH2}(D), $disp^{SH23}$ (E) or cmn^{M82} (F). Loss-of-function mutations in the positive regulators of the *hh* pathway induce eye phenotypes similar to a *hh* mutation.

addition, we isolated multiple alleles of a novel gene, which we name *central missing* (*cmn*) based on the wing phenotype (see below). *cmn* mutant eyes, generated using *eyFLP*, exhibited similar small-eye phenotypes to those caused by mutations in other positive Hh signaling components (Fig. 2).

cmn affects Hh signaling

We identified 9 *cmn* alleles from the mosaic screen and one additional allele by complementation test with previously isolated lethal mutations mapped near the *cmn* locus (see Methods). We used the cmn^{M82} allele for most of our analyses because cmn^{M82} homozygotes exhibited similar phenotypes to cmn^{M82} over deficiency (data not shown), suggesting that cmn^{M82} is a genetically null allele. Wings carrying large clones of *cmn* mutant cells were often smaller and lacked patterning elements in the central region such as vein 2, 3, 4 and 5 (Fig. 3B). These phenotypes are similar to those caused by loss of *hh* in the P-compartment (Basler and Struhl, 1994). Despite the severe patterning defects along the AP axis, the wing margin appears normal, suggesting that *cmn* does not affect Wingless (Wg) signaling.

The similarity of the *cmn* and *hh* phenotypes in both eyes and wings suggests that *cmn* may affect the *hh* pathway. To further test this possibility, we examined whether *cmn* affects the expression of Hh-responsive genes including *dpp* and *ptc*. As *cmn* mutants are pupal lethal, we examined *dpp* and *ptc* expression in late third instar wing discs homozygous for *cmn*. *cmn* mutant discs show reduced levels of *dpp* expression, as indicated by the expression of both Dpp protein and *dpp-lacZ* (Fig. 3D, Fig. 4B). As expected, the upregulation of *ptc* expression at the AP compartment boundary is nearly abolished (Fig. 3F). *cmn* does not regulate *hh* expression because *hh-lacZ* expression is not affected in *cmn* discs (Fig. 3H). These observations suggest that *cmn* acts in the *hh* pathway rather than upstream of *hh* to control its expression

cmn acts upstream of *ptc*

To place *cmn* in the *hh* pathway, we carried out a genetic epistasis analysis. To determine whether *cmn* acts upstream or downstream of *ptc*, we examined Hh responses in *cmn ptc*

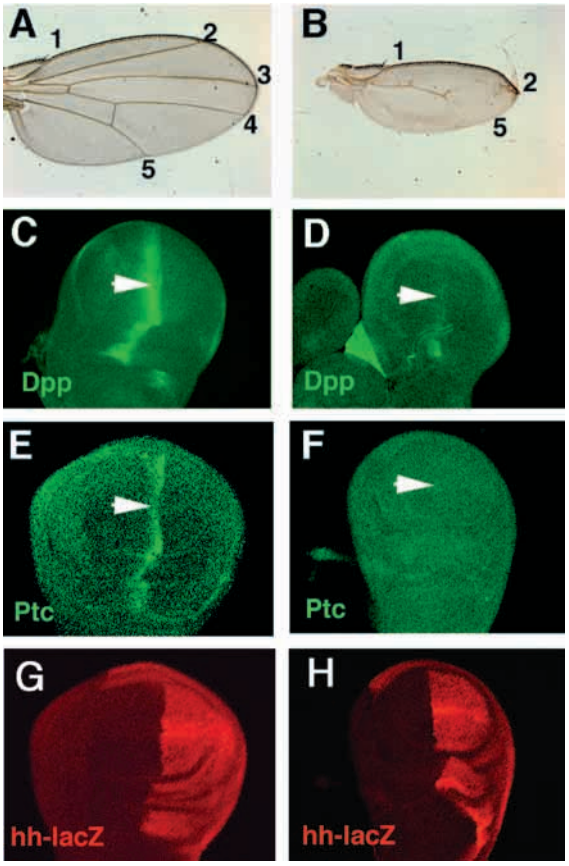

Fig. 3. *cmn* affects Hh signaling. In this and the following figures, all the wing discs are oriented with anterior towards left and ventral up. (A) A wild-type wing and (B) a mosaic wing bearing large *cmn* mutant clones. The central structures of the wing including vein 3 and vein 4 are completely lost. Vein 2 and vein 5 are incomplete. The most anterior (anterior to vein 2) and most posterior (posterior to vein 5) structures are intact. (C,D) Third instar, wild-type (C) and *cmn* homozygous (D) wing discs were stained with anti-Dpp antibody. Dpp expression near the AP compartment boundary is diminished in the *cmn* mutant disc (indicated by the arrowheads). (E,F) Wild-type (E) and *cmn* homozygous (F) wing discs were stained with anti-Ptc antibody. Ptc upregulation is lost in the *cmn* mutant disc (arrowheads) (G,H). Wild-type (G) and *cmn* homozygous (H) wing discs were stained with anti-β-gal antibody to visualize *hh-lacZ* expression. *hh-lacZ* expression appears normal in the *cmn* mutant disc.

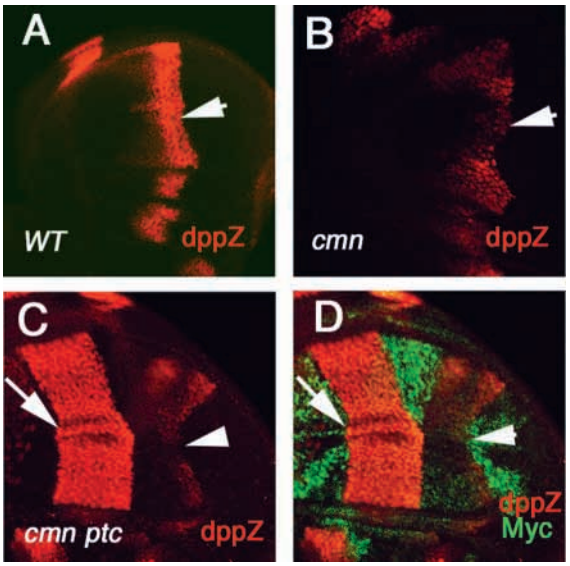

Fig. 4. *cmn* acts upstream of *ptc*. (A) Wild-type wing disc, (B) *cmn* homozygous wing disc and (C,D) *cmn* homozygous wing disc carrying a clone of *ptc* mutant cells, were stained to show *dpp-lacZ* reporter expression (red) and a marker gene (Myc) expression (green in D). *ptc* mutant cells are recognized by the lack of Myc expression. The *cmn* mutant disc exhibits diminished levels of *dpp-lacZ* expression (arrowhead in B). In contrast, *cmn ptc* double mutant cells situated in the anterior compartment express wild-type levels of *dpp-lacZ* (arrow in C and D) Arrowhead in C and D indicates *dpp-lacZ* expression at the AP boundary.

double mutant cells. To do this, we generated *ptc* mutant clones in *cmn* homozygous mutant discs. As shown in Fig. 4, *cmn* singly mutant cells exhibit diminished levels of *dpp-lacZ* expression. In contrast, anteriorly situated *cmn ptc* double mutant cells ectopically activated *dpp-lacZ* at wild-type levels. Thus, *ptc* mutation can bypass the requirement for *cmn* in activating Hh signal transduction pathway, suggesting that *cmn* acts upstream of *ptc*.

cmn is required in the Hh sending cells

Cmn could act upstream of Ptc to regulate Hh movement or as a Hh coactivator in the Hh receiving cells. Alternatively, Cmn could regulate the production or secretion of Hh ligand in the Hh sending cells. To distinguish these two possibilities, we carried out a mosaic analysis in which we generated large clones of *cmn* mutant cells in either the A- or P- compartment and examined their effects on *ptc-lacZ* expression. As shown in Fig. 5, wing discs exhibited normal levels of *ptc-lacZ* expression even though anterior compartment cells near the AP compartment boundary are mutant for *cmn* (Fig. 5A). In contrast, wing discs lost *ptc-lacZ* expression if they contained large P-compartment *cmn* mutant clones that abut the AP compartment boundary (Fig. 5B). These results suggest that Cmn, like Disp, regulates the sending of Hh signal in the Hh producing cells.

Hh is not detected in A-compartment cells of *cmn* mutant discs

We then investigated whether *cmn* affects the secretion of Hh into the anterior compartment by examining Hh distribution in *cmn* mutant discs. To facilitate the detection of the Hh signal, we overexpressed Hh in P-compartment cells using the *hh-gal4* driver line to activate a *UAS-Hh* transgene (Wang et al., 2000). As shown in Fig. 6, wild-type wing discs overexpressing Hh in P-compartment cells exhibited Hh staining in A-compartment cells near the AP compartment boundary. In these cells, Hh colocalized with Ptc in intracellular vesicles (Fig. 6A,A″). A similar pattern of Hh distribution has been described previously (Tabata and Kornberg, 1994). In contrast, *cmn* mutant discs that overexpressed Hh in P-compartment cells exhibit little if any Hh signal in neighboring A-compartment cells (Fig. 6B,B″). This observation suggests that secretion of Hh into the anterior compartment might be impeded in *cmn* mutant discs. *cmn* mutant discs exhibit lower levels of cell surface staining of Hh in the P-compartment

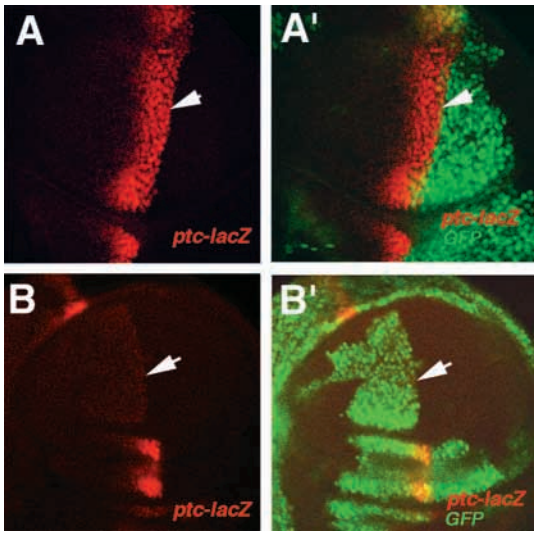

Fig. 5. *cmn* is required in the posterior compartment. (A,A′) A wing disc carrying a large clone of *cmn* mutant cells was stained to show the expression of *ptc-lacZ* (red) and a GFP marker gene (green). *cmn* mutant cells are identifiable by the lack of green staining. Anterior compartment cells near the AP compartment boundary express *ptc-lacZ* (arrowhead) at wild-type levels even though they are mutant for *cmn*. (B,B′) A wing disc carrying a large clone of *cmn* mutant cells (marked by the lack of green staining) in the posterior compartment has lost *ptc-lacZ* expression in adjacent anterior compartment cells (arrowhead).

(compare Fig. 6B with 6A; n>50). Moreover, *cmn* mutant P-compartment cells appear to accumulate more punctate intracellular staining of Hh than wild-type P-compartment cells (Fig. 6A,B). These observations suggest that *cmn* may affect Hh trafficking.

P-compartment cells mutant for *cmn* produce reduced levels of active Hh

It is possible that normal levels of active Hh are produced in P-compartment cells of *cmn* mutant discs but somehow Hh fails to be released into the anterior compartment. If this is true, one would expect that P-compartment cells should activate Hh responsive genes if provided with Ci (Methot and Basler, 1999; Wang et al., 1999). To test this possibility, we used a uncleavable form of Ci (CiU) that requires Hh for its activation (Methot and Basler, 1999). We used a wing-specific Gal4 driver (*MS1096*) to express *UAS-CiU* in wild-type, *disp* or *cmn* discs and examined these discs for the expression of a Hh-responsive gene *col* (Vervoort et al., 1999). In wild-type discs, Hh induces *col* expression in a stripe of cells in the A-compartment abutting the AP compartment boundary (Fig. 7A). This *col* expression is reduced or abolished in *disp* and *cmn* mutant discs (Fig. 7B,C). Consistent with the previous finding that the activity of CiU depends on Hh (Methot and Basler, 1999), expressing CiU in wild-type wing discs ectopically activated *col* only in P-compartment cells (Fig. 7D). Misexpressing CiU in *disp* mutant discs activated *col* in P-compartment cells at levels comparable to those in wild-type discs (Fig. 7E, compared with Fig. 7D). In contrast, P-compartment cells of *cmn* discs expressing CiU expressed diminished levels of *col* (Fig. 7F). We also examined Smo stabilization as a readout for Hh activity (Denef et al., 2000). As shown in Fig. 7, wild-type and *disp* mutant discs stabilized Smo in P-compartment cells at comparable levels (compare Fig. 7G with Fig. 7H). In contrast, *cmn* mutant discs stabilize Smo at levels much lower than wild-type or *disp* mutant discs (Fig. 7I). Taken together, these observations demonstrate that *disp* mutant discs produce normal levels of active Hh in P-compartment cells whereas *cmn* mutant P-compartment cells produce reduced levels of active Hh.

cmn is required for both cholesterol modified and unmodified forms of Hh

Hh is produced as a full-length precursor, which undergoes an auto-processing event to generate a cholesterol-modified N-terminal fragment that functions as a ligand (Porter et al., 1996a; Porter et al., 1996b). To determine if *cmn* affects Hh processing, we tested whether a pre-cleaved form of Hh (HhN) could rescue *cmn* mutant phenotypes (Porter et al., 1996a). We used the *actin>CD2>Gal4* driver line to express *UAS-HhN* uniformly in wild-type, *disp* or *cmn* mutant discs and examined *ptc* upregulation as a readout for the Hh signaling activity. As shown in Fig. 8, indiscriminately expressing HhN in either wild-type or *disp* mutant discs caused ectopic *ptc* upregulation in the entire A-compartment (Fig. 8B,D). In contrast, uniformly expressing HhN in *cmn* mutant discs failed to induce upregulation of *ptc* (Fig. 8F). These results suggest that Cmn does not regulate the cleavage of the Hh precursor into the mature form of Hh. As HhN is no longer modified by cholesterol, this result also suggests that *cmn* is required for the activity of Hh, independent of cholesterol modification.

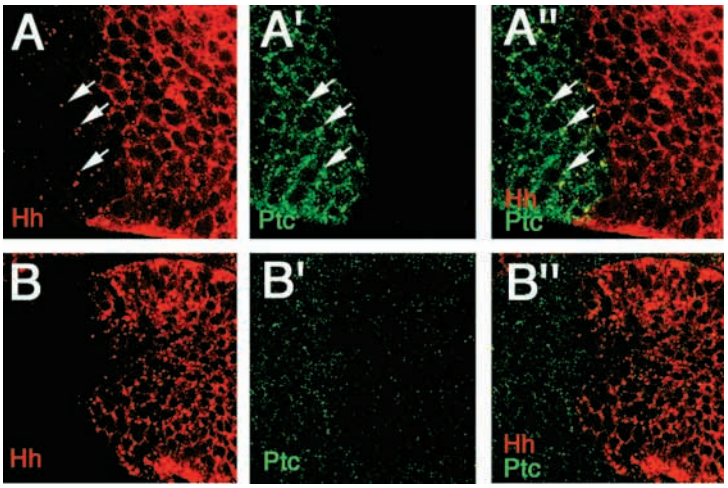

Fig. 6. Hh distribution in wild-type and *cmn* mutant discs. (A,A′,A″) A wild-type wing disc expressing *UAS-Hh* under the control of *hh-Gal4* was stained to visualize Hh (red) and Ptc (green) protein distribution. Hh staining can be detected in anterior compartment cells near the AP compartment boundary. In these cells Hh colocalizes with Ptc in intracellular vesicles (arrows). (B,B′,B″) A *cmn* homozygous mutant wing disc expressing *UAS-Hh* under the control of *hh-Gal4* was stained to visualize Hh (red) and Ptc (green) distribution. Hh staining can only be detected in P-compartment cells, with little, if any, staining in A-compartment cells.

Molecular characterization of *cmn*

As a first step to determine the mechanism by which Cmn regulates the production of active Hh, we carried out molecular characterization of the corresponding gene. We mapped *cmn* to chromosomal region 63B6-63C1 by complementation test with deficiency stocks (see Materials and Methods). Complementation tests with available P elements failed to identify any P element insertion alleles of *cmn*. We decided to use a local hopping strategy to generate P element tagged alleles of *cmn* (Tower et al., 1993). We mobilized a P element inserted at 63B and screened derived hop lines by complementation test with cmn^{M82}. We obtained 3 hop lines that failed to complement the lethality of cmn^{M82}. We focused on one such line, P630, because its lethality over cmn^{M82} could be reversed by precise excision of the inserted P element. Using both plasmid rescue and inverse PCR, we found that P630 harbors a single new insertion between two annotated genes CG14964 and CG12734. However, sequence analysis of *cmn* mutant alleles as well as rescue experiments suggested that none of these two genes corresponds to *cmn* (data not shown). We hypothesized that P630 may affect the expression of *cmn*, which is located further away. We therefore applied RNAi to candidate genes located near the P630 insertion site. One annotated gene, CG11495, drew our special attention because it is related to a gene required for Wg secretion (see below). Injections of double-stranded RNA corresponding to CG11495 into wild-type embryos produced a weak segment polarity phenotype at low frequency (data not shown). Moreover, we found that 5 out 6 EMS induced *cmn* alleles we sequenced have a nonsense mutation in the coding region of CG11495 (Fig. 9B). These results strongly suggest that the phenotypes associated with *cmn* mutations are due to inactivation of CG11495. Of note, cmn^{M82} generates a stop codon at amino acid 163, resulting in a truncated protein that lacks the majority of coding sequence. Thus, cmn^{M82} appears to be a true null mutation.

CG11495 is predicted to encode a protein that is 500 aa long and contains 10 transmembrane domains (Fig. 9B). Blast search identified homologs in mouse and human, which share over 25% sequence identity to CG11495 and the conservation spans the whole proteins (Fig. 9D). Sequence analysis of the CG11495-encoded protein suggests that it belongs to a family of membrane bound acyltransferases (Fig. 9C) (Hofmann, 2000). Interestingly, this family also includes Porcupine, which is required for Wg secretion (Kadowaki et al., 1996; Tanaka et al., 2000).

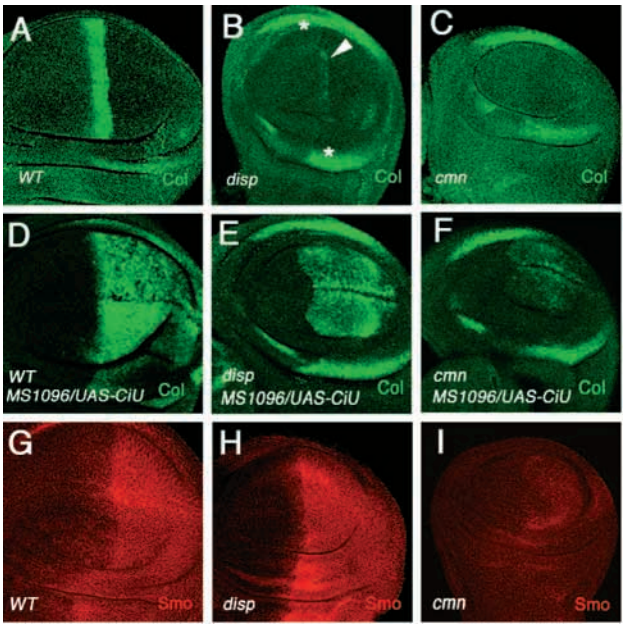

Fig. 7. *cmn* mutant discs produce reduced levels of active Hh in the P-compartment. Late third instar wing discs were stained with anti-Col (green) or anti-Smo (red) antibody to monitor Hh signaling activity. (A-C). Wild-type wing discs activate col in A-compartment cells near the AP compartment boundary in the wing pouch region (A). *disp* mutant discs exhibit diminished levels of Col expression in a narrow stripe of A-compartment cells (arrowhead in B). Note that Col staining surrounding the wing pouch region is not controlled by Hh (indicated by asterisks in B). *cmn* mutant discs fail to express Col at detectable levels near the AP compartment boundary (C). (D-F) Expression of CiU in wild-type (D) or *disp* (E) discs activates Col expression at comparable levels in P-compartment cells, whereas expression of CiU in *cmn* mutant discs (F) induces ectopic Col expression in P-compartment cells at levels much lower than wild or *disp* mutant discs. (G-I). Wild-type (G) and *disp* (H) discs stabilized Smo at comparable levels in P-compartment cells whereas Smo was only slightly stabilized in P-compartment cells of *cmn* discs (I).

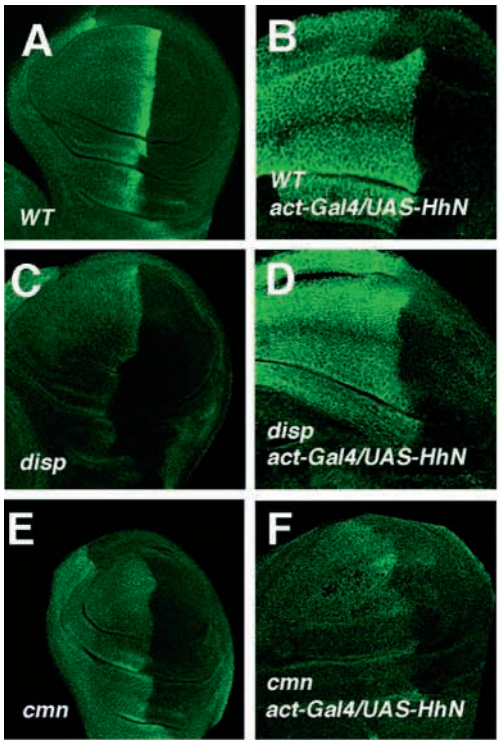

Fig. 8. *cmn* is required for the activity of cholesterol-free Hh. Late third instar wing discs of the following genotypes were stained with anti-Ptc to monitor the up-regulation of *ptc* in response to Hh. (A) Wild type, (B) *act>CD2>Gal4/UAS-HhN*, (C) $disp^{l(3)S037707}$, (D) *act>CD2>Gal4/UAS-HhN*; $disp^{l(3)S037707}$, (E) cmn^{M82}, (F) *act>CD2>Gal4/UAS-HhN*; cmn^{M82}. Misexpressing HhN in both wild-type and *disp* mutant discs induced ectopic *ptc* upregulation (B,D). In contrast, misexpressing HhN in *cmn* mutant disc failed to upregulate *ptc* expression (F).

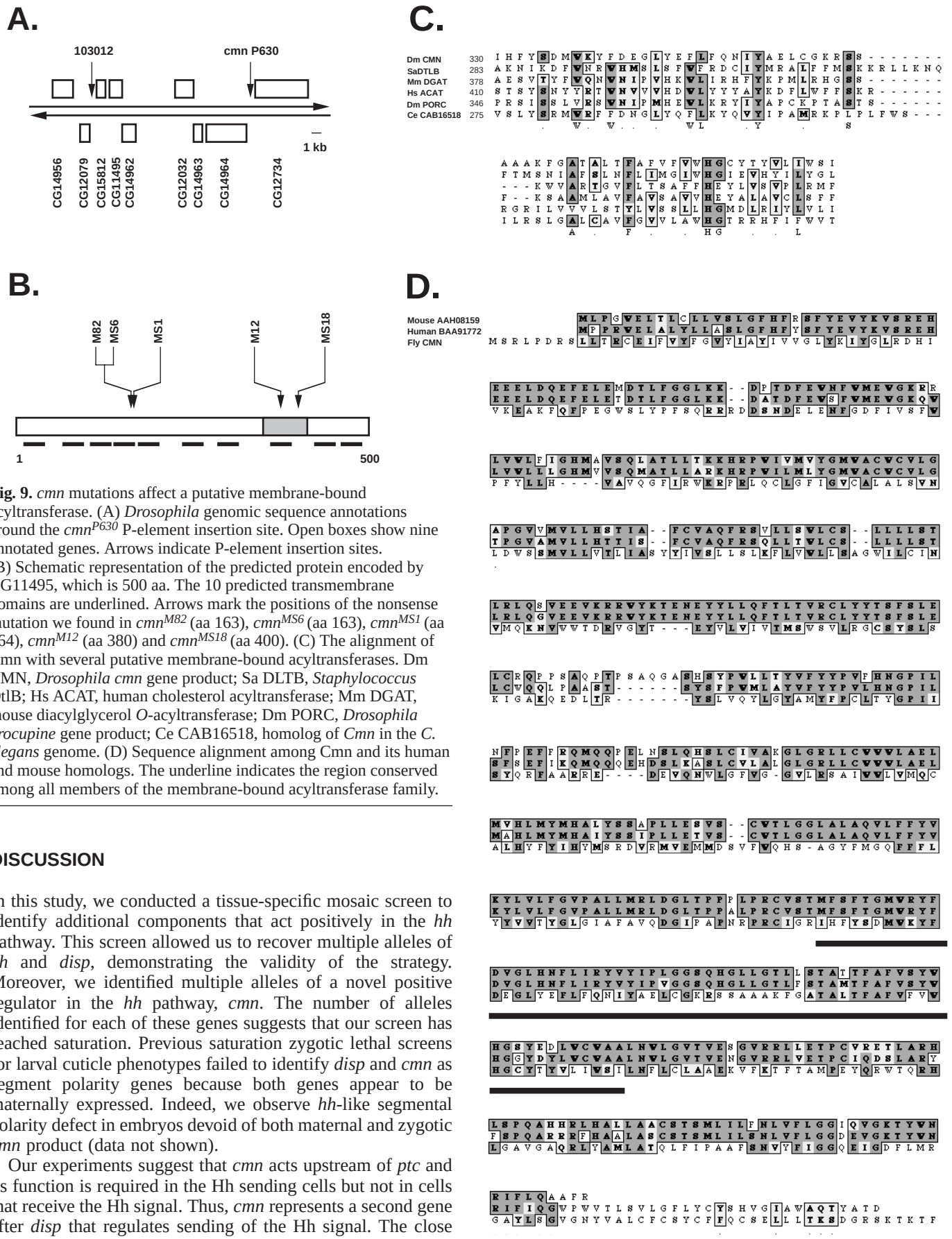

Fig. 9. *cmn* mutations affect a putative membrane-bound acyltransferase. (A) *Drosophila* genomic sequence annotations around the *cmn*^{P630} P-element insertion site. Open boxes show nine annotated genes. Arrows indicate P-element insertion sites. (B) Schematic representation of the predicted protein encoded by CG11495, which is 500 aa. The 10 predicted transmembrane domains are underlined. Arrows mark the positions of the nonsense mutation we found in *cmn*^{M82} (aa 163), *cmn*^{MS6} (aa 163), *cmn*^{MS1} (aa 164), *cmn*^{M12} (aa 380) and *cmn*^{MS18} (aa 400). (C) The alignment of Cmn with several putative membrane-bound acyltransferases. Dm CMN, *Drosophila cmn* gene product; Sa DLTB, *Staphylococcus* DtlB; Hs ACAT, human cholesterol acyltransferase; Mm DGAT, mouse diacylglycerol *O*-acyltransferase; Dm PORC, *Drosophila procupine* gene product; Ce CAB16518, homolog of *Cmn* in the *C. elegans* genome. (D) Sequence alignment among Cmn and its human and mouse homologs. The underline indicates the region conserved among all members of the membrane-bound acyltransferase family.

DISCUSSION

In this study, we conducted a tissue-specific mosaic screen to identify additional components that act positively in the *hh* pathway. This screen allowed us to recover multiple alleles of *hh* and *disp*, demonstrating the validity of the strategy. Moreover, we identified multiple alleles of a novel positive regulator in the *hh* pathway, *cmn*. The number of alleles identified for each of these genes suggests that our screen has reached saturation. Previous saturation zygotic lethal screens for larval cuticle phenotypes failed to identify *disp* and *cmn* as segment polarity genes because both genes appear to be maternally expressed. Indeed, we observe *hh*-like segmental polarity defect in embryos devoid of both maternal and zygotic *cmn* product (data not shown).

Our experiments suggest that *cmn* acts upstream of *ptc* and its function is required in the Hh sending cells but not in cells that receive the Hh signal. Thus, *cmn* represents a second gene after *disp* that regulates sending of the Hh signal. The close

similarities between *cmn* and *disp* phenotypes prompted us to carry out comparative study of these two genes. We found that *cmn* and *disp* affect the sending of Hh in different ways. Whereas *disp* is specifically required for the cholesterol-modified form of Hh, *cmn* regulates Hh activity independent of cholesterol modification of Hh (Fig. 8).

A previous report proposed that *disp* is required for the release of Hh after it reaches the cell surface (Burke et al., 1999). This notion is primarily based on the observation that Hh appears to be trapped in *disp* mutant cells with no apparent change in subcellular localization of Hh (Burke et al., 1999). However, this study did not rule out the possibility that *disp* may affect intracellular trafficking of Hh in a subtle way because imaginal disc cells have very narrow cytoplasmic spaces and it is difficult to distinguish between cell surface staining and cytoplasmic staining. Here we provide evidence that Hh produced in P-compartment *disp* mutant cells can signal normally (Fig. 7), implying that Hh reaches the cell surface.

In *cmn* mutant discs, we did not detect Hh signal in anterior compartment cells near the AP compartment boundary (Fig. 6). However, unlike the case of *disp*, we did not observe accumulation of Hh staining in P-compartment *cmn* mutant cells (data not shown). In contrast, we observed that *cmn* mutant P-compartment cells consistently exhibited lower levels of cell surface staining of Hh with concomitant increase in the number and size of intracellular Hh aggregates as compared with wild-type cells (Fig. 6). This observation implies that *cmn* might affect cellular trafficking of Hh. Consistent with lower levels of cell surface Hh staining, we found that *cmn* mutant P-compartment cells produce lower levels of active Hh as compared with wild-type or *disp* mutant cells (Fig. 7).

In 5 out 6 *cmn* EMS alleles we sequenced, we identified a nonsense mutation in the coding region of CG11495, which encodes a putative membrane-bound acyltransferase (Fig. 9), suggesting that *cmn* phenotypes are caused by inactivation of CG11495. All biochemically characterized members of the membrane-bound acyltransferase family transfer organic acids, typically fatty acid, onto hydroxyl groups of membrane-bound targets (Hofmann, 2000). Although the target for Cmn is not known, a good candidate is Hh. Hh precursor is cleaved to generate the biologically active form that is modified by cholesterol at its carboxyl-terminal end (Porter et al., 1996a). However, our finding that *cmn* affects the activity of both cholesterol-modified and unmodified forms of Hh argues against the possibility that cholesterol modification of Hh is affected by *cmn*.

It has been shown that mammalian Sonic hedgehog (Shh) acquires a palmitoyl modification on an N-terminal cysteine in cell culture (Pepinsky et al., 1998). N-terminal fatty acid modification of Shh appears to enhance its activity in certain developmental settings (Kohtz et al., 2001). In addition, mutation of a conserved cysteine residue in *Drosophila* Hh (C84S-Hh) impairs its function in vivo, implying that Hh may also be modified by palmitoylation at its N-terminal region (Lee et al., 2001). Thus, one possible role for Cmn is to regulate Hh palmitoylation, which may control Hh activity or intracellular trafficking. Indeed, C84S-Hh acts in a dominant negative fashion, implying that it is defective in signaling (Lee et al., 2001). Thus, the lack of detectable Hh signal in A-compartment cells of *cmn* mutant discs could be explained if palmitoyl-free Hh fails to bind and internalize Ptc efficiently.

It is interesting to note that Cmn is related to Porcupine, which is required for the secretion of Wg signal (Kadowaki et al., 1996). Like Cmn, Porcupine also belongs to the membrane bound acyltransferase family (Fig. 9C), suggesting that acylation of secreted proteins may be a more general mechanism than previously thought for regulating the activity or secretion of signaling molecules involved in animal development.

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