

Evidence for a role of vertebrate *Disp1* in long-range Shh signaling

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SUMMARY

Dispatched 1 (*Disp1*) encodes a twelve transmembrane domain protein that is required for long-range sonic hedgehog (Shh) signaling. Inhibition of *Disp1* function, both by RNAi or dominant-negative constructs, prevents secretion and results in the accumulation of Shh in source cells. Measuring the Shh response in neuralized embryoid bodies (EBs) derived from embryonic stem (ES) cells, with or without *Disp1* function, demonstrates an additional role for *Disp1* in cells transporting Shh. Co-cultures with Shh-expressing cells revealed a significant reduction in the range of the contact-dependent Shh response in *Disp1*^{-/-} neuralized EBs. These observations support a dual role for *Disp1*, not only in the secretion of Shh from the source cells, but also in the subsequent transport of Shh through tissue.

KEY WORDS: Dispatched 1, Patched 1, Sonic hedgehog, Polarized epithelium, Morphogen, Neuralized embryoid bodies, Embryonic stem cells, Co-culture

INTRODUCTION

The hedgehog (Hh) family of secreted morphogens is essential for normal development and aberrant activation of the Hh pathway has been implicated in many types of cancer. Mammals have three Hh homologs: sonic hedgehog (Shh), desert hedgehog (Dhh) and indian hedgehog (Ihh) (Echelard et al., 1993). Like other morphogens, Hh proteins are secreted from localized sources in the embryo and can signal over short and long ranges. Patterning of the ventral neural tube is one of the best-studied functions of vertebrate Hh proteins. Shh is secreted initially from the notochord underlying the neural plate and later from the floor plate, the ventral midline of the neural tube, which is crucial for proper dorsoventral patterning of several cell types in the developing spinal cord (Albright et al., 2000). Notochord transplantation and co-cultures of explanted notochord with neural plate tissues have demonstrated that contact is required between the notochord, the source of Shh and neural plate tissue for normal, long-range patterning (Placzek et al., 1993; Placzek et al., 1991), indicating that the establishment of the Shh gradient involves cell-cell contact.

Shh, like other members of the Hh family, undergoes significant processing before release. Shh is generated as a 45 kD precursor, which undergoes autocatalytic cleavage producing 19 kD N-terminus and 26 kD C-terminus fragments (Lee et al., 1994; Porter et al., 1995). Concomitantly, a cholesterol moiety is added to the carboxy terminus of the N-terminus fragment (Porter et al., 1996). In addition, a palmitoyl adduct is added to the amino terminus of the N-terminus fragment by the acyltransferase skinny hedgehog, yielding a mature Shh ligand with dual lipid modifications (Buglino and Resh, 2008; Chamoun et al., 2001; Pepinsky et al., 1998). These modifications probably render the mature Shh protein strictly membrane-associated (Peters et al., 2004).

How an insoluble ligand such as mature Shh signals over a long range is not clear. However, long-range Shh signaling is known to require the action of dispatched 1 (*Disp1*) a twelve-transmembrane, sterol-sensing domain (SSD)-containing protein resembling the Shh receptor, patched 1 (*Ptch1*) (Kawakami et al., 2002; Ma et al., 2002). *Disp1* and *Ptch1* share many characteristics with members of the resistance-nodulation-cell division (RND) family of proton-driven transporters. Not only are the overall structures of *Disp1* and *Ptch1* similar to that of bacterial RND permeases, but aspartic acid residues in the fourth transmembrane span, which are thought to be important for proton translocation, are also conserved (Goldberg et al., 1999; Tseng et al., 1999). It has previously been shown that bacterial RND permeases function as trimers (Nagano et al., 2005). Similarly, it has been shown that *Ptch* is also assembled into trimers (Lu et al., 2006).

Unlike the restricted expression patterns of *Ptch1* and Shh, expression of *Disp1* and *disp* (the *Drosophila Disp1* ortholog) is ubiquitous. Flies lacking *disp* and mice lacking *Disp1* have severe disruptions in Hh signaling (Burke et al., 1999; Caspary et al., 2002; Kawakami et al., 2002; Ma et al., 2002). *disp* and *Disp1* mutants most closely resemble mutants in the Hh signal transduction component smoothed (*Smo*), exhibiting a near complete loss of Hh signaling. Processing and modification of Hh proteins is normal in the absence of *disp*, but Hh is largely retained in source cells, suggesting a role for *disp* in the export of Hh (Burke et al., 1999; Caspary et al., 2002; Kawakami et al., 2002; Ma et al., 2002). *Disp* is also not required for the Hh response, as expression of the Hh target gene decapentaplegic (*Dpp*) is still induced in *disp*^{-/-} clones abutting the anterior-posterior compartment boundary by Hh released from the posterior compartment of the *Drosophila* wing imaginal disc (Burke et al., 1999). However, in vertebrates, Shh signaling has a significantly longer range than in the fly imaginal disc, leaving open the possibility of a role for *Disp1* in the formation of the long-range Shh gradient. Because Shh is retained in source cells in the absence of *Disp1*, it has not been possible to test whether *Disp1* functions exclusively in Shh-expressing cells or whether it is also required in Shh-responding cells for the establishment of the long-range signal.

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Here, we show that, in polarized epithelial cells, *Disp1* mediates the basolateral secretion of Shh, and that *Ptch1* on adjacent cells is required for the uptake of Shh. We further show that the range of Shh signaling is shortened in *Disp1*^{-/-} tissue, even when Shh is produced in *Disp1*-expressing cells. Together, these results support a model in which the reiterated *Disp1*-dependent secretion, coupled to *Ptch1*-dependent uptake, is responsible for the distribution of Shh through a tissue.

MATERIALS AND METHODS

Cell staining

Unless otherwise noted, cells were stained after fixation in 4% paraformaldehyde, placed for 30 minutes on ice and then imaged with an LSM5 Pascal confocal or an AxioObserver Z1 fluorescence microscope (Zeiss).

Generation of *Disp1*^{-/-} ES cells

Disp1^{-/-} cells were derived from *Disp1*^{+/-} cells using established procedures (Mortensen et al., 1991). *Disp1*^{+/-} embryonic stem (ES) cells were selected at 5 mg/ml of G418 (Invitrogen) until individual colonies were visible. After expansion, the genotype of each surviving colony was verified by Southern blotting (Kawakami et al., 2002). We identified two *Disp1*^{-/-} ES cell lines that could be neutralized using established protocols (Wichterle et al., 2002).

Generation of stable Shh-expressing ES cell lines

Wild-type ES cells (murine AB1) stably expressing Shh were derived by electroporating ES cells with a plasmid containing an elongation factor 1 α [*Ef1a* (Eif1a – Mouse Genome Informatics)] promoter, the *Shh* coding sequence followed by an internal ribosome entry site (IRES) and the *Zeocin* (*Zeo*) resistance gene (*Shh*-IRES-*Zeo*). Electroporated cells were selected with 50 μ g/ml *Zeo* (Invitrogen) for 10 days before individual colonies were picked, expanded and analyzed for Shh expression by western blot and immunofluorescence.

Identification of *Disp1* trimers

HEK 293T cells in 12-well dishes were transfected with V5-tagged wild-type canine *Disp1* or *Disp1* mutants. After 24 hours, cells were lysed in 1 \times NativePAGE sample buffer (Invitrogen) containing protease inhibitors (Roche) and 1% n-dodecyl-B-d-maltoside (Invitrogen) on ice for 30 minutes. Lysates were centrifuged at 13,000 *g* for 30 minutes, run on NativePAGE Bis-Tris gels (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen). Membranes were probed with anti-V5 antibody (Invitrogen). Sizes were determined using NativeMark protein standards (Invitrogen).

Shh western blots

Shh-expressing wild-type or *Disp1*^{-/-} ES cells were grown for 24 hours in Optimem (Invitrogen). Supernatants were collected and concentrated approximately 12-fold using Microcon centrifuge filters (Millipore). LDS sample buffer containing DTT (Invitrogen) was added to a final concentration of 1 \times . Cells were rinsed once in PBS, then lysed in 1 \times LDS sample buffer containing DTT. Samples were run on 12% Bis-Tris gels (Invitrogen) and transferred to nitrocellulose. Membranes were probed using a rabbit polyclonal anti-Shh antibody (Cell Signaling).

Generation of *Disp1* deletion mutants and localization in polarized cells

Canine *Disp1* deletions were generated using the QuikChange II XL mutagenesis kit (Stratagene). Madin-Darby canine kidney (MDCK) II cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS (Hyclone) and antibiotics. Three hundred thousand cells were plated onto 12 mm polyester transwell filters (0.4 μ m pore size; Corning). After reaching confluence, cells were transfected with V5-tagged canine *Disp1* constructs. After another 24 hours in culture, the cells were stained with mouse anti-V5 antibody (1:200; Invitrogen) and rabbit anti-ZO1 antibody (1:100; Invitrogen).

Disp1 miRNA analysis

Canine *Disp1* miRNA target sequences were as follows: miRNA1 GACTGGTTACGTGGAATAACA; miRNA2 TTGCGGGTGAAA-GTTTGTAA. miRNAs were cloned into pcDNA6.2-GW/EmGFP-miR (Invitrogen). For Shh localization experiments, *Shh* and either of the two *Disp1* miRNAs were co-transfected into confluent monolayers of MDCK II cells. Thirty-six hours after transfection, cells were fixed and stained for Shh and GFP. To visualize Shh on the apical surface only, anti-Shh 5E1 antibody (1:10; DSHB) was added for 30 minutes at 4°C before fixation, permeabilization and staining.

For western blots, HEK 293T cells were co-transfected with *Disp1* and miRNA plasmids and allowed to grow for 36 hours. Cells were lysed in a small volume of high-salt RIPA lysis buffer (600 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-Cl, pH 7.6), diluted four-fold, run on an 8% Bis-Tris gel and transferred to nitrocellulose. Blots were probed with anti-V5 and anti-GFP antibodies.

KNRK cell staining

KNRK cells (normal rat kidney cells transformed by Kirsten murine sarcoma virus) were grown on glass cover slips and transfected with Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were stained for *Disp1* mouse anti-Myc (9E10) and rabbit anti-Shh antibodies.

Shh Dose Response of *Disp1*^{+/-} and *Disp1*^{-/-} EBs

ES cells were differentiated into neutralized embryoid bodies (EBs) using established procedures (Wichterle et al., 2002) and exposed to Shh N-terminus (ShhN; produced in Baculovirus-infected HiFive cells) at day 2. EBs were grown for an additional 4 days in supplemented DFNB with a medium change after 2 days. On day 6, EBs were stained for Pax7 and Nkx2.2.

EB Co-cultures

EBs were derived as described above from AB1 cells expressing Shh and *Disp1*^{+/-} or *Disp1*^{-/-} cells. Equal numbers of *ShhAB1* and *Disp1*^{+/-} or *Disp1*^{-/-} EBs were co-cultured for 48 hours (for Pax7 analysis) or 72 hours (for HB9 analysis) in a collagen matrix. EB co-cultures were fixed in 4% paraformaldehyde with 4% sucrose for 40 minutes on ice and stained. Ranges of Pax7 repression and HB9 induction were measured in ImageJ at about 20 clearly interpretable EB interfaces and statistic analysis was performed using Prism (GraphPad Software).

Shh transport assay

MDCK II cells were plated on transwell filters as described above. After cells reached confluence, they were transfected with Shh and *Disp1* constructs and grown for 48 hours. For the final 12 hours of growth, 5E1 anti-Shh monoclonal antibody was added to the basolateral chamber 1:1 with normal growth medium. Cells were fixed and stained as described above. Shh retention was quantified by measuring average signal intensity within a circular area slightly bigger than a cell, and secretion was measured using the signal intensity in a 1.5-cell diameter wide circle around each Shh-expressing cell. Over 50 cells and their surroundings were measured, then analyzed with Prism.

RESULTS

Disp1 is a basolaterally localized trimer

To assess the ability of *Disp1* to form trimers, we analyzed lysates of *Disp1*-transfected cells by blue-native gel electrophoresis. Besides a high molecular weight smear, a discrete band of around 480 kD was detected, the expected size for a *Disp1* trimer (Fig. 1A). Similar lysates analyzed on an SDS PAGE gel resolved a single 160 kD band (Fig. 2D). As has previously been shown for *Ptch* and bacterial RND proteins, we found that *Disp1* (and *Disp2*, not shown) assembles into trimers.

During neural tube development, Shh is secreted from two distinct sources: the notochord and the floor plate. Shh is produced in the notochord and signals over a long range to the basolateral side

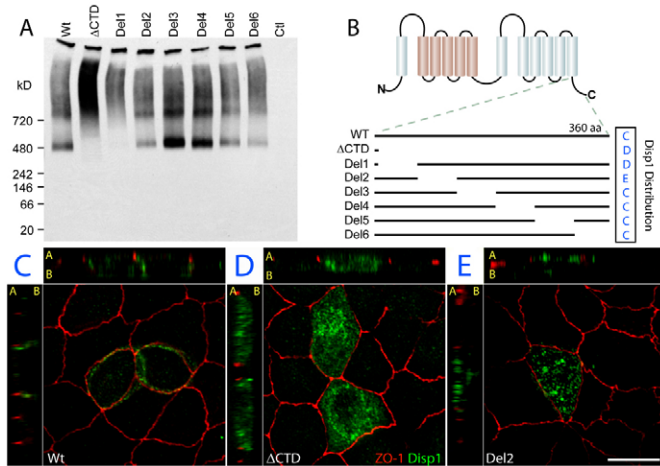


Fig. 1. Disp1 is located basolaterally in polarized epithelial cells and assembles as trimers. (A) Blue-Native gel analysis of *Disp1* transfected HEK 293T cells. The 480 kD band corresponds to the predicted size of *Disp1* trimers. *Disp1* mutants are indicated. (B) Diagram illustrating *Disp1* deletion mutants. In addition to deletion of the entire CTD, a series of six ~60-amino-acid-deletion mutants covering the CTD were generated. The box to the right indicates how each mutant localizes relative to the three *Disp1* forms shown in C-E, and trimer formation is shown in A. (C-E) Visualization of transiently transfected V5-tagged *Disp1* (green) and ZO1 (red) in polarized MDCK cells. ZO1 marks the border between the apical and basolateral aspects of the polarized cells. Wild-type (Wt) *Disp1* (C) is located basolaterally, *Disp1*ΔCTD (D) is located throughout the cell and *Disp1* Del2 (E) is primarily located at or near the apical cell surface. *x,z*-reconstructions are shown on the top and at the left of each panel, yellow As and Bs indicate the apical and basolateral sides, respectively. Scale bar: 20 μ m.

of overlying neural epithelium. Induction of the floor plate occurs in the neural epithelium closest to the notochord, where it is exposed to the highest levels of Shh. The floor plate also expresses Shh and floor plate-derived Shh could be released to the neural epithelium either apically or basolaterally. To determine at which side of an epithelium Shh is transported, we assessed the localization of both *Disp1* and Shh in polarized epithelial Madin-Darby canine kidney (MDCK) cells (Mays et al., 1995). We used antibodies against the tight junction marker ZO1 (Fig. 1C-E, red) to visualize the border between the apical and basolateral domains of the MDCK cells (Nigam et al., 1991).

In MDCK cells transfected with V5-tagged canine *Disp1*, *Disp1* protein accumulates at or near the basolateral surface (Fig. 1C). Similarly, *Disp1* is detected on the lateral and basal sides of cells in the chicken neuroepithelium electroporated with *Disp1* (see Figure S1A in the supplementary material). The carboxy-terminal domain is required for the localization of *Ptch1* (Kawamura et al., 2008) and in analogy we tested the role of the homologous domain in *Disp1*. Complete deletion of the carboxy-terminal domain (Δ CTD; Fig. 1B) resulted in a molecule that neither localized to the surface (Fig. 1D) nor assembled into trimers (Fig. 1A). In addition to deleting the entire CTD, we also generated a series of six smaller (~60 amino acid) deletions spanning the CTD. Deletion of the first 60 amino acids after transmembrane twelve (Del1) also prevented trimerization and surface localization of *Disp1*, and was indistinguishable to Δ CTD (Fig. 1A,B and data not shown). However, deleting the second 60 amino acids after transmembrane 12 (Del2) resulted in a protein that still trimerized but was localized

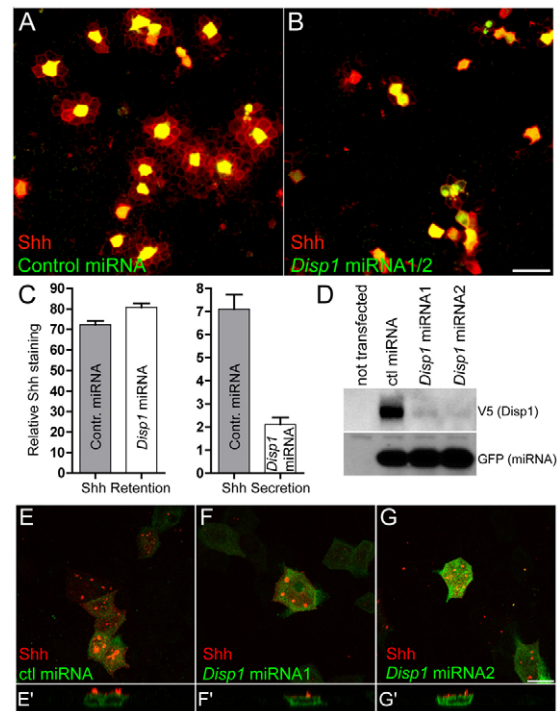


Fig. 2. Disp1 is required for the basolateral secretion of Shh. MDCK cells were grown on 12 mm polyester transwell filters (0.4 μ m pore size) and transfected with Shh (red) and either control *Disp1* miRNA constructs (green, A,E) or *Disp1* miRNA constructs (green, B,F,G). (A,B) Shh was visualized after inclusion of 5E1 in the compartment at the basolateral side of the MDCK cells. Secreted Shh is visible in the cells co-transfected with control *Disp1* miRNA but is severely reduced in cells co-transfected with *Disp1* miRNA1 or 2. (C) Shh staining was quantified by measuring staining intensity in transfected cells (retention) or in a 1.5-cell diameter wide ring around the transfected cells (secretion). Co-transfection of *Disp1* miRNA1 or 2 caused significant increase in retention of Shh, whereas the secretion of Shh was significantly reduced. The y-axis is a relative measure of Shh staining. Error bars are s.e.m., $P < 0.001$. (D) Efficacy of the miRNA constructs. Although canine *Disp1* can be detected when co-transfected with control miRNA, translation is blocked when co-transfected with *Disp1* miRNA1 or 2. (E-G) Shh was visualized after inclusion of 5E1 in the compartment at the apical side. Shh is detected at the surface regardless of the presence of *Disp1* miRNA constructs. (E'-G') *z*-axis reconstructions. Scale bars: 20 μ m in A,B; 10 μ m in E-G.

primarily to the apical surface of the cell rather than the basolateral surface (Fig. 1A,B,E). The remaining four 60-amino-acid-deletion mutants were indistinguishable from wild-type *Disp1* in regard to localization and trimer formation (Fig. 1A,B). Taken together, these results demonstrate that *Disp1* trimerizes and is localized to the basolateral side of polarized epithelial cells. Additionally, sequences within the CTD appear to be required for the basolateral localization and trimerization of *Disp1*.

Lipid-modified Shh is secreted basolaterally in a *Disp1*-dependent manner

To test whether Shh is secreted apically or basolaterally, a polarized epithelium of MDCK cells grown on transwell filters was transfected with a plasmid encoding *Shh* and the distribution of Shh on the apical and basolateral sides was determined by the inclusion of 5E1 anti-Shh monoclonal antibody in the apical or basolateral

compartment of the transwell culture chambers. To deplete endogenous *Disp1* protein, cells were co-transfected with miRNA constructs against canine *Disp1*.

Addition of the anti-Shh antibody to the basolateral side of the culture revealed significant amounts of secreted Shh in a gradient stretching a few cell diameters from the cells producing Shh (Fig. 2A), in agreement with the polarized *Disp1* localization. To determine whether secretion of Shh on the basolateral side of the cell is mediated by *Disp1*, we co-transfected *Shh* with miRNA constructs against *Disp1*. Under these conditions, less Shh could be detected outside of the transfected cells (Fig. 2B), whereas more Shh was retained by these cells as a consequence of significantly reduced levels of *Disp1* (Fig. 2C,D). Shh staining was also present on the apical side of transfected cells (Fig. 2E), and this apically localized Shh was unaffected by co-transfection of *Disp1* miRNA constructs (Fig. 2F,G). The *Disp1*-dependent distribution of Shh away from the source suggests that *Disp1* is required to produce a form of Shh that can be released. In polarized cells this export probably occurs at the basolateral side, as we find the apical localization of Shh to be *Disp1*-independent.

To determine whether the Shh distribution *in vivo* reflects our observations in MDCK cells, we injected 5E1 hybridoma cells into the neural tube lumina of stage 10 chick embryos and visualized the distribution of the anti-Shh antibody 36 hours later. Under these conditions, a significant accumulation of Shh could be detected basally and in the plane of the epithelium, whereas much smaller amounts were associated with the apical surface of the cells (see Fig. S1B,C in the supplementary material). Moreover, although 5E1 is an efficient Shh-blocking antibody (Ericson et al., 1996), no significant reduction in the Shh response was observed (see Fig. S1C in the supplementary material), a further indication that Shh transport in the neural tube is primarily along the basolateral aspect rather than the apical aspect of neural cells.

Several *Disp1* mutants act as dominant-negatives

As *Disp1*, like other RND permeases, forms trimers, we tested whether mutant forms of *Disp1* act as dominant-negative alleles to interfere with *Disp1*-mediated secretion of Shh. To test this, we co-transfected *Shh* with either *Disp1*, *Disp1* lacking the CTD (Δ CTD), or a mutant in which the aspartate residues required for proton translocation were replaced with alanine residues (*AAA*) (Ma et al., 2002). We measured the amount of secreted Shh and the amount of Shh retained by the transfected cells. Compared with cells transfected with wild-type *Disp1* (Fig. 3A), both *Disp1* Δ CTD (Fig. 3B) and *Disp1*AAA (Fig. 3C) inhibited the secretion of Shh and caused accumulation of Shh in the transfected cells (Fig. 3D). These findings suggest that both *Disp1* Δ CTD and *Disp1*AAA interfere with the function of endogenous *Disp1*, consistent with the idea that its multimerization is required for normal *Disp1* function. The observation that *Disp1*AAA acts as a dominant-negative allele suggests that the proton channel is required for normal *Disp1* function. Therefore, *Disp1* is likely to function only in compartments where a transmembrane proton gradient exists, such as in early and late endosomes, trans-Golgi and multi vesicular bodies (MVBs). It seems unlikely that *Disp1* would function at the plasma membrane, however, as the transmembrane proton gradient is unlikely to be large enough.

Disp1 mediates the secretion of non-acylated Shh

Lipid modifications have a profound effect on the signaling range and efficacy of Shh, as measured by the differential responses to the mutants (Lee et al., 2001; Li et al., 2006). We visualized

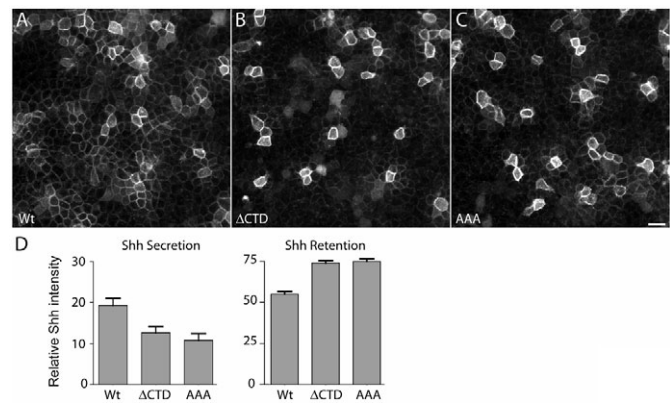


Fig. 3. *Disp1* mutants act as dominant-negatives. MDCK cells were grown on transwell filters and transfected with Shh and wild-type *Disp1* (A), *Disp1* Δ CTD (B) or *Disp1*AAA (C). The anti-Shh monoclonal antibody 5E1 was included overnight in the basolateral compartment and visualized. Both *Disp1* mutants caused a reduction of Shh secretion and an increase in Shh retention. (D) Quantification of Shh secretion by measuring staining intensity in a 1.5-cell diameter wide ring around the transfected cells, and of Shh retention by measuring staining intensity in transfected cells. The mutant *Disp1* co-transfected cells are significantly different from wild-type *Disp1*-transfected cells both for secretion and retention ($P < 0.001$). The y-axis indicates relative levels of Shh staining. Error bars are s.e.m. Scale bar: 20 μ m in A-C.

secreted Shh to assess the effect of the lipid modifications on the range of Shh trafficking and the formation of the Shh gradient. MDCK cells were transfected with Shh mutants lacking either the amino-terminal acylation (C25S), the cholesterol moiety on the carboxy terminus (C*) or both (C25S/C*). Fully lipid-modified Shh could be detected a few cell diameters away from the sites of synthesis (Fig. 4A). Loss of the amino-terminal acyl group resulted in a significant increase in the range over which Shh was distributed (Fig. 4C), although it still appeared to be distributed in a gradient in the extracellular space. By contrast, Shh lacking the cholesterol modification was barely detectable in the extracellular space and not distributed in an obvious gradient (Fig. 4B). A Shh mutant lacking both cholesterol and palmitate anchors was entirely undetectable in our assay (Fig. 4D). These results show that the cholesterol moiety is required for retention of Shh in the extracellular matrix, whereas the acyl modification results in a steeper Shh gradient. Shh without the palmitate anchor still requires *Disp1* function for secretion (Fig. 4E,F), indicating that *Disp1* function is required for the formation of a secreted form of Shh that is retained in the extracellular space. It has previously been shown that Shh without cholesterol is secreted in a *Disp1*-independent manner (Ma et al., 2002). Consistent with this, we observed co-segregation of *Disp1* and Shh, but not of *Disp1* and Shh without a cholesterol moiety, in cells where they are co-expressed (see Fig. S2 in the supplementary material). The results above demonstrate that *Disp1* acts on cholesterol-modified Shh and that Shh secreted in a *Disp1*-dependent manner is retained in the extracellular matrix. We have previously shown that the internalization of Shh is mediated by *Ptch1* (Incardona et al., 2002; Incardona et al., 2000), which itself is a putative RND transporter, leaving open the possibility that the long-range transport of Shh involves the activity of both *Disp1* and *Ptch1*.

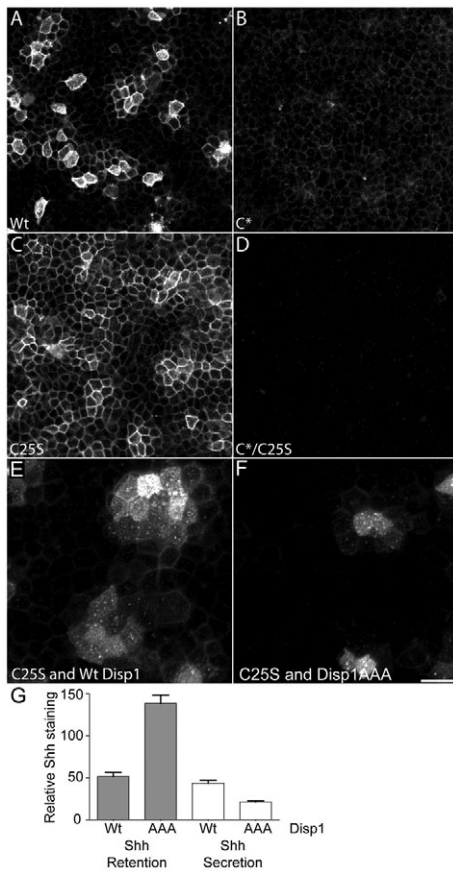


Fig. 4. Non-acylated Shh (C25S) is secreted in a Disp1-dependent manner. MDCK cells were grown on transwell filters and transfected with wild-type (Wt) Shh (A), Shh lacking cholesterol modification (C*) (B), Shh lacking amino terminal acyl modification (C25S) (C) and Shh with neither modification (C*/C25S) (D). The cells were grown overnight with 5E1 included in the basolateral compartment and visualized. The cholesterol moiety (present in A and C) mediates the retention of Shh outside the cells, whereas the loss of the acyl modification resulted in a shallow Shh gradient (C). Shh C25S was co-transfected either with Wt Disp1 (E) or Disp1AAA (F) into MDCK cells that were grown on transwell filters. Anti-Shh monoclonal 5E1 was included in the basolateral compartment for 16 hours and visualized. Disp1AAA caused significant inhibition of Shh secretion from, and a significant increase of Shh retention in, the transfected cells. (G) Quantification of secretion and retention of Shh. Error bars are s.e.m. Scale bar: 20 μm in A-D; 40 μm in E,F.

Disp1 and Ptch1: their roles in intercellular Shh transport

To assess the roles of Ptch1 and Disp1 in the distribution of Shh, we used co-cultures of genetically different cell lines producing and distributing Shh. We generated embryonic stem (ES) cell lines, either *Disp1*^{+/+} or *Disp1*^{-/-}, expressing Shh. Shh expression was not stably maintained in the *Disp1*^{-/-} cells, and Shh synthesized in *Disp1*^{-/-} cells consistently migrated more slowly on SDS PAGE gels, which suggests that Shh undergoes a Disp1-dependent modification (Fig. 5H). As expected based on previous reports, Shh could be detected in the concentrated supernatant of *Disp1*^{+/+} cells, but not in the supernatant of the *Disp1*^{-/-} cells (Ma et al., 2002) (Fig. 5H). By growing *Disp1*^{+/+} (Fig. 5A,C,E), and *Disp1*^{-/-} (Fig. 5B,D,F) Shh cells surrounded by wild-type cells (Fig. 5A,B), *Disp1*^{-/-} cells

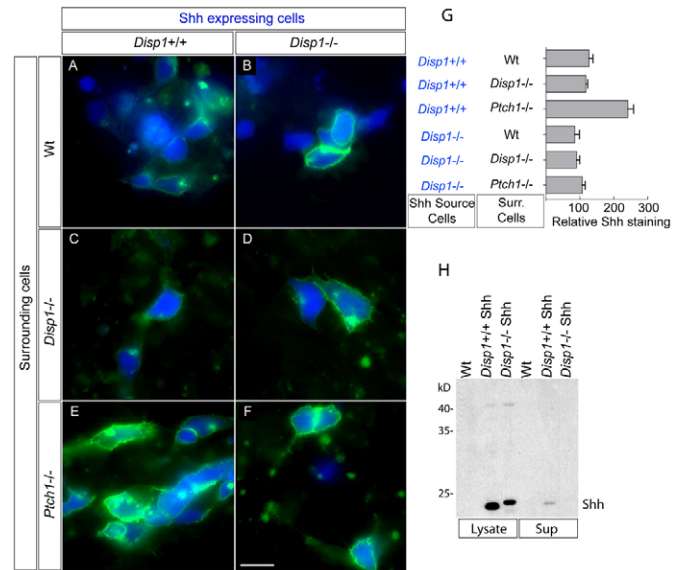


Fig. 5. Ptch1 on neighboring cells mediates the transport of Shh secreted in a Disp1-dependent manner. (A-F) Shh-expressing wild-type (Wt) ES cells (blue, A,C,E) and Shh-expressing *Disp1*^{-/-} cells (blue, B,D,F) were co-cultured with Wt (A,B), *Disp1*^{-/-} (C,D) and *Ptch1*^{-/-} cells (E,F). Shh was visualized using directly conjugated 5E1 included in the live cultures for 1 hour, followed by fixation. When Ptch1 was absent from the surrounding cells more Shh could be detected on cells containing Disp1 (E), whereas this had no effect on the Shh present on cells without Disp1 (F). (G) Quantification of the amount of Shh present on cells cultured under the indicated conditions. Error bars indicate s.e.m. Only the amount of Shh present on Wt cells surrounded by *Ptch1*^{-/-} cells was significantly different from all others. (H) Analysis of the Wt and *Disp1*^{-/-} Shh-expressing ES cells. Shh was easily detected in lysates, but only a small amount of Shh was detected in the concentrated supernatant from the Wt cells, and not at all in the *Disp1*^{-/-} cells. Scale bar: 20 μm.

(Fig. 5C,D) or *Ptch1*^{-/-} cells (Fig. 5E,F), we assessed the role of these proteins in the distribution of Shh away from its source. Visualization of Shh was performed by the addition of 5E1 to the live cultures for 1 hour followed by fixation and microscopy. Shh-expressing cells were labeled with a cell tracker (Fig. 5, blue). As this procedure was performed in the complete absence of detergent, only extracellular Shh was detectable.

Little Shh could be detected in co-cultures of *Disp1*^{+/+} Shh-expressing cells surrounded by *Ptch1*^{+/+} cells (Fig. 5A,C), but significantly more was found when Shh-expressing cells were surrounded by cells lacking Ptch1 (Fig. 5E), indicating that Ptch1 on surrounding cells has an active role in removing Shh from the sites where it is synthesized. Shh was still detected on the cell surface of *Disp1*^{-/-} cells (Fig. 5B,D,F) but the presence or absence of Ptch1 on the surrounding cells had no obvious effect on the accumulation of Shh at the surface of the Shh-expressing *Disp1*^{-/-} cells. This suggests that most of the Shh that reaches the cell surface in the absence of Disp1 cannot be bound efficiently by Ptch1 expressed by adjacent cells (Fig. 5G). Together, these results indicate that Ptch1 on adjacent cells preferentially binds Shh that is secreted in a Disp1-dependent manner, indicating that both Ptch1 and Disp1 play a role in the trafficking of Shh away from the sites of synthesis. However, whereas Disp1 is required in the cells synthesizing Shh (Fig. 2B), Ptch1 is necessary on the neighboring cells (that do not express Shh)

to bind Shh and presumably move it away. An implication of these findings is that the cells surrounding Shh-producing cells affect the distribution of Shh away from the site of synthesis, and that Shh does not diffuse freely, even after secretion mediated by Disp1.

Although these observations suggest a model for the movement of Shh from the source cell to the adjacent cells, it remains unclear how a long-range signal is established. As Shh is distributed several cell diameters away from its source, and because Disp1 is ubiquitously expressed, we next tested whether Disp1 is also required in subsequent events, mediating re-secretion of Shh to cells farther away from the source.

Long-range Shh transport is impaired in *Disp1*^{-/-} cells

To test whether Disp1 function is required outside of Shh-expressing cells for long-range signaling, we measured the range of Shh signaling in embryoid bodies (EBs) derived from ES cells with or without Disp1 function. To exclude the possibility that the loss of Disp1 cause changes in the responsiveness to Shh, we exposed neuralized EBs derived from *Disp1*^{+/-} and *Disp1*^{-/-} cells to various concentrations of soluble Shh N-terminus (ShhN) added to the culture medium. In both cases, the dose-dependent response to ShhN was identical, with repression of Pax7 at 2 nM ShhN and induction of Nkx2.2 at 4 nM ShhN. This demonstrates that there is no inherent defect in the Shh response in *Disp1*^{-/-} cells (see Fig. S3 in the supplementary material).

To test whether Disp1 is required for contact-dependent long-range signaling, wild-type EBs expressing Shh were co-cultured for 48 hours with EBs with or without Disp1 function. Like neural plate explants, neuralized EBs widely express Pax7 (Fig. 6A,B). Repression of Pax7 is one of the most sensitive responses to Shh and immunostaining with Pax7 antibody was conducted to assess the range of Shh signaling. In cases where Shh-expressing EBs contacted *Disp1*^{+/-} EBs, Pax7 staining was significantly reduced throughout the entire EB, on average $174.3 \pm 7.6 \mu\text{m}$ (Fig. 6D,F). By contrast, when *Disp1*^{-/-} EBs are grown in contact with Shh-expressing EBs, the range of Pax7 repression was much shorter, $72.2 \pm 3.3 \mu\text{m}$, and was observed only in cells nearest to the Shh source (Fig. 6E,G).

Consistent with this observation, we found that wild-type Shh-expressing EBs could induce the expression of the motorneuron-specific protein HB9 at an average distance of $38.3 \pm 3.7 \mu\text{m}$ from the source in *Disp1*^{+/-} EBs (Fig. 6H,J), whereas the range of induction EBs lacking Disp1 was $18.2 \pm 2.1 \mu\text{m}$ (Fig. 6I,K). All differences were highly significant ($P < 0.0001$, *t*-test). We obtained similar results using stage 10 chicken notochords as the localized Shh source (see Fig. S4 in the supplementary material).

Although the *Disp1*^{-/-} cells are normally responsive to Shh, they fail to efficiently distribute Shh away from its sites of synthesis, supporting the idea that Disp1 has an important function in both the cells expressing Shh and in the cells involved in the transport of Shh. In addition, Ptch1 plays an equally important role in the distribution of Shh. However, as the *Ptch1*^{-/-} cells have a constitutively activated Shh response, we cannot test the signaling range of Shh in *Ptch1*^{-/-} tissue.

DISCUSSION

Our results suggest that Ptch1 and Disp1 act in concert to mediate the transport of Shh through tissues. The similarities between Ptch1 and Disp1, such as their ability to trimerize (Lu et al., 2006) and their putative proton channel (Ma et al., 2002), indicates that their function might be conserved with that of the RND transporters in

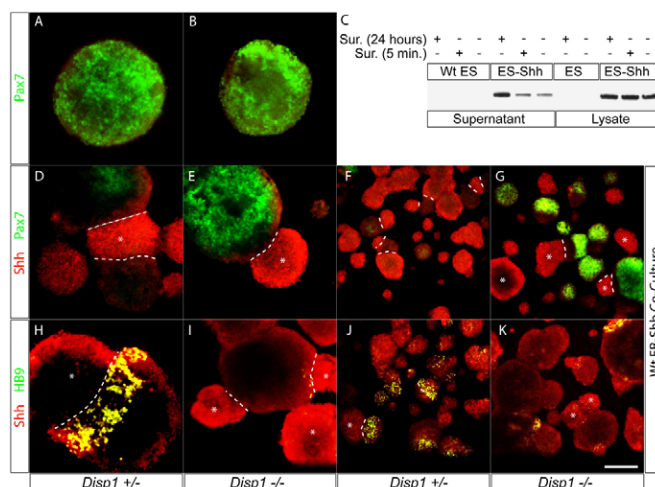


Fig. 6. Long-range Shh signaling is reduced in *Disp1*^{-/-} EBs. To test whether Disp1 is required outside of Shh-expressing cells for a normal long-range response to cholesterol-modified Shh, wild-type (Wt) EBs expressing Shh were co-cultured for 36 or 48 hours with EBs with or without Disp1 function. Repression of Pax7 was used to measure the range of the Shh response. (A,B) In the absence of a Shh source, Pax7 is expressed throughout both *Disp1*^{+/-} and *Disp1*^{-/-} EBs. (C) The level of Shh present in the lysates and supernatants of Shh AB1 cells. Shh accumulated more efficiently in the medium when suramin (Sur) was present. (D,F) When Shh AB1 EBs were co-cultured with *Disp1*^{+/-} EBs, Pax7 was significantly reduced throughout the entire EB. (E,G) In *Disp1*^{-/-} EBs grown in contact with Shh-expressing EBs, Pax7 was only repressed in cells nearest to the Shh source. F and G are low magnification images of the cultures shown in D and E. Induction of HB9 was used to measure the range of Shh response. (H,J) In cases where Shh-expressing EBs contacted *Disp1*^{+/-} EBs, HB9 was induced close to the Shh source. (I,K) In *Disp1*^{-/-} EBs grown in contact with Shh-expressing EBs, very few HB9 positive cells were observed. J and K are lower magnification images of the cultures shown in H and I. As Disp1 is not required for the Shh response per se, these results suggest that Disp1 function is important for transmission of the Shh ligand through the responding tissue. Dashed lines, borders between EBs; asterisks, Shh-expressing EBs. Scale bars: 75 μm in A,B,D,E,H,I; 150 μm in F,G,J,K.

bacteria. In general, the role of Disp1 is in the secretion of Shh, whereas Ptch1 is involved in the uptake of Shh, and the function of both is necessary for long-range Shh signaling. These observations are consistent with a model in which reiterated secretion (by Disp1) and uptake (by Ptch1) are involved in the long-range transport of Shh. The non-directionality of this process, combined with the incomplete secretion of all internalized Shh, would sufficiently distribute Shh in a gradient away from the source.

The ability of 5E1 antibody to recognize Shh on and around live cells has been crucial in our experiments. This method of staining is more sensitive than 5E1 antibody application after fixation and permeabilization. This suggests that either fixation obscures many epitopes, or that the 5E1/Shh complex is more stable than Shh alone, intracellularly or not. Currently, we cannot discriminate between these options, but nevertheless, the distribution of 5E1 in some significant way reflects the range over which Shh is distributed.

All models of how Shh is distributed are currently complicated by the poor understanding of the form of Shh that moves from cell to cell. Biochemical analysis of the Shh in the supernatant of transiently transfected HEK 293T cells reveals that Shh is in a relatively large (~400-1000 kD) complex (Chen et al., 2004; Zeng

et al., 2001) (data not shown). However, the form of Shh that is secreted by Disp1 and internalized in a Ptch1-dependent manner is still unknown. The amount of Shh released by stably expressing ES cells is very small and currently not amenable to further biochemical analysis. Although addition of the polysulfated compound suramin has previously been shown to increase the amount of Shh that accumulates in the supernatant (Yang et al., 1997), we find that this release is Disp1-independent and thus unlikely to represent the normal form of transported Shh (data not shown). Together, our observation that Shh-expressing ES cells release only very small amounts of Shh, combined with our observation that Ptch1 on adjacent cells is required for the transport away from the source, supports the idea that free diffusion of Shh is not responsible for the widespread distribution of Shh, but that in fact Disp1 and Ptch1 are participants in an active transport mechanism where Shh is passed from one cell to the next, mediating the distribution of Shh.

Basolateral signaling and the primary cilium

Signaling from the notochord to the basolateral side of the overlying neural plate, combined with our observation that the Disp1-mediated export of Shh is from the basolateral side of epithelial cells, supports the idea that both the reception and transport of Shh in epithelia occurs basolaterally. Consistent with this finding are observations that in the *Drosophila* wing disc, lipid-modified Hh is found almost exclusively on the basolateral side of cells in the anterior compartment (Callejo et al., 2006). However, in *Drosophila*, conflicting evidence exists about the effect of Disp on the apicobasal distribution of Hh. Although it was originally reported that loss of Disp had no effect on the localization of Hh within Hh-expressing cells (Burke et al., 1999), a later report found that apically localized large puncta of Hh are lost in the absence of Disp function or with cholesterol modification (Gallet et al., 2003). In support of the latter finding, the putative *C. elegans* Disp ortholog, CHE-14, is apically localized and is required for apical secretion from epithelial cells (Michaux et al., 2000). Nevertheless, in MDCK cells it appears that apical Shh localization is independent of Disp1 function, and perhaps more importantly, we show here that the basolateral localization of Disp1 requires sequences present in its carboxy terminal domain, demonstrating that the polarized Disp1 function is a regulated process.

The apically localized primary cilium, is thought to have a crucial role in the response to Shh as many components of the Shh response accumulate in it (Corbit et al., 2005; Rohatgi et al., 2007). Our observation that Shh can traffic to all cell surfaces in a Disp1-independent manner leaves open the possibility that Shh, after internalization at the basolateral side, moves to the apical surface and initiates the Shh response at the primary cilium. This is consistent with our observation that Shh expressing *Disp1*^{-/-} ES cells can transport Shh to the cell surface and that these cells have a significant autocrine Shh response. Importantly, this mechanism would effectively separate the mechanism by which Shh is transported from cell to cell by Disp1 and Ptch1, along the basolateral side of epithelia, from the Shh response at the apical primary cilium. It is possible that a similar phenomenon is present in *Drosophila*, where, despite the absence of primary cilia, basolaterally transported Hh activates the Shh response at the apical aspect.

Our observation that Ptch1 on adjacent cells is involved in transporting Shh away from the sites of synthesis also indicates that contact is required for the intercellular transport of Shh. This makes it probable that Shh transport is along the basolateral side of an epithelium as here epithelial cells are in close approximation. The commonly observed induction of Ptch1 expression in response to

Shh (Marigo and Tabin, 1996) might not only serve as a negative-feedback loop to control the Shh response, but equally important, might also enhance the long-range transport of Shh.

Disp1 and Ptch1 as members of the RND family

It is probable that Disp1 and Ptch1 are bona fide members of the RND family of proton-driven transporters. RND permeases export a variety of substrates from the bacterial inner membrane, including heavy metal ions, antibiotics and detergents (Poole, 2002; Tseng et al., 1999). Ptch1 and Disp1 not only share the characteristic topology with other RND members, but also the aspartic acid residues in transmembrane 4, which are essential for proton translocation across the membrane (Goldberg et al., 1999). This conservation strongly suggests that Ptch1 and Disp1 are proton-driven transporters. Members of the RND family are known to function as trimers (Middlemiss and Poole, 2004; Murakami, 2008; Murakami and Yamaguchi, 2003). Here, we find that like other RND permeases, Disp1 also trimerizes and that this is likely to be crucial to its normal functioning.

Prokaryotic RND family members can export cargo from the outer leaflet of the inner membrane to the extracellular space, similar to the proposed role of Disp1 in the release of membrane-tethered Shh. It is therefore probable that the Disp1 trimer releases membrane-bound Shh using the energy of a proton gradient. This implies that Disp1 functions in an acidic compartment. In the secretory pathway, trans-Golgi vesicles have a pH of roughly 6.0 (Demaurex et al., 1998; Seksek et al., 1995), which would be sufficient to power Disp1. However, when secreted, the lipid modifications of Shh dictate that it must be present in multimers or some membrane-associated form, such as exosomes or lipoprotein particles (Neumann et al., 2007; Panakova et al., 2005), and intriguingly, trafficking of lipoprotein particles involves multivesicular bodies (MVBs). MVBs are specialized endosomes that transit from early/sorting endosomes to late endosomes, where their exosomal cargo is either secreted or sent to the lysosome for degradation. Newly synthesized proteins are also targeted to MVBs (Fomina et al., 2003; Lakkaraju and Rodriguez-Boulant, 2008), allowing the integration of both recycled and newly synthesized proteins into exosomes. The production of exosomes depends on a low pH in MVBs (Iero et al., 2008) and is compatible with the activity of both Ptch1 and Disp1.

Based on these results and previously reported findings, we propose the following model. Disp1 is active in MVBs and mediates the loading of Shh onto exosome/lipoprotein-like particles, which are then secreted. These particles are specifically recognized by Ptch1 at the surface of adjacent cells, which traffics them into early/late endosomes (Incardona et al., 2002; Incardona et al., 2000), where the particles are disassembled. Shh can either be degraded, trafficked to the apical surface or trafficked into MVBs, where it would be loaded onto exosomes again for re-secretion. This model accounts for the putative function of Disp1 as a proton-driven transporter and explains the high molecular weight complex that Shh is found in outside of cells, the pH-dependent action of Disp1 and Ptch1 in the intracellular trafficking of Shh and the role of Disp1 in the re-secretion of Shh.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.043547/-/DC1>

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