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Hedgehog-Mediated Patterning of the Mammalian Embryo Requires Transporter-like Function of Dispatched

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Summary

The dispatched (disp) gene is required for long-range Hedgehog (Hh) signaling in Drosophila. Here, we demonstrate that one of two murine homologs, mDispA, can rescue disp function in Drosophila and is essential for all Hh patterning activities examined in the early mouse embryo. Fibroblasts lacking mDispA respond normally to exogenously provided Sonic hedgehog (Shh) signal, but are impaired in stimulation of other responding cells when expressing Shh. We have developed a biochemical assay that directly measures the activity of Disp proteins in release of soluble Hh proteins. This activity is disrupted by alteration of residues functionally conserved in Patched and in a related family of bacterial transmembrane transporters, thus suggesting similar mechanisms of action for all of these proteins.

Introduction

The pattern of cellular proliferation and differentiation that leads to normal development of embryonic structures often depends upon the localized production of secreted protein signals. Cells surrounding the source of a particular signal respond in a graded manner according to the effective concentration of the signal, and this response produces the pattern of cell types constituting the mature structure (Jessell, 2000; Gurdon and Bourillot, 2001). The importance in embryonic patterning of concentration-dependent cellular responses highlights in turn the importance of mechanisms that influence the effective concentration of the signaling protein. Such mechanisms include the interaction of a signaling protein with extracellular matrix, the presence and action of other interacting secreted proteins, and the sequestration and possible release of the signal within the field of responding cells (Dudley and Tabin, 2000; Teleman et al., 2001).

Among secreted signaling proteins, the Hedgehog (Hh) family (Ingham and McMahon, 2001) is unique in that the mature active form of the信号 (HhNp) is dually lipid modified, with an ester-linked carboxy-terminal cholesterol moiety (Porter et al., 1996b) and an amide-linked amino-terminal palmitate (Pepinsky et al., 1998). Cholesterol addition results from an autoprocessing reaction undergone by the Hedgehog protein precursor (Lee et al., 1994; Porter et al., 1996b), and palmitoylation, which is critical for signaling activity of the Hh protein, requires action of the Skinny hedgehog (Sk) acyltransferase (Chamoun et al., 2001; Lee and Treisman, 2001). Efficient addition of palmitate depends upon prior cholesterol addition, and truncated Hh proteins that are not cholesterol modified show poor activity in embryos (Lewis et al., 2001) unless expressed at high levels (Porter et al., 1996a).

Despite dual lipid modification, which might be expected to firmly anchor HhNp to the membranes of producing cells, Hh signaling extends beyond immediately adjacent cells. In the wing imaginal disc of Drosophila, for example, Hh production in the posterior compartment directly induces expression of the target genes patched (ptc) and decapentaplegic (dpp), in a band extending 5–10 cells beyond the boundary of Hh protein expression (Hidalgo and Ingham, 1990; Basler and Struhl, 1994; Capdevila and Guerrero, 1994). The range of direct Hh signaling is even greater in vertebrate embryos, where the mature form of the Sonic hedgehog protein (ShhNp), also dually lipidated, extends its influence many cells beyond its source to globally influence development throughout a tissue or structure. In the developing neural tube, for example, direct responses to Sonic hedgehog (Shh) are required for induction of diverse neuronal types throughout the ventral half of the neural tube (Roelink et al., 1995; Jessell, 2000; Briscoe et al., 2001), despite production of Shh protein exclusively in the notochord and floor plate (Roelink et al., 1995). The influence of this ventral midline source of Shh signal also extends through the paraxial mesoderm as far as the dorsal somite, where Shh directly induces Myf5 expression in the myogenic precursors of the epaxial musculature (Borycki et al., 1999; Gustafsson et al., 2002). In addition, a long-range effect of direct Shh signaling is evident in the developing limb, where Shh expression at the posterior margin induces gradients of Ptc transcription and of Gli3 transcription and proteolytic processing across the limb bud, encompassing many cell diameters (Goodrich et al., 1996; Marigò et al., 1996b; Wang et al., 2000).

The long-range action of Shh in these tissues raises the question of how a dually lipidated protein signal can escape the membranes of cells in which it is produced to directly stimulate pathway activity in distant target cells. One possible clue to this puzzle derives from the identification of the Drosophila gene dispatched (disp) (Burke et al., 1999), which has a mutant phenotype similar to that of hh and encodes a protein that is similar in sequence and transmembrane topology to the Ptc component of the Hh receptor (Nakano et al., 1989; Hooper and Scott, 1989; Marigò et al., 1996a; Goodrich et al., 1996; Stone et al., 1996; Fuse et al., 1999). Unlike Ptc, however, mutant mosaic studies indicate a requirement for Disp function exclusively in Hh-producing cells,
Figure 1. Characterization of Murine Dispatched Homologs

(A) Sequence alignment between mDispA, mDispB, and Drosophila Disp. Identical residues are in yellow background. The twelve transmembrane spans are underlined, and the GxxxD motifs within TM4 and TM10 are in red rectangles. The intron/exon junctions are indicated by red vertical lines. Blue brackets delimit the sequences used in determining the percentages of sequence identities (see text).

(B) Proposed topology of Disp proteins. Note that the structure probably arose by tandem duplication of a six transmembrane unit. Red letters indicate the three aspartate residues within the GxxxD(D) motifs in TM4 and TM10.

(C) Generation of mDispA null allele. Homologous recombination of the wild-type allele with the targeting vector results in a mutant mDispA allele lacking a large exon coding for 11 of the 12 transmembrane domains (blue vertical lines). The seven coding exons are represented by white rectangles, and green and red vertical lines inside exons 1 and 7 indicate the start and stop codons, respectively. Thymidine kinase (tk, green rectangle) and LoxP (red box) -flanked Neo (gray box) selection cassettes are also shown. Introns of known and unknown lengths are represented by solid and dashed lines, respectively.

with loss of Disp function resulting in accumulation of the Hh signal in the producing cells and restriction of target gene expression to those cells immediately adjacent to Hh-producing cells (Burke et al., 1999). These results suggest that Disp acts by some mechanism to present or release the Hh signal for stimulation of distant target cells.

To investigate the role and mechanism of Disp protein action in mammalian Hh signaling, we characterized two mammalian murine disp homologs. We demonstrate that one of these, mDispA, is required for all detectable manifestations of Hh signaling in the patterning of the early mouse embryo. We also demonstrate in embryonic fibroblasts that a normal response to the ShhNp signal does not require mDispA function, and further show that Shh processing and modification in mDispA mutant cells are normal. In addition, in a direct biochemical assay of Hh export by Disp, the levels of a soluble form of Hh protein released into the medium are increased many fold upon coexpression of mammalian or Drosophila Disp proteins. This activity is disrupted by alteration of residues functionally conserved in Patched and in a related family.
Figure 2. Early Embryonic Expression and Functional Conservation of mDispA, but not mDispB (A–C) mDispA is nearly ubiquitously expressed during early embryonic development, as indicated by whole-mount in situ hybridization of wild-type embryos with antisense mDispA probe at E8 (A), E8.75 (B), and E9.5 (C). (D) shows the gradient of mDispA message level across the hindlimb bud of E10.25 wild-type embryo (anterior is up). (E–I) The disp mutation in Drosophila can be rescued by mDispA, but not by mDispB. (E) Diagram of a wild-type third instar Drosophila wing imaginal disc. Endogenous Hh is expressed in the posterior (P) compartment, inducing a 5–10 cell wide band of anterior (A) compartment cells to express the ptc-lacZ reporter gene (red). Blue color indicates the pattern of en-GAL4 driven ectopic gene expression. The yellow box demarcates the region analyzed in (F–I). (F) shows the wide band of ptc-lacZ expression in wild-type wing disc. In disp wing discs (G), the ptc-lacZ expression is limited to a 1 or 2 cell wide region adjacent to the A-P boundary. This defect can be fully rescued by expression of mDispA (H) but not mDispB (I). disp mutants die at pupal stage (Pupal lethal), and this lethality cannot be rescued by mDispB expression. In contrast, mDispA-expressing disp flies develop to adulthood with normal wing pattern (compare J and K).

Results and Discussion

Identification of Two Murine dispatched Homologs

Using ESTs identified in database searches and by cDNA cloning, we characterized coding sequences for two murine homologs of disp, mDispA, and mDispB (Figure 1A). Based on the experimentally verified topology of the homologous protein, NPC1 (Davies and Ioannou, 2000), we propose a membrane topology for Disp that includes twelve transmembrane spans, with cytoplasmic N- and C-terminal tails (Figure 1B). The predicted amino acid sequences of Disp, mDispA, and mDispB are easily aligned, with greatest similarity noted within a continuous region that includes and extends just beyond the twelve predicted transmembrane spans (TM region; Figure 1A); little similarity occurs outside this region. Overall, the murine proteins, particularly mDispA, have larger N- and C-terminal cytoplasmic domains, whereas the Drosophila protein has relatively larger loops, particularly the extracellular loops between TM1 and TM2, and between TM7 and TM8.

Within the membrane-spanning region, mDispA and mDispB, respectively, display sequence identities with Disp of 36% and 31%, discounting the gaps, whereas sequence identity in this region is 42% between mDispA and mDispB. These relationships suggest that the two murine homologs duplicated after divergence of the insect and mammalian lineages. Consistent with this scenario, the coding sequences of mDispA and mDispB are identically distributed among seven exons, whereas Drosophila Disp coding sequences are differently distributed with only a single intron/exon junction at a point homologous to a junction in the mDisp genes (see Figure 1A).

Early Embryonic Expression and Functional Conservation of mDispA, but not mDispB

Given their probable duplication from a single ancestral gene, we sought to distinguish mDispA and mDispB functionally on the basis of their embryonic expression and their ability to complement disp mutations in Drosophila. By in situ hybridization, we found that the mDispA message is detected throughout the embryo at 7.5 days of gestation (E7.5; data not shown). This nearly ubiquitous expression of mDispA is maintained throughout all stages examined, albeit with some tissue-specific variations in level. Thus, for example, the level of expression at E8 is higher in the somites than in the rest of the embryo (Figure 2A), and a higher relative level of expression is observed in the branchial arches at E8.75
Figure 3. The Laterality Defects of mDispA\(^{-}\) Embryos

(A–C) Overall morphological defects of E9.5 mDispA\(^{-}\) (A), Smo\(^{-}\) (B), and Shh\(^{-}\) (C) embryos compared to wild-type embryos in the same litters. Note the similar phenotypes of mDispA\(^{-}\) and Smo\(^{-}\) embryos. (D and E) Scanning electron micrographs of embryo hearts at E9.5, rostral is to the top. Compared to the normal rightward heart looping of a wild-type embryo (D), heart looping of a mDispA\(^{-}\) embryo (E) is almost completely lost. (F) Whole-mount in situ hybridization for Nodal expression at 5-6 somite stage. Whereas residual Nodal expression is detected in the node of mDispA\(^{-}\) embryo (arrowheads), the normal Nodal expression in the left lateral plate mesoderm (arrow) is completely lost in the mDispA\(^{-}\) embryo.

(Nothing to be added to the document)
Figure 4. The Forebrain and Branchial Arch Defects of Shh−/−, Smo−/−, and mDispA−/− Embryos Examined by Scanning Electron Microscopy

(A–D) Frontal views of E8.25 embryo cephalic region.
(E–H) Frontal views of E8.5 embryo cephalic region.
(I–L) Lateral views of E9.5 embryo cephalic region.
(A, E, and I) Wild-type embryos.
(B, F, and J) Shh−/− embryos.
(D, H, and L) mDispA−/− embryos. The midline defects of E8.25 and E8.5 forebrain are marked by arrowheads and the branchial arch defects at E9.5 are marked by arrows.

alent to those observed in embryos doubly mutant for Shh and Indian hedgehog (Ihh; Zhang et al., 2001). These defects include a failure to establish normal asymmetry along the left/right axis, resulting in a failure of normal embryonic turning and abnormal looping of the embryonic heart; the heart defects are probably responsible for the inflated pericardial sac and early embryonic death.

We found that left/right asymmetry in mDispA−/− embryos indeed is disrupted, as indicated by defective heart looping (compare Figures 3D and 3E). At the molecular level left/right asymmetry defects are apparent even earlier, as the normal asymmetric expression of Nodal in left lateral plate mesoderm (Collignon et al., 1996; Lowe et al., 1996) was disrupted in 4–8 somite stage embryos, with no expression detected in seven mutant embryos examined (Figure 3F). As noted for Smo−/− embryos (Zhang et al., 2001), expression in the node of these same embryos was variable, with three embryos displaying little expression (not shown), three displaying a somewhat higher level of expression on the left side (Figure 3F) as is characteristic of normal embryos, and one displaying a similar level of expression on right and left sides (not shown).

Early Forebrain and Branchial Arch Defects in mDispA Mutants

In addition to defects in left/right asymmetry, mDispA−/− embryos also display defects of the prospective head and face that are characteristic of loss of Hh signaling (Chiang et al., 1996). Thus, by the early head fold stage (E8.25; Figures 4A–4D), Shh−/−, Smo−/−, and mDispA−/− embryos all display common midline defects of the neural plate in the region of the prospective forebrain. These defects include midline fusion of the anterior lips of the heart looping (compare Figures 3D and 3E). At the mo-

node of these same embryos was variable, with three the cyclopic eye in later Shh−/− embryos, and presumably also would do so in Smo−/− and mDispA−/− embryos if their heart defects did not preclude further embryonic development.

Defects of the branchial arches are also evident in mDispA−/− embryos. In particular, the mandibular com-
Figure 5. The Neural Tube and Somite Defects of mDispA<sup>+/−</sup> Mutants

(A and B) Scanning electron micrographs of the neural tubes of E9.5 embryos. The clearly defined morphology of the floor plate in wild-type embryo (A, arrowhead) is lost in mDispA<sup>+/−</sup> mutant (B).

(C–P) Loss of long-range Hh signaling in mDispA<sup>+/−</sup> embryos.

(C) In E9.5 mDispA<sup>+/−</sup> embryos, weak expression of a Hh pathway sensitive reporter (Ptch-LacZ) is retained in tissues that express Hh, such as the notochord (arrowhead) and gut. However, LacZ staining is completely lost in tissues that are farther from Hh sources, such as ventral neural tube and somites (arrow in wild-type embryo).

(D) Pax7 expression in E9.25 embryos.

(E and F) The crosssections marked in (D) showing the ventral expansion of Pax7 expression in both neural tube (arrow) and somites (arrowhead) in mutant embryo (F) compared to wild-type (E).

(G and H) HNF3β expression at E8.25 (G) and E9.25 (H). Whereas gut and weak notochord (arrowhead) expression of HNF3β persists at both stages, ventral neural tube expression (arrow in wild-type embryo) is not detected in mDispA<sup>+/−</sup> embryos.
ponent of the first branchial arch is severely reduced and fused in the midline (Figure 4L, compare to Figure 4J). As seen for left/right axis defects, the branchial arch defects in mDispA−/− embryos are similar to those found in Smo−/− embryos (Figure 4K), and both are more severe than the defects in Shh−/− embryos (Figure 4J), suggesting that normal development of the branchial arches may also involve Hh signaling activity other than that provided by Shh.

**DISPA Is Located within HPE10, a Human Holoprosencephaly Locus**

As in Shh−/− mutants, the cephalic defects in mDispA−/− embryos are reminiscent of holoprosencephaly (HPE) in humans. Holoprosencephaly encompasses a spectrum of brain and facial midline deficits that in extreme cases involve cyclopia and development of the forebrain or prosencephalon as a single undivided vesicle (Muenke and Beachy, 2001). Although heterozygous mutations in the murine Shh gene are silent, human SHH gene function is haploinsufficient and ~5% of HPE, generally at the milder end of the spectrum, can be accounted for by heterozygous SHH mutations (Roessler et al., 1996; Muenke and Beachy, 2001). Indeed, the DISPA gene is located in chromosomal region 1q42 (data not shown), which corresponds to the chromosomal interval 1q42-ter associated with HPE10 (Muenke and Beachy, 2001). It is conceivable that, despite the apparent absence of a heterozygous phenotype in the mouse, haploinsufficiency of the human DISPA gene could account for apparent dominant effects of chromosomal abnormalities associated with HPE10 (Muenke and Beachy, 2001).

**Neural Tube Patterning Defects in mDispA−/− Mutants**

Embryos lacking mDispA function also display patterning defects at more caudal levels. The ventral neural tube at the level of the presumptive spinal cord, for example, fails to take on its characteristic shape with well-differentiated floor plate cells at a sharply defined midline, and instead develops as an undifferentiated epithelial tube (compare Figures 5A and 5B). To further characterize the effects of mDispA loss on patterning of the neural tube and somites, we examined various genes known to depend on Hh signaling for their normal expression, and which in some cases serve as markers for the development of particular cell types.

Perhaps the most universal indicator of Hh signaling activity is the expression of Patched (Ptc) (Hidalgo and Ingham, 1990; Goodrich et al., 1996; Marigo et al., 1996), which encodes a component of the Hh receptor and is induced by Hh signaling. We employed the murine Ptch-lacZ allele (Goodrich et al., 1997) to monitor pathway activity, as fusion of the E. coli lacZ gene in frame to the third codon of Ptch coding sequences provides a sensitive report of Ptch expression through histochemical staining for β-galactosidase activity. The normal expression of β-galactosidase from Ptch-lacZ in the ventral neural tube and somites is completely lost in the mDispA mutant background (Figure 5C), although continued expression in the notochord and portions of the gut suggests that some response to Hh signaling is maintained in tissues that themselves express Hh proteins (see below). We note that heterozygosity for the Ptch-lacZ mutant allele did not noticeably affect the abnormal morphology of mDispA mutant embryos. As an additional sensitive indicator of Hh protein influence, we examined expression of Pax7, normally suppressed in the ventral neural tube by Shh that is produced in the notochord and floor plate (Fan and Tessier-Lavigne, 1994; Ericson et al., 1996). In mDispA−/− mutants, we find that the normal dorsally restricted domain of Pax7 expression extends throughout the neural tube, including the ventral midline, suggestive of a loss of Shh signaling (Figures 5D–5F).

As markers of ventral midline structures, the Shh and HNF3-β genes normally are expressed independently of Hh signaling in the early gut and notochord and are later induced in the floor plate by Shh signaling from the notochord. In mDispA−/− embryos, normal initiation of Shh and HNF3-β expression in the notochord and gut is observed (Figures 5G–5I), but expression does not occur in the neural tube. Later expression of these markers indicates that the notochord begins to degenerate in a discontinuous fashion, much like the degeneration observed in Shh mutants (Chiang et al., 1996). Sections through different levels of the trunk in these embryos (Figures 5J–5L) indeed demonstrate that the notochord is absent in regions lacking Shh expression. Furthermore, these sections clearly demonstrate that no expression of Shh occurs in the neural tube, even at levels of the trunk where notochord is present and is expressing Shh RNA. Thus, in the mDispA−/− mutant background, expression of Shh in the notochord appears to be incapable of inducing normal Shh responses in the adjacent neural tube.

**Somite Patterning Defects in mDispA−/− Mutants**

Shh signaling normally functions to induce sclerotome, marked by expression of Pax1 in the ventral somite (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994; Fan
et al., 1995). Furthermore, Shh also suppresses Pax7 expression in the ventral somite (Fan and Tessier-Lavigne, 1994), restricting it to the dorsal dermomyotome. In addition, Shh signaling contributes to dorsal somite expression of Myf5 in the myotomal precursors of epaxial muscles (Borycki et al., 1999), and this represents one of the longest range effects that can be assigned directly to Shh signaling (Gustafsson et al., 2002).

All three of these activities are disrupted in mDispA^-/- embryos. Thus, sclerotomal Pax7 expression is lost (although Hh-independent expression in the pharyngeal pouches is maintained; see Figure 5M), Pax7 expression expands ventrally throughout the entire paraxial mesoderm (Figures 5D–5F), and the epaxial domain of Myf5 expression in the dorsal somite is lost (Figures 5N–5P), even though a more ventral domain of Hh-independent expression of Myf5 is maintained.

Response of mDispA^-/- Cells to Hh Signaling

Although most aspects of Hh signal response appear to be disrupted in mDispA^-/- embryos, some expression of the Ptch-lacZ reporter is retained in the notochord and in the gut at E9.25 (Figure 5C); this expression is even clearer at E8.5, before the onset of notochord degeneration (Figure 6A). The retention of Ptch-lacZ expression in mDispA^-/- embryos would appear to represent a genuine response to Shh signaling, albeit restricted to cells that themselves express Shh, as this response is absent in Smo^-/- embryos (Zhang et al., 2001). Furthermore, homozygosity for the Ptch-lacZ mutant allele in a Drosophila mutant background produced the abnormal morphology and widespread β-galactosidase expression characteristic of homozygous Ptch-lacZ mutant embryos (data not shown), indicating that mDispA functions upstream of Ptc and of signal reception, possibly in signal production. To further examine the role of mDispA function in Hh signaling, fibroblastic cell lines isolated from E8.5 mDispA^-/- embryos were tested for response to exogenously added ShhNp. As monitored by Gli-luc, a luciferase reporter with tandem Gli binding sites (Sasaki et al., 1997; Taipale et al., 2000), heterozygous and homozygous mutant cells were both responsive to Shh signaling (Figure 6B), and this response was abrogated by the specific Hh pathway antagonist, cyclopamine (Cooper et al., 1998; Incardona et al., 1998; Taipale et al., 2000).

An intact response to Shh signaling in mDispA^-/- cells indicates that the defect in embryonic patterning could be due to inappropriate cleavage and lipidation of the Shh protein or alternatively to a defect in presentation of Ptc and of signal reception or in the processing of Shh protein. To test the ability of mDispA^-/- cells to correctly process Shh, we transfected an expression construct for full-length Shh into mDispA^-/- cells and compared the mobility of the protein produced to that produced by an expression construct for ShhN. The Shh expression construct encodes a full-length protein that undergoes internal cleavage and modification by cholesterol at the newly formed C terminus followed by palmitate addition at the N terminus (ShhNp). The ShhN construct contains a stop codon following the site of internal cleavage and therefore produces a protein (ShhN) containing the same amino acid residues as the processed protein but lacking cholesterol modification. We found that Shh protein expressed in mDispA^-/- cells was efficiently cleaved and that its electrophoretic mobility was slightly greater than that of ShhN (Figure 6C), indicative of normal cholesterol modification (Porter et al., 1996b). This mobility was not altered by cotransfection with expression constructs for mDispA or mDispB, and these results suggest that the signaling defect in mDispA^-/- embryos is not due to a defect in Shh processing.

Figure 6. Cells Lacking mDispA Function Respond Normally to Shh but Fail to Efficiently Stimulate Responding Cells

(A) mDispA^-/- cells are capable of responding to Hh signals, as indicated by the expression of a Hh-sensitive Ptch-lacZ reporter in the notochord (arrow) and gut in a E8.5 mDispA^-/- embryo.

(B) mDispA^-/- primary fibroblasts respond normally to exogenous Shh. mDispA^-/- (yellow bars) and mDispA^-/- (red bars) primary fibroblasts were transfected with Shh-sensitive Gli-luc reporter. Luciferase expression was induced in both cell lines in response to exogenously added ShhNp (10 nM), and the responses were blocked by cyclopamine (5 μM), a specific inhibitor of Shh signaling.

(C) Shh autoprocessing and cholesterol modification do not require mDispA function. mDispA^-/- cells were transfected with the expression constructs indicated, and after 3 days, the electrophoretic mobilities of Shh proteins were analyzed by SDS-PAGE followed by immunoblotting. Migration of Shh N-terminal domain derived from full-length Shh construct (Shh) was not affected by coexpression of mDisp proteins and was faster than that of protein derived from truncated Shh construct (ShhN). Because these proteins are identical in amino-acid sequence, the faster migration is indicative of cholesterol modification.

(D) Shh-expressing mDispA^-/- cells fail to efficiently stimulate Hh responsive reporter cells. mDispA^-/- cells were transfected with the expression constructs indicated and mixed with Shh-sensitive reporter cells (Shh-LIGHT2) in the absence or presence of cyclopamine. Transient expression of Shh alone elicited low level of response probably due to contact-dependent signaling. Coexpression of mDispA increased the response of the Shh-LIGHT2 cells to a level approaching that elicited by soluble ShhN. However, mDispB expression had no effect, consistent with the observation that mDispB cannot rescue Drosophila disp^-/- mutant. Fold induction is normalized to the respective cyclopamine controls.
Defective Export of ShhNp from mDispA<sup>+/−</sup> Cells

To explore whether the mDispA<sup>+/−</sup> signaling defect is due to an inability to present or release a signaling-compotent form of ShhNp, we transfected mDispA<sup>+/−</sup> mutant cells with expression constructs for full-length Shh or for ShhN and mixed these cells with stably transfected cells carrying the Gli-luc reporter (Shh-LIGHT2 cells; Taipale et al., 2000). We found that the ShhN-transfected cells induced a greater response in the Shh-LIGHT2 reporter cells (Figure 6D), consistent with previous studies demonstrating that ShhN protein is readily released from cells (Porter et al., 1996a). We tested the ability of Disp proteins to increase the amount of ShhNp available for signaling by cotransfecting the Shh expression construct into mDispA<sup>+/−</sup> cells with a construct for expression of mDispA or mDispB. We found that mDispA but not mDispB expression significantly increased the response of Shh-LIGHT2 cells (Figure 6D), suggesting that mDispA expression can increase the amount of signaling-competent Shh protein available to other cells. These experiments did not, however, distinguish between the possibilities that mDispA mediates release of ShhNp or instead mediates more efficacious presentation to responding cells on the surface of presenting cells.

To directly measure release of processed Hh proteins, we inserted coding sequences for Renilla luciferase in-frame into the amino-terminal signaling domains of full-length Hh and Shh coding sequences (Figure 7A). The point of insertion was a peripheral loop within the structure of ShhN (Hall et al., 1995), and the ability of Hh-Rluc and Shh-Rluc proteins to undergo processing was preserved (data not shown). We then transfected expression constructs for these modified Hh proteins together with expression constructs for Disp proteins and assayed the release of Renilla luciferase activity into the culture medium. Upon testing of several cell lines for Disp protein enhancement of protein export, the best results for Hh-Rluc and Shh-Rluc proteins were obtained in Drosophila S2 cells. In typical assays (Figure 7B), Disp proteins increased export efficiency ~3–10-fold, and enhancement of export by mouse or fly Disp was greatest for the Hh protein of the corresponding species. No enhancement of export was observed for mDispB (Figure 7B). These results indicate that Disp and mDispA, but not mDispB, can mediate the release and extracellular accumulation of Hh proteins in soluble form, and further suggest that the physiological role of Disp activity is to release Hh proteins from cells.

Functional Conservation in Disp Proteins of Residues Critical for Function of RND Transmembrane Transporters

Sequence comparison of Disp to other proteins in the database clearly shows sequence similarity not only to Ptc (Burke et al., 1999), but also to the prokaryotic RND permease superfamily. The majority of these proteins are prokaryotic efflux pumps involved in conferring resistance to drugs or heavy metals or in the secretion of endogenous molecules. These proteins appear to have arisen by tandem duplication of a six transmembrane unit (Tseng et al., 1999) to give rise to the twelve transmembrane spans of the full structure, including large extracellular loops at homologous positions in the two units, between TM1 and TM2, and between TM7 and TM8 (see Figure 1B). Biochemical studies have indicated that the RND superfamily proteins function as proton-driven antiporters (Nies, 1995; Tseng et al., 1999). Members of the RND family of proteins have a conserved GxxxD motif in the middle of TM4 (Tseng et al., 1999), and the aspartate residue within this motif is important for antiporter function and has been proposed to be the proton binding site (Goldberg et al., 1999). An expanded form of this motif, GxxxDD, is present in the middle of TM4 of Disp and mDispA, and the GxxxDD motif is also present within TM10, the homologous position within the presumptive intramolecular duplication; mDispB in contrast lacks Asp within TM4 (Figure 7C).

The presence of three acidic Asp residues in the middle of the bilayer in Disp and in mDispA and the experimentally demonstrated requirement for one of these residues in the function of bacterial efflux pumps (Goldberg et al., 1999) suggests the possibility that Disp and the bacterial proteins might act by a similar mechanism. Consistent with this possibility, 2 of 3 of the Asp residues conserved in Disp and mDispA are absent from mDispB, which is unable to complement Drosophila disp mutations and unable to release Hh proteins in cultured cell assays. To further test whether these conserved Asp residues are important for the function of Disp proteins, we introduced Asn or Ala substitutions at these sites and tested expression constructs encoding these altered proteins for their ability to export Hh proteins from S2 cells. We found that neither Disp nor mDispA in either of their altered forms were able to increase export efficiency of Hh proteins when tested in the S2 cell assay (Figure 7D). To further test these altered proteins and to validate the S2 cell export assay, we tested both altered Disp proteins for their ability to rescue the Drosophila disp mutation in vivo. Similarly, we found that neither Disp protein carrying the Asn or the Ala substitutions was able to rescue disp mutant function in the wing imaginal disc (Figures 7G–7H, compare to E–F).

Conclusions

Perhaps the most striking and unexpected aspect of our results is the extreme nature of the pattern disruptions in mDispA<sup>+/−</sup> embryos. The mDispA<sup>+/−</sup> mutant phenotype is more severe than that of mutations in any single gene encoding a Hh protein (Bitgood et al., 1996; Chiang et al., 1996; St-Jacques et al., 1999). In contrast, the Drosophila disp mutant phenotype is less severe than the hh phenotype (Burke et al., 1999). This discrepancy appears in part due to disruption of signaling by multiple Hh proteins, as the mDispA<sup>+/−</sup> phenotype resembles that of Smo<sup>−/−</sup> and that of the Shh<sup>−/−</sup>/Ihh<sup>−/−</sup> double mutant (Zhang et al., 2001). However, the phenotype also appears to owe its severity, at least in part, to a distinct balance in the relative importance of long-range and short-range signaling in Drosophila and in the mouse. In Drosophila disp mutants, short-range Hh signaling is intact and contributes to maintenance of target gene expression and to patterning. In mDispA<sup>+/−</sup> embryos, some signal response is retained in cells that express Shh (Ptch-lacZ is expressed in the notochord), but this
response appears not to contribute to the morphological pattern. The null function phenotype for the mDispA gene thus permits dissection of the relative importance of long- and short-range Hh signaling in mouse embryos and reveals a near absolute dependence on long-range signaling in patterning of the mouse embryo.

Mechanisms and processes proposed to play a specific role in long-range signaling (reviewed in Teleman et al., 2001) include the specific transport of protein signals to distant cells by movement through intervening cells (transcytosis), the sensing of signals produced at distant locations by long cytoplasmic extensions (cytonemes), and the movement of signaling proteins through tissues in membrane fragments termed argosomes (Greco et al., 2001). We can not rule out a role for Disp in helping to form these structures or mediate these processes, and these mechanisms may very well play some role in long-range action by other signaling proteins. However, the ability of Disp proteins to release soluble Hh proteins into the medium of cultured cells presents the relatively simple alternative of catalyzed signal release as a primary mechanism for initiating communication with distant cells.

The severe phenotype of the mDispA mutant indicates that little Hh protein is released in the absence of signals to distant cells by movement through intervening cells (transcytosis), the sensing of signals produced at distant locations by long cytoplasmic extensions (cytonemes), and the movement of signaling proteins through tissues in membrane fragments termed argosomes (Greco et al., 2001). We can not rule out a role for Disp...
teryl and palmitoyl adducts into the lipid bilayer. Membrane release of Hh protein represents a novel activity for bacterial RND transporters, the family to which mDispA belongs. A precedent for transmembrane molecular transporter acting in release of lipid-modified proteins from membranes is that of the bacterial ATP binding cassette (ABC) transporter, the LoICDE complex of E. coli (Yakushi et al., 2000). Membrane release of lipoproteins also represents a novel activity for ABC transporters, as other members of this family are involved in transport of substrates across the membrane (Linton and Higgins, 1998). Like these other ABC transporters, activity of the LoICDE complex is coupled to hydrolysis of ATP, which presumably supplies the energy required for membrane release. Transmembrane transport by RND family members also requires energy in the form of an electrochemical gradient, and it will be interesting to learn whether release of Hh by Disp also depends upon such a gradient, perhaps with the use of our cultured cell system that releases soluble Hh protein. Physical characterization of protein from this system should also illuminate the question of why soluble lipidated Hh protein in the medium does not reassociate with cells and should permit comparison to other soluble forms of lipidated Hh protein (Zeng et al., 2001).

Finally, it is interesting to note that another RND family member, the Ptc protein, is a Hh pathway component that plays a central role not in Hh signal release, but in regulating response to the Hh signal by modulating the activity of Smo. Like Disp, Ptc proteins also contain a functionally conserved GxxxD motif within the predicted TM4 domain, and this motif is affected in three of the six known missense mutations that cause Basal Cell Nevus Syndrome (also Gorlin’s syndrome) (Taipale et al., 2002). The Ptc protein thus also appears to function by a mechanism similar to that of Disp and RND transporters, although unlike Disp, its substrate remains unclear. These two members of the RND family with apparently similar mechanisms thus appear to have evolved to play essential yet very different roles in the Hh signaling pathway.

Experimental Procedures

Cloning and Sequence Alignments

Expressed sequence tags (ESTs) corresponding to the two murine disp homologs were identified by database search, and the initially identified ESTs were used to screen mouse cDNA libraries. mDispA cDNA was isolated as a single cDNA clone from a mouse testis cDNA library, and the full-length mDispB cDNA was assembled from two independent clones isolated from a mouse lung cDNA library. The accuracy of the full-length sequences was confirmed both by PCR reactions and intron/exon organization. The sequence alignment was generated using Pileup (GCG software package) and the identities between the sequences were marked by the SeqVu program.

Drosophila Stocks and Immunostaining

The disp mutant (I3/S03770), the UAS-disp transgene, the en-Gal4 driver, and the ptc-lacZ reporter gene are described in Burke et al., 1999. mDispA, mDispB, myc-tagged dispNINN, and dispAAA sequences were cloned into pUAST vector (Brand and Perrimon, 1993) and the corresponding UAS-transgenes were generated through P-element mediated germline transformation. disp−/− larvae for dissection were identified by the absence of the Tubby genotype present on the balancer chromosome TM6b. Third instar wing disc fixation and fluorescence labeling was performed as in Burke et al., 1999.

Generation and Identification of mDispA+/− and Smo+/− Mutant Alleles

The last coding exon of the mDispA gene was replaced by a loxP-flanked PGK-neo cassette through homologous recombination in the R1 ES cell line. Correctly targeted ES clones were identified by genomic Southern blot (See Supplemental Data available at http://www.cell.com/cgi/content/full/111/1/63/DC1) and chimeras were generated by blastocyst injection of CS7/B6L host. Germline transmission was confirmed by genotyping PCR reactions and the mutant allele was maintained in a C57/B6L:129SvJ mixed genetic background. The Smo+/− mutant allele was generated by replacing part of the first coding exon of Smo gene including the start codon and the signal peptide by a loxp site through CRE-mediated site-specific recombination of a conditional Smo allele (to be described elsewhere). Segregation of the phenotypes with the genotypes was established using genotyping PCR reactions (See Supplemental Data available at above URL).

Probes, In Situ Hybridization, and β-Galactosidase Histochemistry

In situ hybridization and β-galactosidase histochemistry were performed essentially as described (Hogan et al., 1994; Knecht et al., 1995). Ptc-lacZ allele was from Dr. M. Scott. The in situ hybridization probes were as follows: two probes, AA(V3-L184) and AA(R660-Y901), were used for mDispA which gave essentially the same pattern; Myf5 probe was provided by Dr. R. Emerson, and Pax1 and Pax7 probes were from Dr. C. Fan. HNF3(3′-UTR), Shh(3′AA L373-437), and Nodal(3′-UTR) probes were generated by PCR reactions with mouse genomic DNA as template.

Cell Culture Based Assays

mDispA−/− and mDispA+/− embryonic fibroblasts were isolated directly from E8.75 embryos and maintained in DMEM supplemented with 10% fetal bovine serum (FBS). Drosophila S2 cells were maintained in Schneider’s Drosophila Medium supplemented with 10% FBS. Fibroblasts and S2 cells were transfected using Fugene 6 (Roche) and calcium phosphate coprecipitation, respectively. For reporter assays, cell culture medium was replaced 48 hr after transfection with fresh low serum medium (DMEM with 0.5% bovine calf serum and 5 mM cyclopamine where indicated, and after 72 hr further incubation, the cells were lysed and reporter activities measured (Dual Luciferase, Promega). For Hh/Shh-Rluc export assays, cells were maintained by centrifugation 3 days after transfection and lysed directly in Passive Lysis Buffer (Promega). Conditioned medium was further cleared by centrifugation at 21,000 × g, and reporter activities in the cell lysates and conditioned medium were assayed as above. Similar results were observed using ultracentrifugation (100,000 × g for 1 hr). The relative export efficiency index was calculated using the following formula:

\[
\text{Renilla luciferase Activity in conditioned medium} / \text{Renilla luciferase activity in cell lysate} = \text{export efficiency index}
\]

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