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Vertebrate Hedgehog is secreted on two types of extracellular vesicles with different signaling properties

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Hedgehog (Hh) is a secreted morphogen that elicits differentiation and patterning in developing tissues. Multiple proposed mechanisms to regulate Hh dispersion includes lipoprotein particles and exosomes. Here we report that vertebrate Sonic Hedgehog (Shh) is secreted on two types of extracellular-vesicles/exosomes, from human cell lines and primary chick notochord cells. Although largely overlapping in size as estimated from electron micrographs, the two exosomal fractions exhibited distinct protein and RNA composition. We have probed the functional properties of these vesicles using cell-based assays of Hh-elicited gene expression. Our results suggest that while both Shh-containing exo-vesicular fractions can activate an ectopic Gli-luciferase construct, only exosomes co-expressing Integrins can activate endogenous Shh target genes HNF3 β and Olig2 during the differentiation of mouse ES cells to ventral neuronal progenitors. Taken together, our results demonstrate that primary vertebrate cells secrete Shh in distinct vesicular forms, and support a model where packaging of Shh along with other signaling proteins such as Integrins on exosomes modulates target gene activation. The existence of distinct classes of Shh-containing exosomes also suggests a previously unappreciated complexity for fine-tuning of Shh-mediated gradients and pattern formation.

The Hedgehog (Hh) family of proteins encodes highly conserved morphogens involved in patterning of developing tissues. Hh proteins are post-translationally modified by cholesterol at the C-terminus and palmitate at the N-terminus^{1,2} and these lipid modifications act as their cell surface anchors. In several developmental contexts as well as in cancers, mutually exclusive populations of cells produce and respond to Hh. Therefore, release, capture and transport of these membrane-anchored proteins are important regulatory steps controlling morphogen dispersion and signaling. Homo-oligomerization of Hh proteins also appears to be a prerequisite for its long-range paracrine signaling³.

Hh activates signaling in the recipient cells by binding to its receptor Patched (Ptch). Binding of Hh to Ptch abrogate its inhibition on Smoothed (Smo) and activates signaling via Gli transcription factors. Subsequent studies suggest that binding of Hh to Ptch is not sufficient for target gene activation, several co-receptors namely, HSPGs, BOC, CDO, Gas-1, LRP2 are also essential for Hh signaling^{4,5}.

Mechanisms of morphogen release into the extracellular milieu have been intensively investigated and a number of different processes have been described. In addition to the secretion of unmodified (lipid-free) Hh⁶, release of Hh via lipoprotein-mediated⁷⁻⁹ and exosome-mediated pathways^{10,11} has been proposed. Lipid modifications of Hh proteins are essential determinants of the dispersion range and signaling function¹²⁻¹⁵, and must be masked for Hh transport in the hydrophilic extracellular milieu. Much of our understanding of Hh dispersion comes from studies in *Drosophila*. In the lipoprotein-mediated delivery model, Hh is proposed to be inserted into circulating lipoprotein particles that are not synthesized by Hh-producing cells. This model leaves little room for controlled release by Hh-producing cells. Moreover, Hh proteins on lipoprotein particles seem to possess only partial signaling activity, being able to stabilize Ci155 and Smo and increase phospho-Fused levels in the *Drosophila* wing imaginal disc⁹, but failing to activate downstream target genes. By contrast, exosome-associated Hh is able to activate the pathway, including downstream target genes such as Ptc-promoter-trap::GFP reporter as well as endogenous Ptc and Collier expression in *Drosophila* wing imaginal discs^{11,16}. Exosomes are secreted-vesicles derived from the endocytic compartment by packaging in intraluminal vesicles



of multi-vesicular bodies (MVBs) and release into the extracellular space¹⁷. Evidence for exosome-mediated Hh release comes from perturbation of genes involved in endocytosis and biogenesis of MVBs such Rab5, Shbire, Tsg101, Hrs and Vps4, which reduces the Hh gradient and target gene activation in the wing disc^{11,16}. Vesicular release of Shh has also been reported during left-right determination by the ventral node in developing embryos¹⁸. Like other morphogens^{19,20} extracellular Hh and Shh particles are also transported to signaling competent cells in *Drosophila* wing and chick limb bud along cytonemes^{16,21,22}, thin actin-based filaments that extend between cells. However, the nature of the transported particles is not well understood and perhaps consistent with either lipoprotein or exosomal forms.

Little is known about the secretion of Hh in vertebrates, particularly, it is unclear whether the mechanisms described in *Drosophila* are conserved. Here we report on the vesicular forms of vertebrate Shh derived from cultured human cells and primary chick notochord cells. Perturbation of endocytic regulators in Shh-expressing cells disrupts ShhNp release, confirming the endosomal origin and therefore the exosomal nature of these extracellular vesicles. We have probed the origin, composition and physiological functions of vertebrate extracellular vesicles containing Shh. We find that Shh is released as distinct exosomal fractions that can be separated by ultracentrifugation and exhibit overlapping as well as distinct protein and miRNA composition. Functional assays using Shh-induced differentiation of mouse embryonic stem cells to ventral neuronal progenitors suggest that only those exosomes that carry Shh along with additional signaling partners, such as Integrins, are able to activate endogenous target genes efficiently. Our results suggest that distinct exosome classes may be a mechanism for fine-tuning the range and/or signaling capability of morphogen gradients.

Results

Shh is secreted efficiently by HEK293T cells on distinct classes of extracellular vesicles. To examine the release of Shh, HEK293T cells were transfected with full length Shh, which undergoes dual lipid modification to generate biologically active ShhNp. A truncated form of Shh, which fails to undergo cholesterol addition (ShhN), was used as a control, as it is thought to be released more efficiently. Conditioned medium from Shh-HEK cells was collected 2 days post transfection, cells and debris were removed and the cleared conditioned medium was used to isolate extracellular vesicles/exosomes (Fig. 1A). Both ShhNp as well as ShhN were detected in the growth medium (Fig. 1B). As expected, relatively higher levels of ShhNp were seen in the cellular fraction than ShhN, consistent with greater lipid-mediated membrane retention of the dually lipidated form². Absence of endoplasmic reticulum (Calnexin), Golgi (GM130), endosome (Rab5, Rab7, Rab 11) and cytoplasmic proteins (Zyxin-GFP, GAPDH, Vps4B) in the supernatant provided the evidence that the extracellular detection of ShhNp and ShhN were not a result of cell lysis but due to specific secretion mechanisms (Fig. 1C).

This characterized culture supernatant was further processed to analyse various forms of secreted ShhNp. Since the Hh family of proteins is reported to be released as exosome-like vesicles^{10,11} the supernatant was subjected to ultracentrifugation at 150,000 g to isolate the exosomal fraction (P150 fraction; Fig. 1A). ShhNp has also been reported to be secreted on lipoprotein particles which are much smaller and less dense than exosomes⁸. We therefore subjected the S150 fraction to additional ultracentrifugation at 450,000 g as a means of collecting all possible particulate forms of secreted ShhNp. We observed that while ShhNp was indeed detected in the bonafide exosome-fraction (P150), it was also present in the fraction pelleted at 450,000 g (P450) (Fig. 1D). Further, the two extracellular particulate pools were vesicular as they both floated at a density range typical of exosomes on sucrose density gradients (from 1.09 to

1.20 gm/ml) (Fig. 2A). Thus, while P150 represented the well-known extracellular form, P450 could potentially represent an as yet uncharacterized form of secreted ShhNp.

To further characterize the two vesicular fractions, we probed for components known to be associated with exosomes. As observed by western blot analysis, P150 and P450 fractions shared some typical exosomal proteins such as Tsg101 and CD9, but the P450 fraction lacked Flotillin1 and Flotillin2 (Fig. 1D). The differential detection of some exosome-associated proteins between P150 and P450 fractions suggested that there might be more differences in the protein content between the two vesicular pools. To evaluate the protein composition of the two vesicular pools, we used the unbiased approach of mass spectrometry. P150 and P450 fractions were isolated, purified on sucrose density gradients and samples enriched for Tsg101 (~1.12 gm/ml) as confirmed using immuno-blotting (data not shown) were profiled by LC-MS/MS following in-gel digestion (Fig. 2B). Differences in the protein content between the P150 and P450 pools were evident on the SDS-PAGE gel (Supplementary Fig. S1A). Serum vesicles as well as BSA, a major serum component were depleted from the preparations by using a modified exosome production medium. Peptides obtained by mass spectrometric analysis of the two fractions were compared to the ExoCarta database for the presence of extracellular vesicle proteins. Majority of the proteins identified in P150 and P450 pools (76% & 78% respectively) were reported in the database (Supp. fig 1B–C). Importantly, presence of Flotillin-1 and Flotillin-2 proteins was again detected only in the P150 fraction confirming our initial findings using western blot analysis (Supplementary Table S1) and supporting the distinction between the fractions. The peptide lists obtained by MS analysis in the two fractions were further analyzed to identify unique and common proteins. While a significant overlap was observed between the P150 and P450 fractions, the P150 fraction contained more unique proteins than the P450 fraction (Fig. 2C). This difference in the protein content between P150 and P450 (Table 1 and Supplementary Table S1–S2) was validated using western blot analysis of selected proteins (Fig. 2D). We found that cell surface or extracellular proteins such as Flotillins, Annexins, Integrins were only detected in the bonafide exosomes (P150) by LC-MS/MS based profiling; while proteins such as LRP2, Laminin alpha3, Proteoglycan 4 and CD109 were detected only in the P450 pool (Fig. 2D, Table 1 and Supplementary Table S1–S2). The classification of cytoskeletal, endocytic and cytoplasmic proteins as “extracellular” in these databases reflects the finding that these proteins are reproducible components of exo-vesicles. Taken together, these observations indicated that P450 vesicles may represent a sub-population of exosomes whose protein content largely overlapped with classical exosomes, but also displayed some unique components.

Shh is a key morphogen secreted by the developing notochord to pattern the early embryo. To determine whether the different exovesicular forms secreted by HEK cells over-expressing Shh in culture were also produced by a physiological source, we used primary notochord cells derived from early chick embryos. Notochords were dissected from HH stage 21–24 chick embryos and cultured in notochord culture medium (NCM) (Fig. 2E). Using the methods developed for the HEK cells, P150 and P450 secreted fractions were derived from the notochord conditioned medium after 48 hrs of culture in notochord EPM. Western blot analysis confirmed the presence of endogenous ShhNp in both fractions (Fig. 2F). Consistent with the experiments using ectopically expressed Shh in HEK293T cells, again Flotillin-1 was only found in P150 fraction (Fig. 2F). We therefore concluded that the two different vesicular pools of ShhNp were a physiological feature of endogenous Shh-producing cells.

To further confirm that both pelleted fractions were vesicles, we analysed the Shh-HEK isolated pools by electron microscopy. Negative staining showed clear vesicular structures (Fig. 3A, top

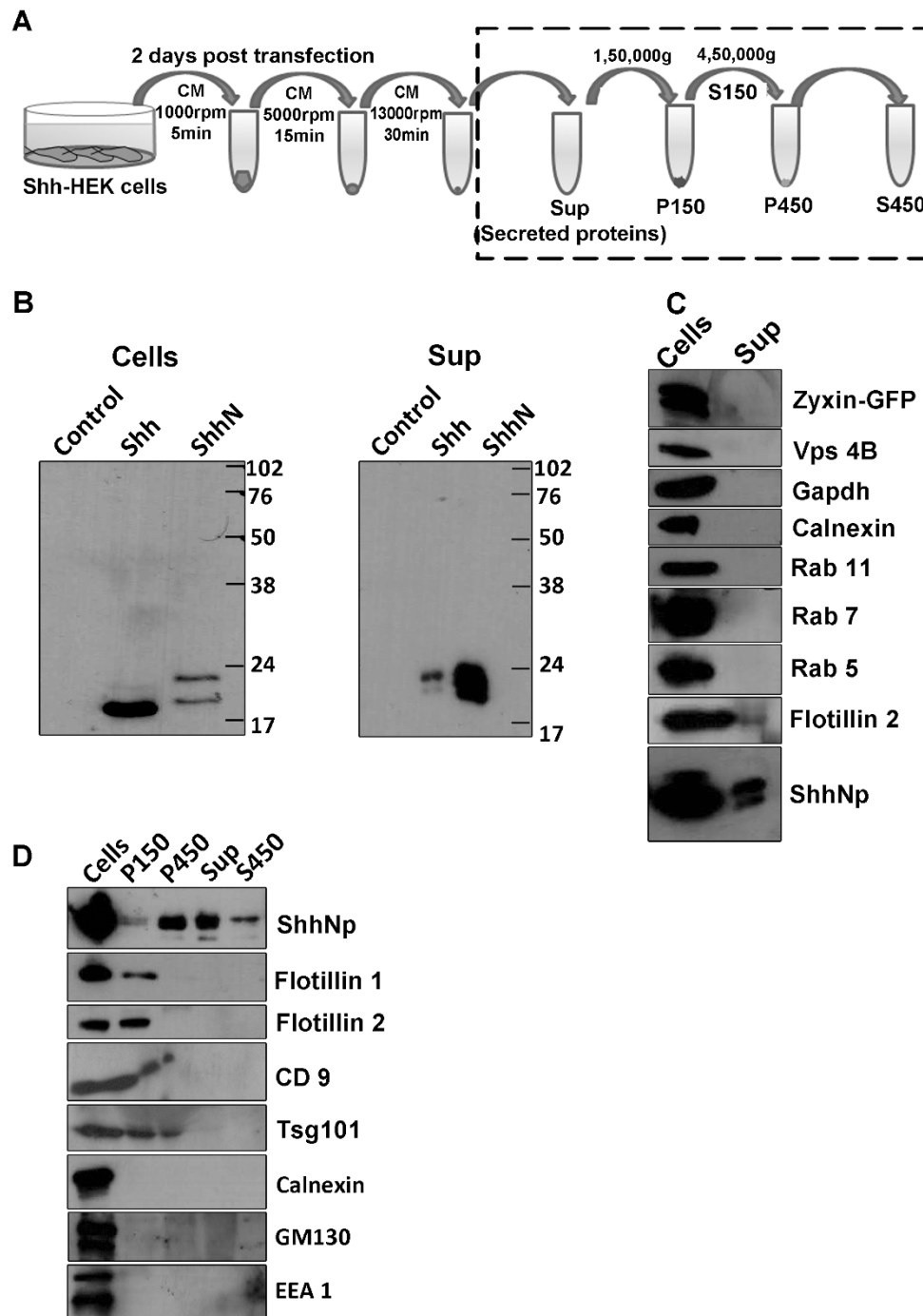


Figure 1 | Shh is secreted in different forms by HEK-293T cells. HEK-293T cells were transfected with Shh or ShhN. Cells and supernatant were analysed by western blot analysis. (A) Schematic showing the protocol used for derivation of the different extracellular pools containing ShhNp. Conditioned medium (CM) was collected after two days from Shh-HEK cells, cells and debris were removed by differential centrifugation before assessing the presence of secreted proteins in the resultant supernatant (Sup). Exo-vesicles were isolated by centrifugation at 1,50,000 g (P150), subsequently the S150 was centrifuged at 4,50,000 g to isolate other secreted forms (P450). (B) Western blot using anti-Shh antibody indicated presence of ShhNp and ShhN proteins in cell lysate as well as Sup of transfected HEK cells. (C) Absence of cytosolic and intracellular organelle-specific proteins in the Sup was evidence for negligible cell lysis. Note that none of the cellular proteins whether overexpressed (Zyxin-GFP) or endogenous (Rab5, Rab7, Rab11, Calnexin, Vps4B, GAPDH) were seen in the supernatant, unlike Shh or Flotillin 2. (D) Western blot analysis of proteins present in different fractions. Flotillin 1 and Flotillin 2 were only detected in P150 where as ShhNp, Tsg101 and CD9 are seen in both the fractions. Calnexin (ER marker), GM130 (Golgi marker) and EEA1 (Early endosomal marker) were not detected in either secreted fraction.

panel). Exosomal marker CD63, a tetraspanin (Fig. 3A middle) as well as ShhNp (Fig. 3A, bottom) were detected on both vesicular fractions by immuno-EM. Given that the two pools were isolated by differential ultracentrifugation, it might be expected that the size range would be reflected in the two isolated fractions.

However, while the P450 pellet also contained vesicles smaller than 30 nm, and the P150 pellet contained vesicles >50 nm, most vesicles in both pools were in the size range reported for exosomes¹⁷ i.e. 30–50 nm (Fig. 3B). Thus, the distinct pelleting behavior of the two vesicular fractions may depend on features other

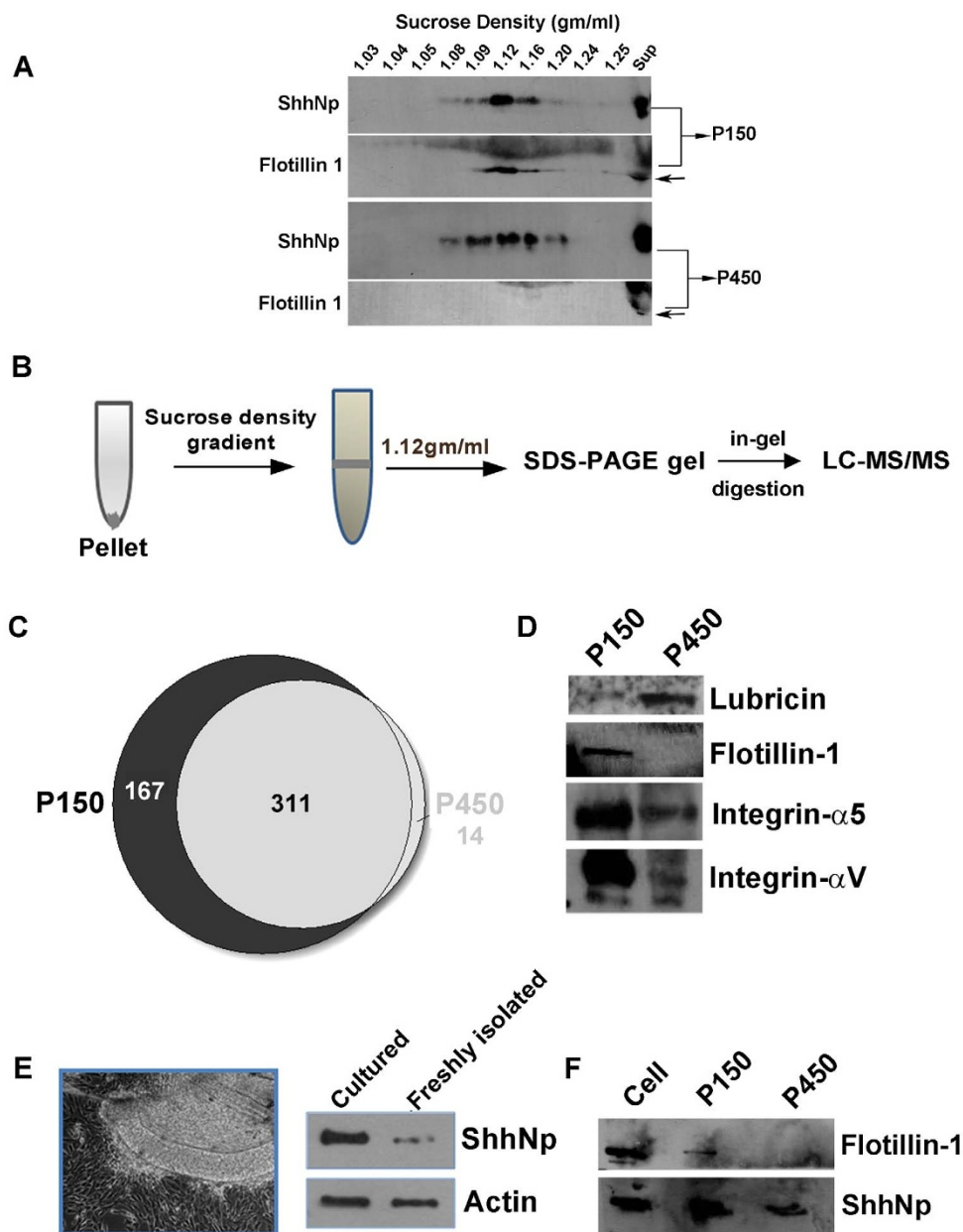


Figure 2 | Shh is secreted on exosomes of overlapping and unique protein composition. (A) Sucrose density gradient centrifugation was performed using P150 and P450 pellets. Fractions were analysed by western blotting to identify the fractions containing Shh and Flotillin-1, a marker for exosomes. Flotillin- and Shh-positive fractions derived from the P150 pool float at similar density (1.09 to 1.12 gm/ml). Shh-positive fraction derived from the P450 pool also floats at similar density but lacks Flotillin-1. (B) Mass spectrometry-based comparison of the two vesicular forms. Schematic representing the derivation of samples for LC-MS/MS. (C) Peptides obtained by LC-MS/MS analysis were analysed for shared and unique peptides in each fraction using Proteome Discoverer software 1.4 and are represented as a Venn diagram. Majority of proteins were shared between the two vesicular pools. LC-MS/MS was performed on two biological replicate samples and only peptides identified in both runs were analyzed further. (D) Western blot analysis of Shh-HEK derived P150 and P450 fractions validating the differential detection of selected proteins identified using LC-MS/MS based profiling. Equal amount of total protein was loaded. (E) Phase contrast micrograph of notochord cells derived from HH 21-24 stage chick embryos in culture. Western blot analysis confirmed the expression of Shh in freshly isolated as well as cultured notochord cells. (F) Western blot analysis of the P150 and P450 pellets derived from cultured notochord cells. Equal amount of total protein was loaded for each sample. ShhNp was present in both P150 and P450 pellets unlike Flotillin 1, which was detected only in P150.

than size per se, and may reflect the presence of unique combinations of proteins.

Both extracellular vesicular pools are of endocytic origin. Exosomes are extracellular vesicles released from the cell surface by the fusion of MVBs with the plasma membrane²³. Budding of plasma membrane as seen in case of viruses^{24–26} may also release vesicles with similar content. Since the formation of both

membrane buds and exosomes share some common regulators and may also share several protein constituents, we decided to investigate the origin of the two subpopulations of ShhNp extracellular vesicles. Exosomes are generally assumed to be of endocytic in origin^{23,27} and Rab proteins are known to regulate endocytosis as well as MVB biogenesis, but are not reported to be involved in plasma membrane budding. We first probed the endocytic fate of ShhNp using ectopic expression of both Shh and


Table 1 | List of unique cell surface or extracellular proteins identified in P150 and P450 pools, involved in cell-communication or development

| No. | P150- Unique Proteins | P450-Unique Proteins |
|-----|---|--|
| 1. | 14-3-3 protein beta/alpha | Laminin subunit alpha-3 |
| 2. | 14-3-3 protein eta | Low-density lipoprotein receptor-related protein 2 |
| 3. | 14-3-3 protein gamma | Proteoglycan 4 |
| 4. | 14-3-3 protein zeta/delta | |
| 5. | 2',3'-cyclic-nucleotide 3'-phosphodiesterase | |
| 6. | A disintegrin and metalloproteinase with thrombospondin motifs 13 | |
| 7. | Annexin A2 | |
| 8. | cDNA FLJ78024 | |
| 9. | Complement component C6 | |
| 10. | Disintegrin and metalloproteinase domain-containing protein 10 | |
| 11. | Flotillin-2 | |
| 12. | Guanine nucleotide-binding protein G(s) subunit alpha isoforms XLas | |
| 13. | Integrin beta-1 | |
| 14. | ISTI homolog | |
| 15. | Leukocyte surface antigen CD47 | |
| 16. | Nuclease-sensitive element-binding protein 1 | |
| 17. | Ras-related protein Ral-A | |
| 18. | Syntaxin-4 | |

Protein list is generated using "Proteome Discoverer 1.4" software (ThermoScientific). LC-MS/MS analysis was done on the exosomal pools in duplicates. Only proteins detected in both the biological repeats were analysed for differences.

GFP-tagged Rab5a to track endosomes. We found several endosomes containing internalized Cy5-labelled ShhNp overlapped with the endosomal tracer GFP-Rab5aWT protein (Fig. 4A; top panel). By contrast, cells expressing a Rab5a dominant negative mutant -GFP-Rab5aS34N- showed marked reduction in the number of ShhNp endosomes (Fig. 4A; bottom panel), suggesting that interfering with Rab5a function inhibits the endocytosis of ShhNp. To confirm that endocytic internalization of ShhNp was tracked into MVBs for secretion, we followed secretion in eGFP-tagged Rab5a-WT and Rab5a-S34N overexpressing cells. We found that the secretion of both P150 as well as that of P450 was inhibited by ectopic expression of either Rab5a-Wt or Rab5a-S34N as compared to control eGFP overexpression (Fig. 4B & 4C). These observations were consistent with the known role of Rab5 in exosome secretion^{11,28}. Rab5a-WT overexpression may inhibit endosomal maturation from early endosome to MVBs leading to reduced exosome biogenesis²⁹. We therefore concluded that both P150 as well as P450 correspond to subpopulations of membrane vesicles of endosomal origin. The presence of several Rab proteins in the P150 as well as P450 fractions using LC-MS/MS analysis (Supplementary Table S1) supported this interpretation. The endosomal origin and composition of these vesicular fractions indicated that both vesicular pools could be exosomes.

The two vesicular forms contain distinct miRNA profiles. The results thus far suggest that the two exosomal pools share an endocytic origin but display some distinct features as evidenced by the distinct protein profiles. To further distinguish the two pools, we used miRNA profiling of small RNAs purified from the exosomal fractions derived from Shh-HEK cells. Exosomes are known to contain miRNAs^{17,30} and are proposed to deliver these regulatory molecules between cells. To ensure that RNA isolated from the secreted fractions was contained within the vesicles rather than associated non-specifically on the surface, each vesicular pool was treated with RNase A before RNA isolation (Supplementary Fig. S2A). Bioanalyzer readings indicated that unlike fresh culture medium, conditioned media containing the two classes of secreted vesicles contained RNA (Supplementary Fig. S2B–E). We generated small-RNA libraries from the two vesicular pools separately and sequenced them using next generation sequencing. Of ~76 million reads, 77.5% represented sequences of 18–24 nt, of which 85% of P150 and 90% of P450 reads aligned to the human genome. Small

RNA profiling showed strong enrichment of sequences of 21–22 nt, which suggests enrichment of either endo-siRNAs and miRNAs in the small RNA population (Supplementary Fig. S3A). The entire 18–24 nt population was aligned to the miRNA database to identify the miRNAs packaged in the different vesicular pools. Both the exosomal fractions contained substantial numbers of miRNAs (Supplementary Table S3). Reads that mapped to individual miRNAs were normalized to the total number of mappable reads from each sample. We found 693 miRNAs that have at least one read from either P150 or P450 fraction (Supplementary Table S3). We selected the top 20 highly enriched miRNAs in P150 and P450 samples for pathway analysis of the potential target mRNAs. Using miRpath³¹ for miRNA target pathway prediction we found that these miRNAs mostly target cancer and other signaling pathways (Corrected p value < 0.01) (Supplementary Fig. S3B–C & S3D–E). Our data clearly demonstrates the enrichment of distinct miRNAs in two ShhNp-exosomal pools whose protein composition also differs.

To examine if vesicles derived from endogenous Shh-expressing cells also contain miRNAs, RNA was isolated from chick notochord derived P150 and P450 fractions (Supplementary Fig. S2F–H) and processed for small RNA library preparation and sequencing. Primary notochord vesicular fractions also contain miRNA (Supplementary Table S4). Specific miRNAs in the notochord-derived P150 and P450 pools were validated using Taqman real-time PCR analysis (Supplementary Fig. S4A–B). Pathway prediction of the notochord miRNAs revealed that FGF signaling and Wnt pathway constituted some of the top targets of miRNAs contained in P150; while axonal guidance and the nervous system were the top targets of miRNA in P450 fraction (Supplementary Fig. S4C–D). As with the protein profiling, there was substantial overlap of miRNA sequences in the P150 and P450 fractions. However, differential enrichment of a group of miRNAs in each fraction supports the notion that they represent distinct pools. The prediction of potential target mRNAs that might be affected by the delivery of these miRNAs was also distinct and suggests new possibilities for Shh effects.

The finding that ShhNp-containing extracellular vesicles can also ferry miRNAs as cargo substantially broadens the potential of morphogen-mediated signaling to affect target cell function.

Both exosomal pools of Shh can activate a canonical Gli reporter. LC-MS/MS based profiling and western blot analysis confirmed that the P150 fraction comprises several unique peptides representing

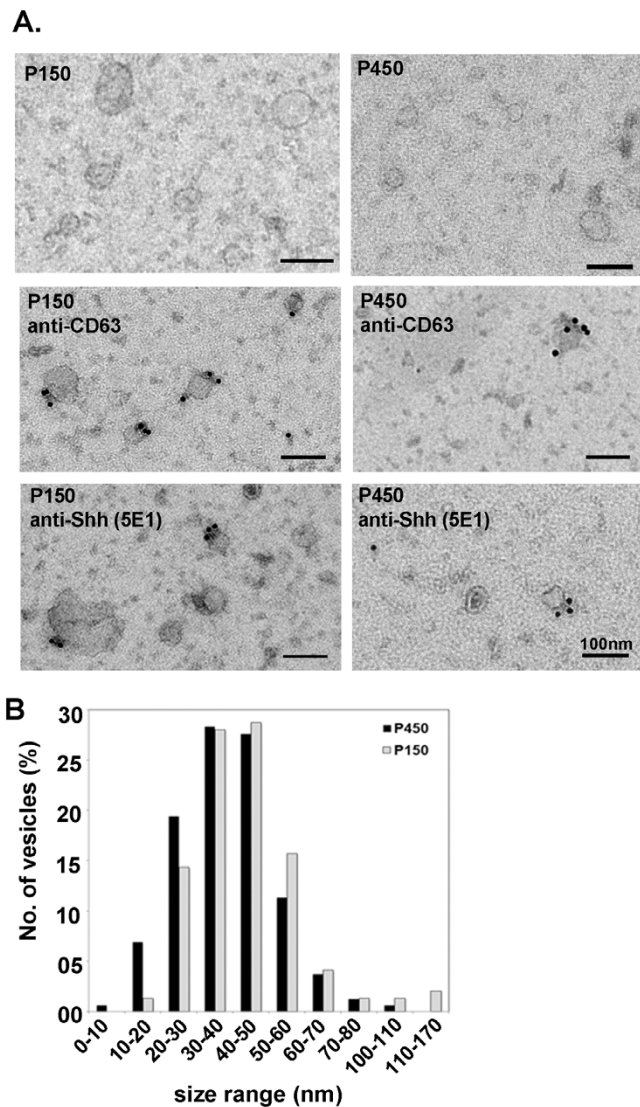


Figure 3 | Verification of exosomal vesicles by immuno-EM. (A) P150 and P450 pellets were negatively stained with uranyl acetate (top panel) and immunolabelled using anti-CD63 (exosomal marker; middle panel) and anti-Shh antibodies (bottom panel, 5E1). Scale bar, 100 nm. (B) Graph representing size range of exosomes in P150 and P450 pellets. Size bins and their frequency were derived from the immunoEMs. Image Analysis and quantification of exosome size and number was done using the iTEM software (Soft Imaging System, SIS, Germany). For exosome size estimation, $n = 150$.

extracellular and cell surface proteins known to play a role in cell communication during development and disease (Table 1 and Supplementary Table S1–S2). In addition to the Shh cognate receptors and co-receptors, ShhNp capture in signal-receiving cells is also dependent on other ECM and cell surface proteins. Thus, the finding that proteins such as $\beta 1$ -integrin (in the P150 pool) and Lrp2/Proteoglycan-4 (in P450 pool) were differentially enriched suggested the possibility that the two pools might exhibit different functional abilities with respect to Shh signaling.

To evaluate the functional properties of the distinct exosomal forms of ShhNp, we used a Gli-reporter assay in Shh-light2 cells³². This construct reports for canonical Hh-signaling that activates its target transcription factor Gli binding to multimerized Gli-binding sites. Cell equivalent volumes of the P150 and P450 fractions derived from the same preparation could activate luciferase activity (Fig. 5A, B). Further, we confirmed that the Gli-reporter activity induced by

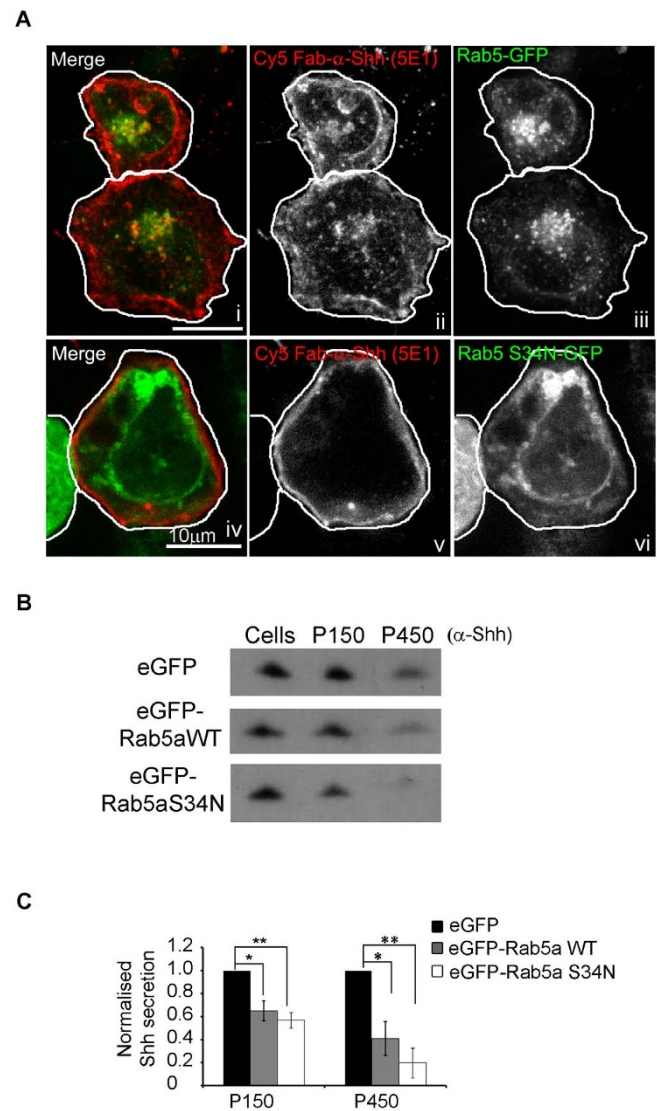


Figure 4 | P150 and P450 pools are endocytic in origin. (A) Confocal images of the endocytic pool of ShhNp as marked by Cy5-labelled Fab fragments derived from 5E1 antibody; 30 min endocytic pulse (top panel: ii & red in colour merge; bottom panel: v & red in colour merge represents endocytic pools of ShhNp). eGFP-Rab5aWT expression in Shh-HEK cells (top panel: iii & green in colour merge represents eGFP-Rab5aWT shows colocalization with ShhNp on several endosomes. eGFP-Rab5aS34N expression in Shh-HEK cells (bottom panel: vi; green in colour merge) reduces the endocytic uptake of ShhNp. All images represent merged confocal stacks. Scale bar, 10 μm . (B) Western blot comparing cellular ShhNp expression and secretion of ShhNp in P150 and P450 pools. eGFP expressing Shh-HEK cells were used as control for ShhNp secretion by eGFP-Rab5aWT and eGFP-Rab5aS34N expressing cells. (C) Quantification of relative amount of ShhNp in P150 and P450 pools was done using densitometry. Secreted fractions were normalized to cellular expression and then compared with eGFP control. Values represent mean \pm SD from two independent experiments. * $P < 0.05$, ** $P < 0.01$.

each exosomal pool was Shh-dependent, since an Shh blocking antibody (5E1), as well as Cyclopamine, a pharmacological inhibitor of Shh signaling, were both able to block the activation to control levels (Fig. 5A). Posthoc analysis of the ShhNp content in the cell equivalent volumes used for the analysis revealed a ~ 10 fold difference in the amount of ShhNp between the two vesicular pools (P150 fraction contains more ShhNp than P450) (Fig. 5B). Under these assay conditions, both P150 and P450 pools activated a sensitive reporter of

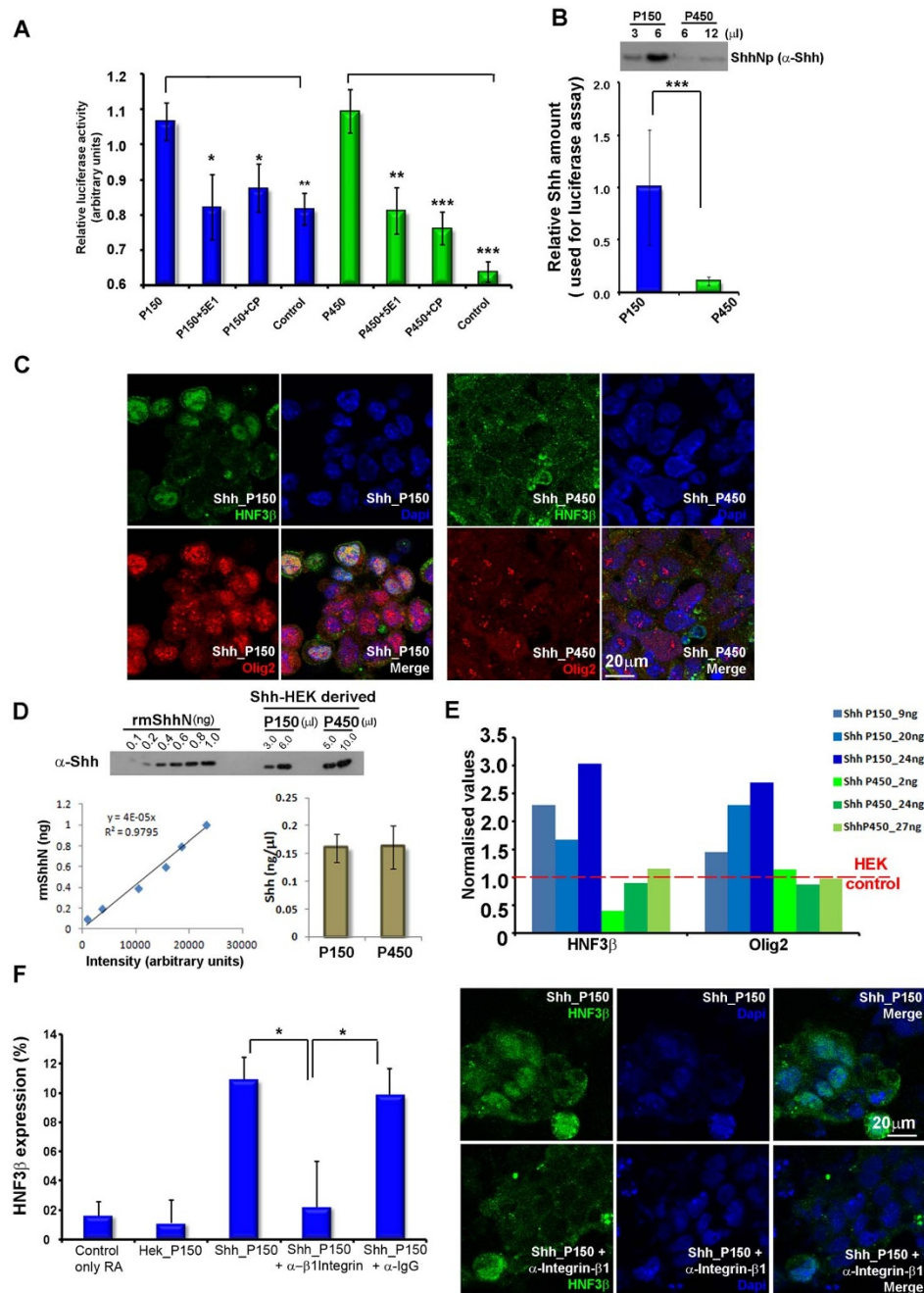


Figure 5 | Two Shh-exosomal pools have different signaling efficiencies. (A) Gli-luciferase reporter assay shows that ShhNp secreted from HEK293T cells is biologically active. Cell-equivalent volumes of vesicular pool were supplemented for the assay. “Control” represents Shh-light2 cells incubated with P150 or P450 pools derived from un-transfected HEK293T cells. Graph represents mean \pm s.d. of one representative experiment. (B) Graph depicts post-hoc densitometric quantitation of the relative amounts of ShhNp in P150 and P450 fractions (shown in the western blot) used for Gli-luciferase assay. Note: Vesicles were resuspended in equal volumes, equal volumes were used for the Gli-luciferase assay. (C) ES to motor neuron progenitor differentiation assay shows ShhNp-dependent activation of HNF3 β and Olig2. Single confocal plane represented, nuclei are marked with DAPI in blue, HNF3 β staining is represented in green and Olig2 in red. (D) Western blot showing the quantitation of ShhNp content in Shh-HEK derived P150 and P450 fractions. Different amounts of commercial recombinant mouse ShhN (rmShhN, R&D Systems) were used to generate the standard curve to quantitate ShhNp in the two fractions. Bar graph represents the amounts of ShhNp in P150 and P450 pellets derived from the standard curve and used for ES to MN assay for equivalent ShhNp in the two fractions (24 ng each, ref Fig 5E). (E) Bar graph shows relative HNF3 β and Olig2 expression scored from three different experiments with differing ShhNp content in the two fractions. Three different sets include P150_9 ng & P450_2 ng (cell equivalent ShhNp), P150_24 ng & P450_24 ng (equal ShhNp) and P150_20 ng & P450_27 ng. Relative expression of HNF3 β and olig2 are obtained by normalizing results from RA + Shh-HEK_P150 or RA+Shh-HEK_P450 samples with RA+ respective vesicles from untransfected HEK control. 300–400 cells were counted per sample. (F) Graph representing HNF3 β expression upon inhibition of β 1-Integrins. Percentage of cells expressing HNF3 β in ES to MN differentiation assay reduces upon incubation of the Shh-P150 vesicles with anti- β 1-integrin blocking antibody (A2BII, DSHB) as scored by HNF3 β staining. Representative images presented. Blue –DAPI; green—HNF3 β . (A, B, D & F) Values represent mean \pm SD, from 2 or more independent experiments. *p-value < 0.05, **p-value < 0.005, ***p-value < 0.0005.



canonical Hh signaling to a similar extent despite the difference in the ShhNp content may suggest differences in the signaling capabilities of the two pools imposed by other (unique) components of the fractions.

Differential ability of the two exosomal pools in activating endogenous target genes. To further investigate our hypothesis that the differential distribution and identity of ECM and cell surface proteins in the two pools might affect exosomal ShhNp function, we used a motor neuron differentiation assay. Exposure of mouse embryonic stem cells (mESC) to Shh in the presence of retinoic acid (RA) triggers their differentiation into ventral neuronal progenitor cells^{33,34} and ShhNp is known to activate distinct neuronal target genes in a concentration-dependent manner. To evaluate the signaling potential of the different vesicular pools, undifferentiated mESC were placed in suspension for two days to generate embryoid bodies (EBs), in the presence of leukemia inhibitory factor (LIF) to prevent spontaneous differentiation. EBs were then transferred to differentiation medium containing either RA alone or RA along with ShhNp-containing vesicles derived from Shh-HEK cells. Vesicles isolated from untransfected HEK cells were also used as controls. Suspension-cultured EBs were harvested and dissociated on day 3, and plated on gelatinized dishes to detect the presence of neuronal progenitors using antibody staining. Specificity of the antibodies used to detect different neuronal progenitors was established by immuno-staining cryo-sections of early chick embryos (Supplementary Fig. S5). Interestingly, we found that when cell equivalent volumes of P150 and P450 were used in this assay (ShhNp P150_9ng & ShhNp P450_2ng), unlike what was observed in the Gli-luciferase assay, only the P150 fraction was able to activate expression of endogenous target genes HNF3 β (floor plate marker and highest threshold target), Olig2 (intermediate target) and Nkx6.2 (Supplementary Fig. S6; lower threshold target) (Fig. 5C, E). By contrast, even with equivalent amounts of ShhNp in both the vesicular pools, the P450 fraction was unable to activate any of the neuronal progenitor markers (Fig. 5C, E). The amount of ShhNp present in the vesicular pools to be used for the assay was quantitated by western blotting against standards (Fig. 5D).

The finding that the two pools show differential signaling capability with respect to endogenous gene activation contrasted with the earlier observation, where both pools could activate an ectopic canonical Hh reporter despite unequal ShhNp amounts (Fig. 5A, B). The distinct protein profiles displayed by the two fractions present a likely explanation. Since the P150 exosomes contain β 1-integrin, a key determinant of adhesive interactions, it is conceivable that while different types of vesicles might incorporate ShhNp, only vesicles containing potentiating/synergizing protein factors may be efficient activators of downstream endogenous target genes. To test this hypothesis, we incubated the vesicles with inhibitory antibodies to β 1-integrin that was reproducibly detected only in the P150 pool. Indeed, when P150 vesicles are pre-incubated with β 1-integrin blocking antibodies, they fail to activate expression of HNF3 β in the motor neuron differentiation assay, unlike an IgG control (Fig. 5F). This result suggests that presence of ShhNp is necessary but not sufficient for efficient target gene activation, and that interactions mediated by other exosome components such as integrins are critical for full signaling capability.

Discussion

During development, morphogens are produced by select groups of cells and elicit effects in target cells at a distance to pattern the growing embryo. The long-range effects of lipid-modified signals such as Wnt and Hh proteins require mechanisms to transport them between their sites of production and action. Thus, packaging, release, capture and export of membrane-anchored morphogens

are major regulatory nodes for efficient signal transduction. While cholesterol modification is thought to restrict the release of Hh proteins, it is probably also required for packaging Hh proteins in a signaling-competent manner. In this report, we show that vertebrate ShhNp is released by producing cells on two operationally defined classes of extracellular vesicles derived by sequential ultracentrifugation. These ShhNp-displaying vesicles show features of exosomes based on three criteria—firstly, they are secreted membrane-bound structures in the size range (30–50 nm) reported for exosomes; secondly, they possess >70% of proteins earlier reported to be present on exosomes, and, thirdly, their release into the extracellular milieu requires efficient endocytic pathways, consistent with their endosomal origin. Exosomes have been reported to represent a heterogeneous population of vesicles issued from the same or distinct endosomes^{17,35}. We also show that while the two classes of ShhNp-exosome share a great majority of their constituents, the P150 and P450 fractions can be distinguished not only by unique protein profiles, but also by their unique miRNA profiles. We demonstrate that despite relatively equivalent ability to activate an ectopic canonical reporter of Hh signaling in target cells, the two pools differ in their ability to activate endogenous target genes during motor neuron differentiation from stem cells. Finally, we demonstrate that the signaling functions of the ShhNp transport exosomes depend not only on the presence of ShhNp itself, but also on the presence of accessory proteins typical of one class of exosomes. Specifically, only the P150 exosome fraction that is marked by the presence of β 1-integrin is able to efficiently activate endogenous target genes. Taken together, these results establish a new framework for understanding paracrine morphogen action and identify new molecular players in the process.

Extracellular vesicles: Origin and signaling potential. Given the importance of Hh in patterning the early embryo, as well as in paracrine functions that control tumor progression, the release and transport of Hh has been extensively studied, predominantly in flies. Secreted Hh is proposed to participate in at least two forms of macromolecular complex-circulating lipoprotein particles^{7–9} and vesicular exosomes^{10,11}. Our study provides strong support for the packaging of ectopically expressed Shh into exosomes, but does not preclude the existence of other compartments such as lipoprotein particles. The form of the ShhNp-decorated structures detected in immuno-EM is clearly vesicular, and the demonstration that perturbation of endocytic regulators in producing cells disrupts ShhNp packaging/release is also consistent with a derivation from MVBs, the precursors of exosomes.

Despite the differential centrifugation applied to pellet them, the two pools of ShhNp-exosomes are apparently of largely overlapping size distribution (30–50 nm). Yet, there are substantial differences in composition. While most of the proteins of P450 fraction are also present in P150 fraction, including components such as CD9, Tsg101, CD81, Rab35, Rab8, Rab11 and heat shock proteins (Fig. 1D, and Supplementary Table S1), proteins such as LRP2, Laminin alpha3, Proteoglycan 4 and CD109 are detected only in the P450 pool (Fig. 2D, and Supplementary Table S1). By contrast, several bona fide exosome-associated proteins such as Flotillin1, Flotillin 2, Annexins and Tetraspanins are selectively sorted into the P150 fraction along with Integrins. It is conceivable that the lipid domain segregation observed on the plasma membrane³⁶ and on the limiting membrane of MVBs³⁷ might be responsible for this selective segregation of proteins, generating exosomes of distinct composition, but different mechanisms of exosome biogenesis³⁵ may also play a role.

Unlike lipoprotein release or membrane budding, exosome secretion is dependent on the endocytic pathway. Over-expression of dominant-negative Rab5 interferes with the formation of early endosomes—precursors of MVBs. In *Drosophila*, Rab5 dominant negative overexpression in Hh-producing cells leads to accumulation of



apical Hh and reduces signaling range of Hh¹¹, but its effect on Hh secretion remained undetermined. We hence investigated the impact Rab5 dominant negative on secretion of both vesicular pools. Our results indicate that ectopic expression of a dominant negative Rab5a not only disrupts endocytosis of ShhNp but also secretion of both vesicular pools of ShhNp (Fig. 4). This finding complements the protein profiles of the two pools to underscore the identification of the classical P150 fraction and the newly described P450 fraction as bona fide exosomes, albeit of varying composition. Although our current estimates of size based on EM suggest similarities in the two pools, their distinct protein composition could significantly impact the distance to which each class of exosome disperses within a developing tissue. Thus, the morphogenetic field influenced by secreted ShhNp may contain a set of overlapping fields generated by populations of exosomes with different properties, and may form part of the explanation for the observed graded gene activation in development. Indeed, ECM and cell surface proteins are known to play an important role in protein-protein interactions that would be predicted to influence the retention or dispersal of the extracellular vesicles that bear them. Further, these accessory protein components of exosomes are themselves capable of activating signaling cascades and thus orchestrating cellular behavior and tissue patterning^{17,38}.

While the Gli-luciferase assay proved that ShhNp displayed on both types of vesicle is intrinsically signaling-competent in its ability to efficiently activate a canonical reporter (Fig. 5A), the activation of endogenous ShhNp-dependent promoters was restricted to the P150 exosomal pool. Shh in presence of RA is able to direct differentiation of mouse ES cells to ventral neuronal progenitors^{33,39}. Although it does not mimic the spatial dispersal inherent in the developing embryo, this *in vitro* assay captures the concentration-dependent graded response to ShhNp where high (HNF3 β) and intermediate (Olig2) threshold response elements can be evaluated simultaneously. We find that only ShhNp in the P150 pool is able to activate the different threshold endogenous target genes (Fig. 5C–E). Interestingly, differential activation of transfected and endogenous target genes by lipoprotein-associated Shh has been demonstrated earlier⁹; lipoprotein-associated Hh can activate Shh-light2 cells and stabilize Ci155 activator but is unable to activate endogenous target genes in *Drosophila*⁹. Further, a moderate increase in Wntless signaling (via Wg^{S239A}—a lipidless variant)—that had little or no effect on patterning, failed to activate high threshold target genes but was able to promote cell proliferation in gradient-independent manner⁴⁰. We propose that the Shh-P450 pool which fails to activate the different threshold targets might also be involved in Shh gradient-independent functions. It is also possible that the presence of the P450 pool in the extracellular milieu modifies the integrated response to the P150 pool by competing or otherwise interacting with the cell surface receptors and co-receptors that capture the bona fide signaling exosomes.

Cell surface proteins on extracellular vesicles and their role in Shh signal transduction. Hedgehog signaling appears to require the combinatorial involvement of several co-receptors in addition to its cognate primary receptors. Vertebrate Hh proteins are known to interact with cell surface receptors Patched1 and Patched 2 (Ptch1/2) to activate a signaling cascade in target cells. However, it is now well accepted that presence of Ptch proteins is not sufficient for activating Hh signaling. Co-receptors such as Proteoglycans⁴, BOC/CDO/Gas-1^{41,42} and LRP2⁴³ are also essential for Hh signaling. Given our finding that Shh-exosomes include the previously implicated co-receptors proteoglycans and Lrp2, it is conceivable that this pool of co-receptors modifies Shh signaling outcomes. We propose that the differential signaling ability of the P150 and P450 pool is a function of accessory exosomal proteins. Exosomal integrins have been previously implicated in adhesion and signal activation in B cells³⁸. Since the P150 pool is uniquely able to activate endogenous targets of Shh, we probed the role of β 1-integrin

that is uniquely detected on the P150 exosomes. When pre-incubated with anti-integrin β 1 blocking antibodies, the Shh-dependent signaling activity of the P150 pool of exosomes is reduced to control levels (Fig. 5F). Our findings suggest that Shh is necessary but not sufficient for efficient activation of endogenous target genes, and that accessory partners like Integrins are necessary for full signaling capability.

The size of exosomes is consistent with the possibility for a single vesicle to carry more than one Shh molecule along with other proteins like integrins and proteoglycans on their surface. Whether the full signaling capability requires clustering of integrins and Shh in the exosome membrane and the generation of a complex signaling synapse with Patched/Lrp2/integrin ligands on the target cell plasma membrane is an avenue for future investigation. The molecular mechanism for co-receptor/accessory protein function is also unclear and may be a function of increased efficiency of capture or stabilization of the signaling complex (Fig. 6). In this context, it is possible that the lipid anchors of the membrane-tethered morphogens like Shh and Wnt are important not only to restrict their release, but also to ensure the correct sorting and packaging into exosomes along with the right partners for efficient downstream target gene activation. β 1-Integrin knock-out mice do show phenotypes similar to Hh signaling-deficient animals, however this defect has been attributed to reduced Hh expression in these animals⁴⁴. Recent reports also suggest a role for integrin-linked kinase in activation of Shh signaling by mediating translocation of Smo in to the primary cilium⁴⁵. Whether the integrin-mediated interaction promotes cell surface retention of Shh containing vesicles or activates a separate or synergistic signal to contribute to Shh-dependent neuronal marker induction remains to be determined.

Implications of extracellular vesicular miRNA for Shh signaling. miRNAs have received significant attention as modulators of gene expression in development, homeostasis, regeneration and disease. These small RNAs bind to sites in target mRNAs and modulate either stability or translation usually by inhibiting gene expression, but sometimes activating the target⁴⁶. Uncovering this post-transcriptional gene regulatory layer has led to intense efforts to translate the power of miRNA regulation for disease modulation. The discovery that exosomes contain miRNA³⁰ has profound implications for our understanding of control mechanisms, as the influence of a cell may stretch beyond its protein secretome. However, the evidence for the non-cell autonomous effects of specific miRNAs remains controversial and much remains to be learned about the modes of operation of these potent riboregulators beyond cell boundaries. Our finding of a complex set of miRNAs in specific morphogen-transporting vesicles extends the potential signaling impact of morphogens to a new level. Our work shows that not only exosomes derived from cell lines ectopically expressing Shh but also those exosomes derived from primary notochord culture expressing endogenous Shh (Supplementary Table S3–S4, Supplementary Fig. S3 and Supplementary Fig. S4), carry miRNAs. The reproducible detection of overlapping and unique sets of miRNAs particularly in the two classes of notochord exosomes suggests a degree of specificity in miRNA packaging into the extracellular vesicles.

Prediction of the targets of miRNAs in the two systems suggests that there are differences in miRNA targets between Shh-HEK and notochord-derived exo-vesicles. Interestingly, the notochord-derived exo-vesicles contain miRNAs that target developmental pathways such as Wnt, FGF, axon guidance and neuronal development while HEK-derived exosomes appear to target mainly cancer pathways. Thus, the exo-vesicular miRNA content is dynamic, depends on the cells from which they are derived, and importantly, reflects the key signaling features of the source tissue. While the physiological role of secreted miRNA remains unresolved, our findings

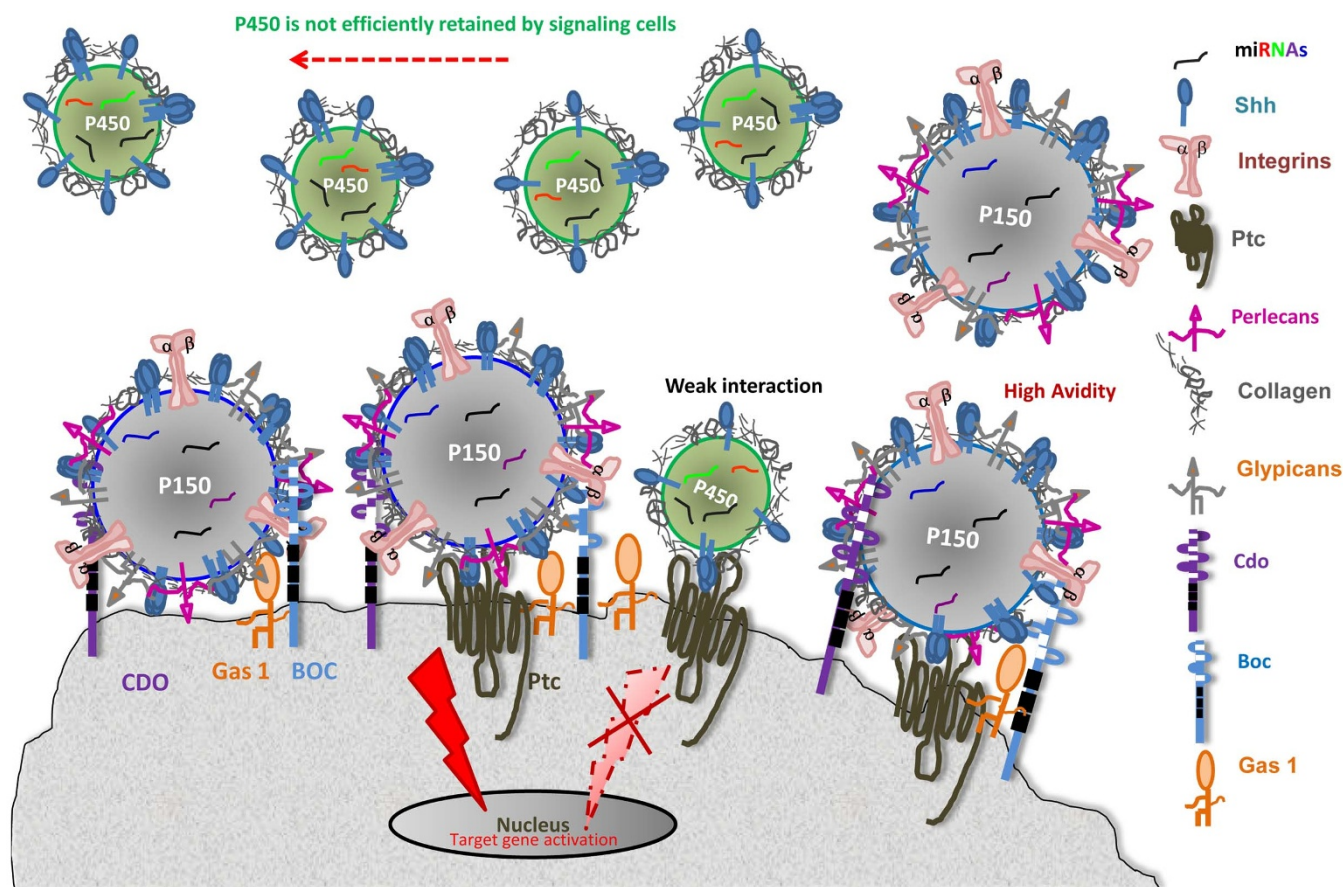


Figure 6 | Model for the observed difference in the signaling competence of the two exosomal pools and importance of accessory proteins. Model represents the predicted difference in the ability of the two pools to activate downstream signal transduction. P150-vesicles contain different cell surface proteins and hence might be retained more efficiently at the cell surface by interaction with the different known interactors of Shh such as Boc, CDO and Gas-1, consequently activating the downstream signaling cascade. P450-pool fails to interact efficiently with the cell surface integrators and hence fails to activate efficient downstream signaling.

suggest a previously un-appreciated complexity in the influence of secreted morphogens like Shh. Thus, apart from activating downstream signaling mediated by the binding of Shh itself to its cognate receptors and co-receptors/accessory modulatory proteins, Shh-exosomes might also be cargo vehicles for miRNAs involved in fine-tuning many parallel pathways within target cells. However, the actual delivery of miRNAs from producer to target cells is yet to be demonstrated. Further, unlike Shh signaling which can be activated by cell surface interaction of these vesicles with Shh receptor & co-receptors, paracrine miRNA delivery will require their fusion with target cells. We suspect that the P150 pool might be involved in closer range Shh-dependent target gene activation, while the P450 pool may travel further and may fine tune other parallel developmental signals operating at a distance via miRNA delivery. Several questions emerge from our studies: How are the alternate fates of exosomes (fusion vs. binding to receptors and co-receptors) regulated? Does interaction of vesicular Shh with its cognate receptor and co-receptors facilitate vesicular fusion? Do the two different vesicular pools differ in the efficiency of miRNA delivery? Answers to these questions will radically change our understanding of morphogen action. In conclusion, our findings reveal that Shh is secreted on two distinct exosomal pools that not only carry signal-modulatory accessory proteins but also ferry cellular miRNAs. Such complex paracrine signaling could have profound implications for development, disease progression and therapeutic intervention.

Methods

Cell culture and transfections. HEK293T cells and Shh-light2 cells were maintained in DMEM medium (Invitrogen) supplemented with 10% FBS (Gibco), 100 mg/ml

penicillin, 100 mg/ml streptomycin and 750 mg/ml Glutamax (Invitrogen). Shh, ShhN, Zyxin-GFP plasmids were transfected using Lipofectamine LTX transfection reagent (Life Technologies) according to manufacturers protocols.

Neuronal differentiation medium: Dulbecco's Modified Eagle Medium: Nutrient mixture F12 (1:1) (DMEM/F12; GIBCO), 1X Penicillin/Streptomycin (GIBCO), 1X GlutaMAX™ (GIBCO), 1X N2supplement (GIBCO), 0.1 mM β-mercaptoethanol (GIBCO), 10% KNOCKOUT™ Serum Replacement (GIBCO).

Neuronal maintenance medium: 1X F12 + GlutaMAX Nutrient mixture (Ham) (GIBCO), 5%, Horse serum (GIBCO), 1X B-27 Supplement (GIBCO), 1X N2 Supplement (GIBCO), 1X Penicillin/Streptomycin (GIBCO).

Notochord culture medium (NCM): 1X MEM Alpha (Minimum Essential Medium), 15% Horse Serum (GIBCO), 2.5% Chick embryo extract (Seralab), 1X Penicillin/Streptomycin (GIBCO), 1X GlutaMAX™ (GIBCO).

Exosome Production Medium (EPM): was modified from the reported protocol⁴⁷ by spinning the fresh culture medium at 450,000 g using Type 90.1 rotor, overnight at 4°C. The resultant supernatant was supplemented with 1X Penicillin/Streptomycin (GIBCO), 1X GlutaMAX™ (GIBCO) and termed as “modified EPM”.

Mouse Embryonic Stem cell line (G4) maintenance medium: Knockout DMEM (GIBCO), 18% Benchmark™ FBS (Gemini Bio-products), 1X Non essential aminoacids (GIBCO), 1X Penicillin/Streptomycin (GIBCO), 1X GlutaMAX™ (GIBCO), β-mercaptoethanol (sigma), LIF was derived from condition medium of Cos-7 cells transiently transfected with LIF cDNA⁴⁸.

Shh secretion assay. Shh-HEK293T stable pool or cells transiently co-transfected with ShhN and Zyxin-GFP were grown to 70%-90% confluency in growth medium. Spent medium was then replaced either by fresh growth medium or modified EPM. Conditioned medium (CM) was collected after 48 hours. Cells and cell debris were removed by sequential centrifugations (at 4°C) at 1000 rpm, 5 mins followed by 5000 rpm, 15 mins and finally at 13,000 rpm for 30 mins. An aliquot of conditioned medium (CM) was processed further to derived different vesicular pools. CM was subjected to 150,000 g for 1 hour 30 minutes at 4°C. The pellet after this spin is the bonafide exosomal pellet (P150). The resultant supernatant (S150) was then spun at 450,000 g for 1 hr 30 minutes at 4°C to isolate the second exosomal pool (P450). The supernatant post this spin is termed as S450.



Protein estimation of samples was done by Bradford Protein Estimation method, (Bradford reagent, Biorad), as per manufacturers protocol.

For western blot, primary antibodies used were α -Shh antibody (Mouse, Invitrogen/Rabbit, CST; 1:500), α -Vps4B (Rabbit, Abcam, 1:1000), α -Rab11 (Rabbit, CST, 1:500), α -GFP, α -EEA1, α -GAPDH, α -Rab 5 and α -Rab 7 (Rabbit, Abcam, 1:500), α -CD 9, α -Tsg101, α -Flotillin 1 and α -Flotillin 2 (Rabbit, Sigma, 1:500), α -Calnexin (mouse, BD, 1:500), α -GM130 (Rat, BD, 1:500). HRP-conjugated secondary α -mouse and α -rabbit (Jackson ImmunoResearch, 1:5000) were used to detect primary antibody binding.

Sucrose gradient density centrifugation: The two vesicular pools derived from the CM were layered on sucrose density gradients ranging from 85% to 10% prepared in 10 ml tubes, centrifuged overnight at 100,000 g, 4°C using Type 90.1 rotor. 1 ml fractions were collected, diluted with 5 volumes of PBS and pelleted at 1,50,000 g for P150 pool and at 450,000 g for P450 pool to purify the vesicles from sucrose solution. The pellets thus obtained were used for subsequent western blot analysis, LC-MS/MS or EM/immuno-EM analysis.

EM/ImmunoEM. Negative EM and immunoEM were performed as described earlier⁴⁷. Antibodies used were α -Shh antibody (mouse, 5E1, DSHB), α -CD63 (mouse, abcam, 1:100). For electron microscopy of the isolated exosomes, a drop of exosomes suspended in PBS was deposited on Formvar-carbon-coated electron microscopy grids, immunolabeled, fixed with 2% PFA and stained as described for ultra-thin cryo-sections⁴⁹. All samples were analyzed using a FEI CM120 electron microscope (FEI Co.), and digital acquisitions were made with a numeric camera (Keen View; Soft Imaging System, SIS, Germany).

Mass Spectrometry. The two different vesicular fractions were isolated from CM of Shh-HEK293T cells as described above. Each vesicular pellet fraction was floated on sucrose gradients and fractions containing the highest amount of Shh (sucrose density 1.12 gm/ml) were first identified by western blot. The peak Shh fractions (~4 mg of total protein) were resolved on 12% SDS-PAGE gel, stained using Coomassie Blue R250, and each Coomassie stained lane was cut into 10–15 different bands and analysed by LC-MS/MS (C-CAMP) after in-gel digestion⁵⁰. For protein/peptide identification MS/MS data was searched against the Uniprot/Swissprot amino acid sequence database (version of Jan 2013) using an in-house MASCOT server (version 2.4; <http://www.matrixscience.com>). The search was set up for full tryptic peptides with a maximum of three missed cleavage sites. Carbamidomethyl and oxidized methionine were included as variable modifications. The precursor mass tolerance threshold was 10 ppm and the maximum fragment mass error was 0.8 Da. The significance threshold of the ion score was calculated based on a false discovery rate of < 1% estimated by the peptide validator node of the Proteome Discoverer software 1.4 (ThermoScientific).⁵¹

Small RNA library preparation and analysis. Total RNA was isolated from the vesicular pools using Trizol (Invitrogen). Vesicles were treated with RNase A, 30 min before resuspending them in Trizol. Small RNA libraries were prepared using the Truseq small RNA library preparation kit (Illumina). Small RNA libraries were subjected to Next generation sequencing using a Hi-seq1000, Illumina platform (C-CAMP). The small RNA reads obtained from HEK cells and chick notochord were mapped to human and *Gallus gallus* genome respectively using the UCSC genome browser. The reads were also mapped to the miRNA database from human and *Gallus gallus* miRNA database from Mirbase. The number of reads mapped to individual miRNAs in each sample was normalized to the total number of mapped reads from the whole sample.

Pathway analysis. The top 20 enriched miRNAs from human P150 & P450 samples were used for target mRNA pathway prediction using mirPath. Experimentally validated miRNA interaction database (Tarbase) and predicted target database (micro-TCDS) both were individually used to predict targets and the overlap between them computed. Results were filtered the data based on FDR corrected p-value ($p < 0.05$). Similarly, the top 20 enriched miRNAs from chick P150 & P450 were also subject to bioinformatics analysis. Since miRNA target database for chick is not available, we used miRanda algorithm to scan selected enriched miRNA against *Gallus gallus* (galGal4) 3'UTRs downloaded from UCSC genome browser, to identify putative targets. The targets were filtered based on the complementarity score (score ≥ 140) and free energy ($\Delta G \leq -14$ Kcal/mol) calculated from miRanda. For the pathway analysis, the targets obtained from *Gallus gallus* were used to fetch corresponding human homologs (orthologs) using the lift up tool, Homologene (<http://www.ncbi.nlm.nih.gov/homologene>). The human orthologs were then fed into GSEA/MsigDB⁵¹ to annotate pathways. Top 20 pathways with FDR q-value < 0.05 were represented as a histogram.

Notochord culture. Notochords were derived from the Day 4 chick embryos and collagenase treated for 15 min at 37°C to dissociate the chords, washed in 1X PBS, resuspended in fresh Notochord Culture Medium (NCM) and plated on pre-gelatinized plates. For western blot analysis of the P150 and P450 pellets derived from notochord cells in culture, equal amounts of total protein was loaded.

Gli-luciferase activity. Shh-light2 cells were plated in 24-well plate and grown to ~90% confluency. Cells were then exposed to different secreted forms of Shh derived from the Shh-HEK293T CM in DMEM containing 0.5% serum, and incubated for

40 hrs before isolation of lysates for luciferase assay (Dual luciferase reporter assay, Promega).

ES to MN differentiation assay. ES cells (G4 strain) were cultured in ES maintenance medium, in suspension for 2 days to induce embryoid bodies (EB) formation. EBs were then cultured in differentiation media supplemented with all-trans-Retinoic Acid (2 μ M RA) or 2 μ M RA plus P150 or P450 derived from the Shh-HEK293T CM and 2 μ M RA plus P150 or P450 derived from HEK293T CM was also used as control. An aliquot of the Shh containing pools was used for estimation of Shh in each fraction by western blotting and densitometry. After 3 days in neuronal differentiation medium, the EBs were disaggregated using trypsin and the single cell suspension was plated on 0.1% gelatin coated coverslip dishes and cultured overnight in neuronal maintenance medium for attachment and spreading to facilitate immuno-staining. Primary antibodies used for immunostaining include HNF3 β (Goat, Santa Cruz, 1:50), Olig2 (Rabbit, Millipore, 1:50). Secondary antibodies Donkey anti-Goat (A488, 1:250, Molecular Probes) and Donkey anti-Rabbit (A568, 1:250, Molecular Probes).

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Author contributions

N.V. conceived the ideas, designed all the experiments, performed and analyzed all experiments except E.M. and miRNA analysis. A.W. helped N.V. in ES to MN differentiation assays. D.T. helped N.V. in biochemical experiments. D.B. and D.P. prepared miRNA libraries. V.L. and D.P. analyzed the NGS data. A.L. and G.R. performed EM analysis. N.V. and J.D. wrote the paper. A.W., D.T., A.L., G.R. and D.P. reviewed the manuscript.

Additional information

Raw data obtained from small RNA sequencing are submitted to NCBI, SRA database. SubmissionID: SUB715604, BioProject ID: PRJNA263979.

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