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### Opening new doors: Hedgehog signaling and the pancreatic cancer stroma

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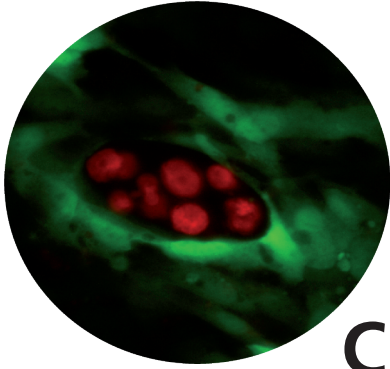
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# CHAPTER

# 4

## **Blocking Hedgehog release from pancreatic cancer cells increases paracrine signaling potency**

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## ABSTRACT

Members of the Hedgehog (Hh) family of morphogens play crucial roles in development, but are also involved in the progression of certain types of cancer. Despite being synthesized as hydrophobic dually lipid modified molecules, and thus being strongly membrane-associated, Hh ligands are able to spread through tissues and act on target cells several cell diameters away. Various mechanisms that mediate Hh release have been discussed in recent years, however, little is known about dispersion of this ligand from cancer cells. Using co-culture models in conjunction with a newly developed reporter system, we were able to show that different members of the ADAM family of metalloproteinases strongly contribute to the release of endogenous, bioactive Hh from pancreatic cancer cells, but that this solubilization decreases the potency of cancer cells to signal to adjacent stromal cells in direct co-culture models. These findings imply that under certain conditions, cancer cell-tethered Hh molecules are the more potent signaling activators and that retaining Hh on the surface of cancer cells can unexpectedly increase the effective signaling range of this ligand depending on tissue context.

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**Key words:** Sonic Hedgehog, shedding, ADAM, pancreatic cancer

## INTRODUCTION

Morphogens are locally secreted proteins that spread from producing cells and define the cell fate of receiving cells at different distances from the source [1]. In both vertebrates and invertebrates, many diffusible signals are essential during development, and dysfunction in the dispersion of these proteins gives rise to severe developmental defects. One of the best-studied group of such molecules is the Hedgehog (Hh) family, consisting of Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh) in mammals [2]. Hh proteins are essential for processes like patterning of the ventral neural tube and specifying digit identities in the limb bud, as well as cell growth and differentiation [3]. Given this wide array of functions, it is not surprising that aberrant activation of the Hh pathway has been implicated in many types of cancer. An especially notorious example of this is pancreatic cancer, in which Shh produced by tumor cells activates the pathway in adjacent stromal cells to aid in the activation of this compartment [4].

Hh ligands are generated as a 45-kDa precursor proteins, which undergo autocatalytic cleavage to produce a 19-kDa N-terminal peptide that has a cholesterol moiety attached to its C-terminus. In addition, a palmitoyl residue is attached at the N-terminus by the transferase Skinny Hedgehog (Hhat), yielding a dually lipid-modified ligand that is strongly hydrophobic and hence associated with membranes. This has long raised questions as to how it can reach cells located at a distance from the cell of origin. Notably, both lipid modifications are essential for normal biological activity [5-7].

Several molecular mechanisms of how Hh ligands travels long distances are currently under intense debate. Proposed models of Hh solubilization include release through the transmembrane protein dispatched 1 (DISP1), oligomerization of proteins and formation of 'micelle-like' structures, co-transport with lipoprotein particles, and proteolytic release by metalloproteinases [8-13]. The latter mechanism was described only recently and involves the A desintegrin and metalloproteinases (ADAM) family proteins.

The family of ADAM proteins is important for the release of proteins from producing cells, a process termed shedding. Substrates include members of the EGF receptor family ligands, Notch receptors, TNF $\alpha$  and the amyloid precursor protein (APP) [14]. Not surprisingly, ADAMs have been implicated as playing an important role in diseases such as Alzheimer's, chronic inflammation, and cancer [14]. ADAMs have also been shown to mediate shedding of Hh ligands by cleavage of Hh oligomers from the surface of Hh producing cells, leading to the formation of biologically active signaling complexes [12, 15]. These initial studies were based on overexpression systems and it is still unknown whether ADAMs can be involved in processing and release of endogenously expressed Hh molecules.

In the present study, we define the role of ADAM metalloproteinases in the release of endogenous Hh from pancreatic cancer cells. Using chemical stimulation of sheddase activity, profiling of cancer tissue and cells, and knockdown studies, we show that Hh release from cancer cells can be mediated by ADAM10, ADAM12 and ADAM17. We further show that blocking the release of Hh leads to an increase in cell bound Hh

protein, which results in both an enhancement of juxtacrine signaling as well as an expansion of the signaling range.

## RESULTS

### **Pancreatic cancer cells activate Hh-responsive fibroblasts that are several cell diameters away**

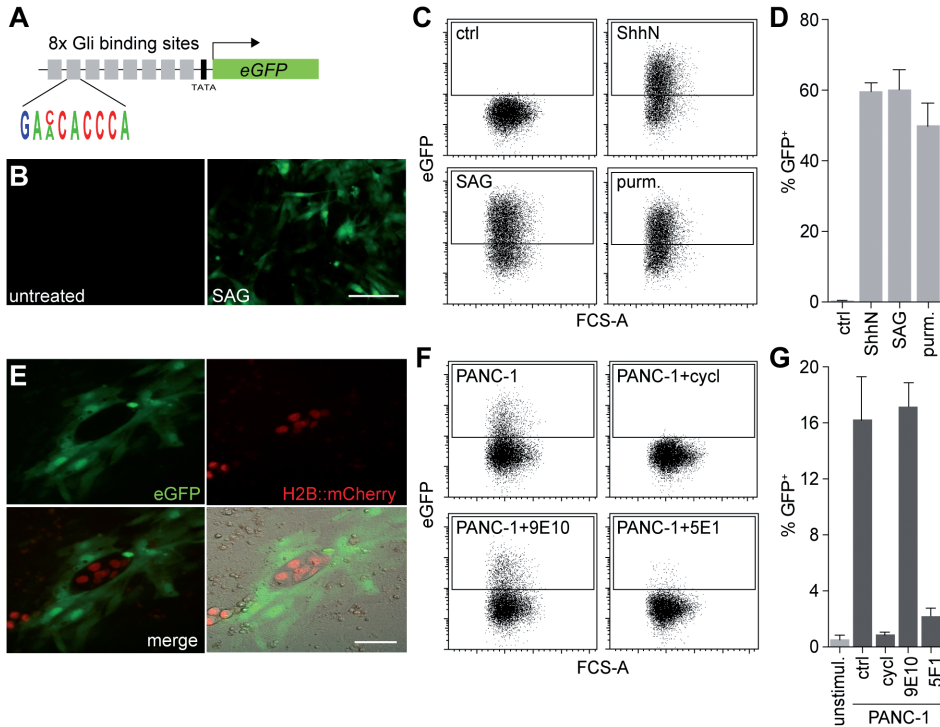
Developmental biology has yielded elegant models to study Hh distribution, like the neural tube and the *Drosophila* imaginal discs. These tissues can be imaged to reveal the spread and functional consequences of Hh ligand as it forms a gradient. For tumor biology, such imaging was not previously available. To monitor Hh pathway activity in a spatial and temporal manner, we developed a lentiviral reporter construct that expresses eGFP under the control of a promoter containing eight concatemeric Gli-binding site (GBS) motifs and a TATA box (Fig. 1A). Mouse embryonic fibroblasts (MEFs) transduced with the reporter construct (GBS-GFP MEFs; denoted GGMs), showed a wide dynamic range of reporter activity. Almost no GFP expression was observed under unstimulated conditions, as measured by fluorescence microscopy (Fig. 1B) and flow cytometry (Fig. 1C), indicating very low basal Hh pathway activity in GGMs. Treatment with ShhN (a C-terminally truncated form of Shh lacking the cholesterol modification) or Smo (Smo, a downstream effector of Hh signaling) agonists SAG and purmorphamine led to a strong induction of GFP expression (Fig 1C, D).

To test whether this reporter system could also be used to study the activity of Hh endogenously expressed in pancreatic ductal adenocarcinoma (PDAC) cells, we co-cultured GGMs with the PANC-1 cell line and assessed the proportion of GFP-positive cells by flow cytometry. PANC-1 cells robustly activated the reporter construct in GGMs and co-administration of the Smo inhibitor KAAD-cyclopamine or the Hh blocking antibody 5E1 strongly diminished this activation, showing that GFP activity in the fibroblasts was specifically mediated by Hh derived from PANC-1 cells (Fig. 1F, G).

Intriguingly, we did not only observe Hh pathway activation in cells directly adjacent to cancer cells with mCherry-tagged histone 2B (H2B), but also three to four cell diameters away from the source of Hh ligand in a gradient-like manner, with the GGMs closest to the cancer cells having stronger GFP signal (Fig. 1E; supplementary material Fig. S1). Hence, this system can be used to quantitatively assess the diffusion capacity of cancer-cell-produced forms of Hh ligand.

### **Sheddases can release Hh protein from pancreatic cancer cells**

Several mechanisms for the release of Hh protein have been described in recent years. In overexpression systems it has been shown that one of these release mechanisms involves the catalytic activity of members of the ADAM family of metalloproteinases, especially ADAM17 [12]. The process of metalloproteinase-mediated cleavage and release of membrane bound substrates, is a regulated process that can be rapidly stimulated, for example by activation of protein kinase C (PKC) by phorbol 12-myristate 13-acetate (PMA), and via G-protein-



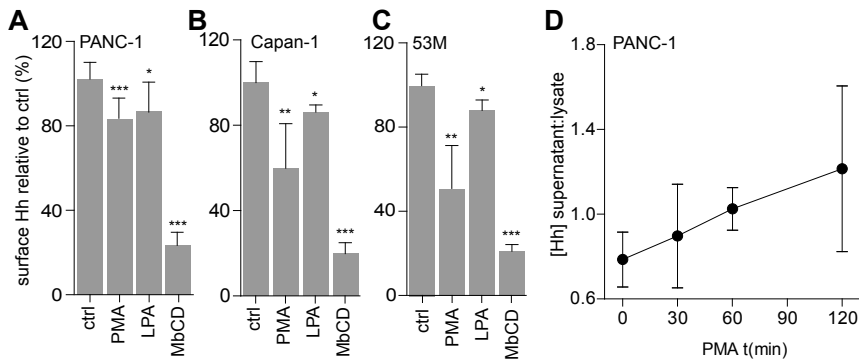
**Figure 1.** Pancreatic cancer cells activate the HH pathway several cell diameters away from the source in an eGFP reporter cell line. (A) Schematic representation of the lentiviral reporter construct containing 8x Gli-binding site (of which the consensus sequence is shown) driving the expression of eGFP. Construct was used to create the stable MEF reporter cell line GGM. (B) Representative fluorescence images of GGMs treated for 72h with 500nM SAG. Scale bar: 100  $\mu$ m. (C) FACS plots of reporter MEFs treated for 72h with conditioned ShhN supernatant, 500nM SAG or 1  $\mu$ M purmorphamine (purm.). (D) Mean percentage of GFP positive reporter cells from treatment experiments as shown in panel C. Results are mean  $\pm$  s.d.,  $n \geq 8$ . (E) 2000 H2B-mCherry-labeled PANC-1 cells were seeded on top of confluent GGMs and imaged after 72h. Scale bar: 50  $\mu$ m. (F) GGMs and PANC-1 cells were co-cultured, and 100nM KAAD-cyclopamine (cycl) or 2  $\mu$ g/ml Hh-blocking antibody 5E1 was added for the duration of the experiment to show specificity of the pathway activation. 9E10 is an anti-Myc antibody clone. (G) Mean percentage of GFP positive reporter cells from treatment experiments as shown in panel F. Shown is mean  $\pm$  s.d.,  $n \geq 7$ . All experiments were performed in 0.5% FCS.

coupled receptor activation through the addition of lysophosphatidic acid (LPA) [16-18]. To test whether ADAM sheddases can play a role in solubilization of Hh from pancreatic cancer cells, we treated PANC-1 cells with known stimulators of sheddase activity and measured the amount of surface bound Hh protein by flow cytometry. All tested stimuli significantly reduced the levels of Hh on these cells (Fig. 2A). As a positive control, methyl- $\beta$ -cyclodextrin, which extracts cholesterol and as a consequence sterolated Hh from the cell surface, was included [19]. In addition, this compound has been demonstrated to activate sheddases [12]. Treatment with cyclodextrin resulted in a near complete loss of Hh on the cell surface. Similar results were obtained with Capan-1 cells, and the patient derived primary cell line 53M, (Fig. 2B, C).

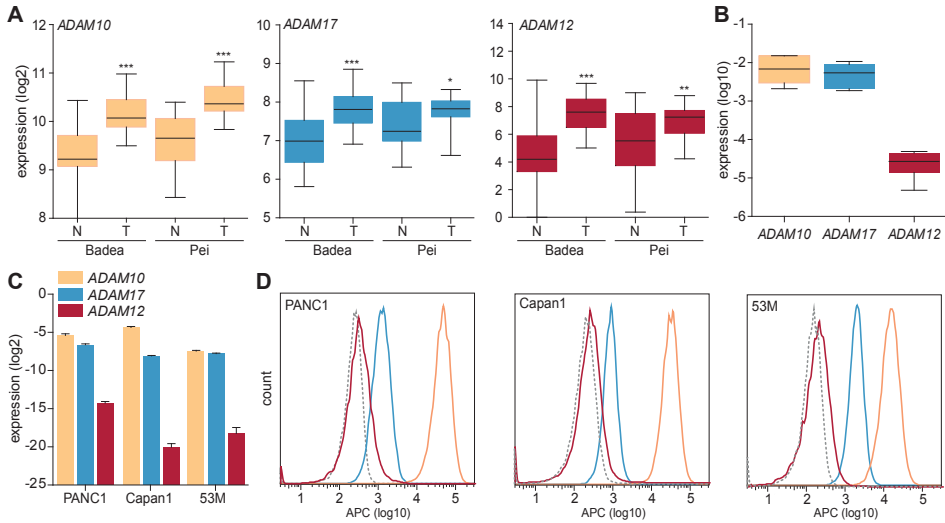
To demonstrate that Hh is indeed released into the medium of treated cells after sheddase stimulation, we collected the supernatant of PANC-1 cells treated with PMA and quantified the amount of Hh protein in the supernatant and cell lysates. Indeed, the supernatant to lysate protein ratio increased over time (Fig. 2D), showing that the addition of PMA results in a displacement of Hh ligand from the cell membrane to the aqueous extracellular environment. To formally assess the involvement and requirement for ADAMs in the observed release of Hh, we decide to pinpoint and ablate the exact molecules involved.

### Expression of ADAMs in pancreatic cancers, patient-derived xenografts and cell lines

To identify which ADAM proteins could mediate the release of Hh from pancreatic cancer cells, we used two publically available microarray datasets from PDAC patients [20, 21] to profile the expression of ADAM10, ADAM12 and ADAM17, which have been previously implicated in shedding of overexpressed Hh from cells [15]. All proteins studied were expressed in healthy pancreatic tissue, but were significantly upregulated in tumor tissue, consistent with previous observations [22, 23](Fig. 3A). We also found similar expression of these ADAMs in pancreatic cancer patient-derived xenografts (PDXs) measured by quantitative real-time PCR (qPCR), with ADAM10 and ADAM17 being expressed considerably highly than ADAM12 (Fig. 3B). The pancreatic cancer cell lines used for the shedding assay (Fig. 2) displayed comparable expression levels to the PDX tumors (Fig. 3C).



**Figure 2.** Hedgehog protein can be released from the surface of pancreatic cancer cells in response to stimulation of sheddases. Pancreatic cancer cell lines PANC-1 (A) and Capan-1 (B), or primary pancreatic cancer cells 53M (C) were treated with 100 ng/ml PMA, 10  $\mu$ M LPA, or 1 mg/ml methyl- $\beta$ -cyclodextrin (MbCD) for 1 h in serum-free medium. Surface expression of HH ligand was detected by flow cytometry using the anti-Hh antibody 5E1 and results are displayed as geometric mean fluorescence intensity (gMFI) after subtraction of isotype gMFI relative to control treatment. Data show the mean  $\pm$  s.d.,  $n \geq 3$ , \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . (D) PMA-mediated release of endogenous Hh into serum-free supernatant was measured over time. Plot shows quantification of Hh protein levels in supernatant (sup) relative to that in lysates of PANC-1 cells treated with 100 ng/ml PMA for 30, 60, and 120 min. Supernatant was subjected to 5E1 immunoprecipitation before detection of Hh protein by western blotting. Cells were lysed directly prior to blotting. Data show the mean  $\pm$  s.d.,  $n \geq 3$ .



**Figure 3.** ADAM sheddases are expressed in pancreatic cancers, patient-derived xenografts and pancreatic cancer cell lines. (A) Box plots showing log<sub>2</sub> transformed gene expression values from two microarray datasets of pancreatic cancer patients comparing normal (N) and tumor tissue (T). Badaea set (GSE15471); n=36 paired biopsies, Pei set (GSE16515); n=15 (normal), n=36 (tumor). \*P<0.05, \*\*P<0.01, \*\*\*P<0.001. (B) The levels of the indicated transcript levels measured on seven different patient-derived xenografts by qPCR using species-specific primers. The results show the distribution of ADAM transcripts relative to human *GAPDH*, with the box indicating the 25<sup>th</sup>-75<sup>th</sup> percentiles, with the median indicated. The whiskers show the range. (C) qPCR analysis of ADAM gene expression relative to human *GAPDH* in PANC-1, Capan-1 and 53M cells. Data show the mean  $\pm$  s.d., n=3. (D) Surface expression of ADAM10, ADAM12 and ADAM17 on cell lines was confirmed by flow cytometry using antibodies directed against the ectodomain of these proteins. Representative histograms are shown. Colors represent proteins as indicated in the panel C legend. Grey dashed line shows isotype control measurements.

For ADAMs to be able to act on their substrates, they must be present in an activated form on the plasma membrane [24]. To measure this active pool of ADAMs, protein levels at the cell surface were determined by staining with ectodomain specific antibodies in flow cytometry assays (Fig. 3D). All ADAMs measured were present on the surface of pancreatic cancer cells and could thus potentially mediate Hh release.

### ADAM knockdown leads to retention of biologically active Hh protein on the surface of cells

Having identified ADAM10, 12, and 17 as proteins that could potentially be involved in Hh shedding from PDAC cells, we proceeded to ablate these proteins in cancer cells by stable short hairpin RNA (shRNA) transductions, and assess the amount of Hh protein on the surface of these cells. Successful knockdown was confirmed by qPCR and flow cytometry (supplementary material Fig. S2). Cells carrying an shRNA directed against Sonic Hedgehog (SHH) were included as a control for the specificity of staining as well as subsequent signaling assays. A positive control for the effects of a blocked Hh release came



from the knockdown of Dispatched 1 (DISP1). Although the exact working mechanism of this protein is not fully understood, its involvement in the spread of lipid modified Hh from mammalian cells is well established [11, 25, 26] and indeed, knockdown of DISP1 led to an increase in surface levels of Hh as measured by immunofluorescent staining (Fig. 4A) and flow cytometry (Fig. 4B). Knockdown of any of the three ADAM proteins implicated in the shedding of Shh had similar (ADAM10), or even stronger effects (ADAM12 and ADAM17), on the retention of Hh on the cell surface compared to knockdown of DISP1 (Fig. 4A,B). This suggests that these proteases function to release Hh from the surface of tumor cells. Surface retention of Hh was also observed in the absence of serum (data not shown).

To assess whether the pool of Hh that accumulates on the cell surface following silencing of ADAM17 (or DISP1) is still amenable to dispersal by DISP1, and to confirm that the effects we observe are not due to subcellular sorting artefacts that affect Hh distribution prior to its presentation at the surface, we overexpressed DISP1 in these cells and assessed whether this could reduce the accumulated surface Hh. Indeed, overexpression of DISP1 was found to rescue the effect of ADAM17 and DISP1 silencing (Fig. 4C), and this effect was most obvious in cells with the highest levels of surface Hh. A form of DISP1 that carries a mutated residue in the fourth transmembrane region that is crucial to its antiporter function was included as a control. This protein (D99Y) was unable to rescue the effect of ADAM silencing, and in fact was found to result in increased Hh levels in control silenced cells. This is probably a consequence of the dominant negative action such a mutant molecule could have in the previously described trimers that DISP1 functions in (see [25]). These results show that the fraction of Hh that accumulates on the cell surface in the absence of ADAM17 can still be transferred by DISP1 to some other machinery that disperses Hh from the cell surface. This in turn implies that in the absence of ADAM17, DISP1 is a rate-limiting step in the redirection of Hh and that the two proteins act on the same pool or fraction of Hh.

The strong surface retention of Hh observed in DISP1 or ADAM knockdown cells suggests that less Hh is released into the medium from these cells. To confirm this to be the case, we compared the amount of Hh in these supernatants and the corresponding lysates. Indeed, ablation of DISP1 or ADAM resulted in a strongly reduced supernatant:lysate ratio of Hh (Fig. 4D) and again, ADAM17 knockdown had the strongest effect. What was also obvious from these experiments was that a large fraction of the total Hh present in the cell culture resided in the supernatant as compared to the cell. This seems to suggest that these cells are very actively transporting Hh from the cell surface into the medium, for instance by the action of ADAMs. This notion is further supported by the finding that although the amount of Hh detected by Western blot or FACS in the SHH knockdown cells was very low, some residual ligand could still be detected in the supernatant. At the very least, these data strongly suggest that in PDAC cells, ADAMs function to shed or otherwise move Hh from the surface into the medium.

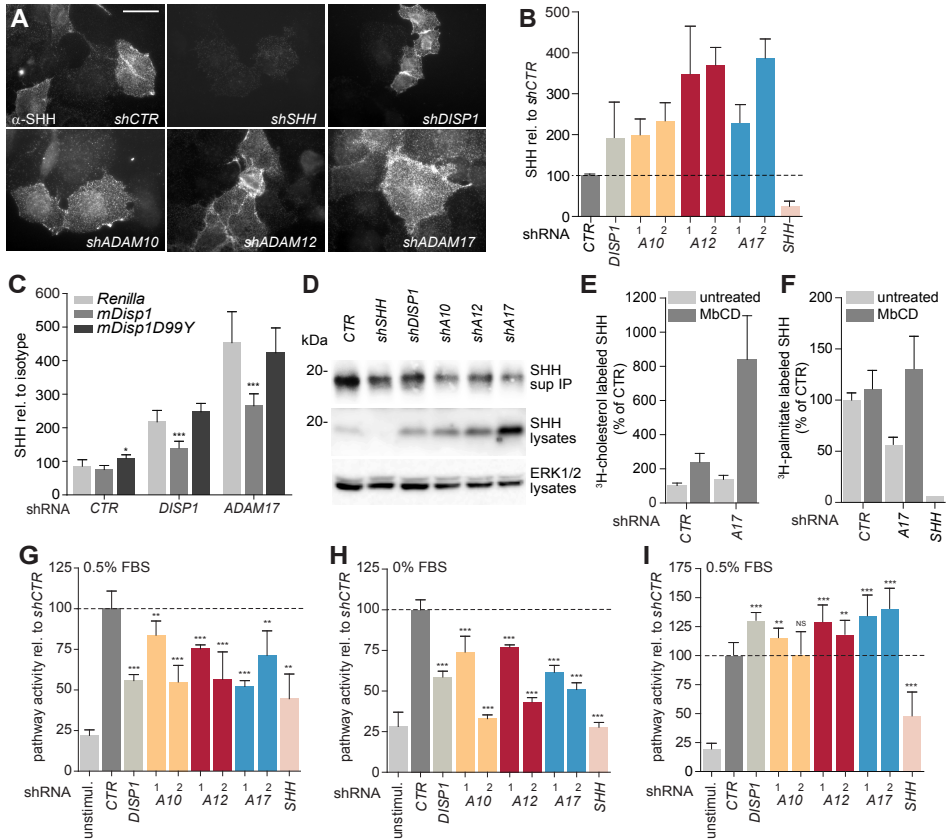
ADAM-mediated release of Hh has been reported to involve proteolytic processing of Hh proteins, resulting in the removal of the cholesterol moiety [15]. A consequence of this model holds that any Hh that is medium-borne in the absence of ADAM function should still

be sterolated. To formally confirm this to be the case in our experimental setup, we loaded PDAC cells with [<sup>3</sup>H]-cholesterol and assessed the amount of sterolated Hh in the supernatant. Although the total amount of Hh protein in the supernatant from ADAM17 knockdown cells was found to be strongly reduced (Fig. 4D), the amount of sterolated Hh in the supernatant was found to be equal or even higher compared to control, suggesting that indeed ADAM17-mediated processing involves removal of cholesterol from the mature Hh protein (Fig. 4E). Addition of cyclodextrin to the medium mobilized sterolated Hh from the cell surface, resulting in a strong increase in sterolated Hh in the supernatant (Fig. 4E, dark grey bars). This effect was especially apparent in the ADAM17 knockdown cells. In addition, this confirms that the effect of cyclodextrin on Hh release from the surface is at least in part due to extraction of the protein from the membrane owing to its cholesterol modification [19], rather than acting solely through the activation of sheddase activity [12]. To assess whether palmitoylated Hh is also extracted from the cell surface by cyclodextrin, we loaded PANC-1 cells with [<sup>3</sup>H]-palmitate and repeated the experiment (Fig. 4F). In control silenced cells, palmitoylated Hh was not extracted by cyclodextrin, suggesting that the fractions of sterolated and palmitoylated Hh do not necessarily overlap. In ADAM17 silenced cells, however, palmitoylated Hh was extracted by cyclodextrin. This suggests that in the absence of ADAM function, the SHH accumulating on the cell surface is more likely to have both lipid modifications.

To assess the functional consequences of Hh solubilization by ADAMs, we treated Shh-LIGHT II reporter cells (stably transfected 3T3 cells with GLI-driven firefly luciferase and constitutive TK-promoter-driven *Renilla* luciferase expression)[27] with conditioned medium from the different knockdown cell lines. As expected from the reduced Hh protein levels in the supernatant following ADAM ablation, we also observed a significant decrease in signaling activity of the conditioned medium of all the tested knockdown cells (Fig. 4G). These findings were confirmed in the complete absence of serum (Fig. 4H). However, when we performed direct co-culture experiments with the knockdown PANC-1 cells and luciferase reporter cells to allow juxtacrine signaling we observed a clear increase in Hh pathway activity with cells displaying high Hh levels on the surface (Fig. 4I). Interestingly, despite the apparent abundance of Hh ligand in the medium, the supernatants were less effective inducers of pathway activity than the co-cultured cells. The experiments mentioned above show that cells can release biologically active Hh into the medium, and that stopping this release strongly reduces activity in medium transfer experiments; they also show that enhancing the presence of cell-tethered forms of Hh increases the direct signaling capacity of cells in co-culture. This effect is apparently strong enough to overcome the effects of reduced signaling by solubilized Hh in the system.

### **Blocking release of Hh leads to an increase in signaling range**

To confirm our finding that the surface bound form of Hh is a more potent pathway activator than shed Hh, we performed co-cultures of different knockdown cells with the GGMs, which in contrast to the luciferase reporter system allow us to determine the position of responding cells relative to the source of ligand. Consistent with the previous



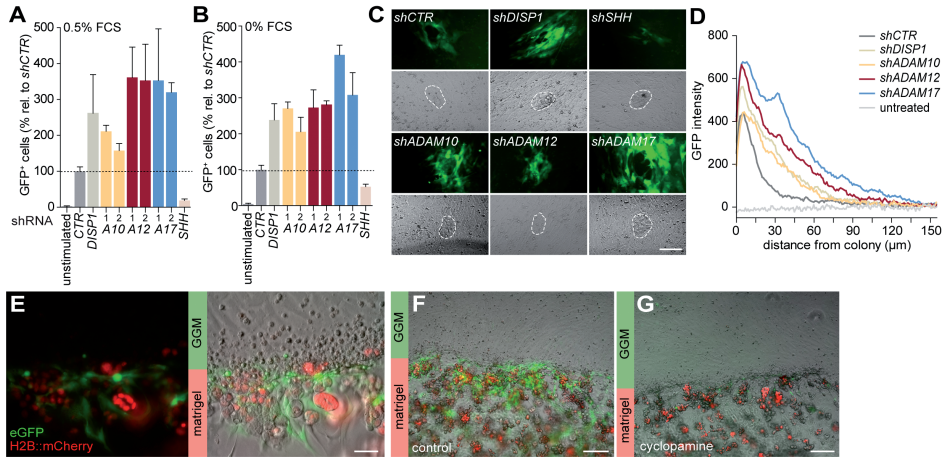
**Figure 4.** Knockdown of ADAMs in pancreatic cancer cells leads to retention of HH on the cell surface and increased juxtacrine signaling. (A) The indicated genes were silenced in PANC-1 cells and following confirmation of knockdown, cells were seeded on coverslips, grown in 8% FCS, and immunofluorescence using 5E1 on live cells was performed. Detection, mounting, and imaging were performed after fixing. shRNA against *SHH* was included as a control for the specificity of the staining. shCTR, control shRNA. Scale bar: 30  $\mu$ m. (B) Hh surface levels of the cells used in panel A were measured by flow cytometry analysis using 5E1. All shRNA clones targeting the ADAM proteases (A10, A12 and A17 represents *ADAM10*, *ADAM12* and *ADAM17*, respectively) as well as *DISP1* showed an accumulation of Hh protein on the surface. The isotype-corrected gMFI of several experiments is plotted relative to control silenced cells (CTR). Data show the mean  $\pm$  s.d.,  $n \geq 3$ . Experiments were performed in 8% FCS. (C) Cells silenced for *DISP1*, *ADAM17*, or control were cotransfected with eGFP and *Renilla* luciferase (control), mouse *DISP1* (*mDisp1*), or mouse *DISP1* D99Y (*mDisp1D99Y*) as indicated. After 3 days, cells were harvested and stained for surface Hh. Data show the average isotype corrected geometric mean of the GFP<sup>+</sup> population,  $\pm$  s.d.,  $n=6$ . Significance was calculated compared to respective *Renilla* control in every cell line. \* $P < 0.05$ , \*\*\* $P < 0.001$ . Experiments were performed in 8% FCS. (D) Serum-free conditioned supernatant (sup) from cells silenced for the indicated genes for 48 h was subjected to immunoprecipitation (IP) before western blotting. Lysate was also analyzed from cells lysed directly in Laemmli buffer. Note that the membranes-containing lysates or immunoprecipitated samples were run simultaneously, but imaged separately, and that the loaded fraction of total protein was ~threefold higher in the immunoprecipitation blot (10% of lysate ran vs. 33% of immunoprecipitate eluate). (E) Control or *ADAM17* silenced PANC-1 cells were seeded, loaded with [<sup>3</sup>H]-cholesterol or [<sup>3</sup>H]-palmitate (F) and serum-free conditioned supernatant from cells that had been cultures for 4 days with addition of 300  $\mu$ g/ml methyl- $\beta$ -cyclodextrin (MbCD) during the last 24 h. Supernatant was subjected to immunoprecipitation

- with 5E1 and radioactivity was measured in the precipitated protein. Graph shows disintegration events per minute relative to untreated control cells. Shown is mean  $\pm$  s.d., n=3. (G) Medium containing 0.5% FCS or 0% FCS (H) was conditioned on the indicated cells and supernatant was prepared. Shh-LIGHT II cells were treated with diluted supernatants (1:2) for 24 h after which pathway activation was measured by assessing the firefly luciferase activity normalized to constitutive *Renilla* luciferase, and expressed relative to results from supernatant from control silenced PANC-1 cells. Data are shown as mean  $\pm$  s.d., n $\geq$ 3, \*P<0.05, \*\*P<0.01, \*\*\*P<0.001. (I) Shh-LIGHT II cells were co-cultured with indicated PANC-1 cells in 0.5% FCS and pathway activity was measured after 24 h. Data are displayed relative to control silenced cells and show the means  $\pm$  s.d., n $\geq$ 6, \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

observation in the luciferase assays (Fig. 4I), we found that co-culture with cancer cells silenced for DISP1 or ADAMs lead to a strong increase in the percentage of GFP positive reporter cells measured by flow cytometry (Fig. 5A, B), showing that this effect is independent of the readout used for Hh pathway activation. Again, these data seem to suggest that cell-tethered Hh is a strong signaling form and that its release from the cell surface is detrimental to its signaling capacity. These findings are corroborated by the lack of responsiveness of GGMs to conditioned supernatant from PANC-1 cells (data not shown) but their high sensitivity to cell-tethered ligand (Fig. 5A, B).

To test whether this increase in signaling potential of cells with high Hh surface expression is related to the range of signaling, we performed the same co-cultures and imaged these. DISP1 and ADAM knockdown cells showed clearly more GFP-positive fibroblasts around the cancer cell colonies with the reporter cells close to the source displaying higher GFP intensity compared to control silenced cells (Fig. 5C). To measure the actual distance of the signaling range in distance, we quantified the spread of GFP signal from at least 20 colonies of each condition (Fig. 5D). We found that especially interference with ADAM17 and ADAM12 increased the intensity and range of Hh pathway activity in the cells adjacent to the cancer cells. While co-culture with control silenced cells yielded a signal reaching around four cell diameters, the range in shADAM17 cells was doubled to eight cell diameters, showing that increase in direct transsignaling ability of cells with high Hh surface levels can contribute to an increased signaling range away from the ligand source. The effects of ADAM silencing on the signaling capacity of cancer cell-derived Hh was confirmed in a primary line (supplementary material Fig. S3).

These data again suggest that, at least in this model, cell-tethered Hh is a more effective form of this ligand as opposed to its soluble counterparts. To confirm this in another imaging setup that more strictly distinguishes between cells in close proximity and medium-borne crosstalk, we devised a co-culture model in which there was a fairly strong physical separation between the producer and responder cells. By first placing cancer cells, mixed in a Matrigel cushion, in a culture dish and subsequently placing GGMs on top, we found that the latter cell type only contacted the cancer cells after migrating into the Matrigel laterally (Fig. 5E). Following this, we could observe reporter activity only in those GGMs in or under the Matrigel cushion, but not in those that were merely in close proximity to the border of this structure (Fig. 5E, F). Adding cyclopamine to the cultures abrogated the response, proving the observed GFP expression was mediated



**Figure 5.** Cell-membrane-tethered Hh protein increases the range of signaling to adjacent reporter cells. (A) Cells silenced for the indicated genes were seeded on top of GGM reporter cells as for Figure 4, and after 3 days cells were harvested and GFP positive cells were quantified by flow cytometry. Data are displayed as percentage relative to control silenced PANC-1 cells, and shown is the mean  $\pm$  s.d.,  $n \geq 14$ . Experiments were performed in 0.5% FCS. (B) As for panel A, but using 0% FCS. (C) Representative images of co-cultures 72 h after seeding. Upper panels show GFP positive cells activated by Hh ligand from the tumor cells. Lower panel show the corresponding bright field image with cancer cell colonies demarcated by a dashed line. Scale bar: 100  $\mu$ m. (D) Images of co-cultures with indicated PANC-1 cells were taken on a fluorescence microscope and GFP intensity was quantified using ImageJ as a function of the distance away from the cancer cell colonies. One cell diameter equals 15  $\mu$ m on x-axis. Graph represents measurements of over 20 cells. (E)  $4 \times 10^5$  H2B-mCherry labeled PANC-1 cells were seeded in 50  $\mu$ l growth factor reduced Matrigel and after 48 h, GGMs were seeded on top. Representative image of co-cultures 3 d after seeding is shown. Green and red bars indicate the border of the Matrigel cushion. Scale bar: 50  $\mu$ m (F) As for panel D, 6 d after seeding. (G) As for panel E, but including 100nM KAAD-cyclopamine. Scale bar: 200  $\mu$ m (E-G). Experiments were performed in 0.5% FCS.

through Smo rather than some non-canonical activation of the Gli transcription factors as a consequence of the seeding conditions (Fig. 5G). Thus, we conclude that Hh that is released from cancer cells will not activate signaling in the GGMs and again argues in favor of a model of Hh distribution that does not include solubilization.

## DISCUSSION

A wide range of release and transport mechanisms for Hh proteins from source cells have been described over the years in different, mainly developmental, model systems. It appears that Hh secreting cells can make use of not one, but several of these mechanisms to achieve differential biological output over short and long distances, and that the mode of Hh distribution can be influenced by the environmental context [28]. However, in the setting of malignancies in which the ligand is aberrantly expressed in tumor cells and mostly signals to adjacent stroma, such as pancreatic cancer [4], no studies have addressed the exact signaling range of Hh. By using chemical inducers of sheddase activity together with interference of

ADAM activity on a transcriptional level, a clear role of ADAM sheddases in the release of endogenous Hedgehog from pancreatic cancer cells has now become apparent.

All three ADAMs previously reported to release overexpressed Hh from Bosc23 cells were found to be involved in Hh release from pancreatic cancer cells, but some interesting things were noted in these experiments: silencing of the various paralogs did not result in equally strong surface retention of Hh, and the silencing of some ADAMs seemed to affect Hh surface levels to an extent that it was incompatible with a possible redundancy between the three ADAMs tested. One explanation for this could be the difference in expression levels of the various ADAM proteins. For instance, the very high expression levels of ADAM10, even after knockdown, could be responsible for some residual catalytic activity of this protein, resulting in incomplete surface retention of Hh. Conversely, ADAM12 was expressed at much lower levels, and as a consequence it was reduced to almost undetectable, and possibly inactive, levels following knockdown. A second explanation comes from a potential cross-regulation in our knockdown experiments: the activity or surface localization of one particular ADAM might depend on the presence of other proteases. Indeed, the surface localization and activation of matrix metalloproteinase 14 (MMP-14) has recently been shown to depend on ADAM12 [29], and conceivably such an interaction could also exist between the different ADAM family members in our system. A third possibility is that proper processing of Hh relies on the sequential action of several ADAMs, and that taking out one of the ADAM proteins will affect this sequential action, resulting in more surface retention of Hh than would be expected. Although these considerations make it more difficult to pinpoint the exact roles of the individual ADAMs, they do not detract from the conclusion that ADAM sheddases are involved in the release of Hh from pancreatic cancer cells.

Previous studies regarding the activity of proteins involved in Hh transport or dispersion have relied on biochemical methods such as size-exclusion chromatography. However, the amount of endogenously expressed Hh in our studies was rather limited and currently not amenable to in-depth biochemical analysis to determine, for instance, the cleavage sites or aggregate status. As a consequence, the use of the conformational epitope antibody 5E1 in conjunction with flow cytometry has been crucial to this study and we found it to be more sensitive and less technically challenging than conventional biochemical methods. It also allows easy quantification on overall cell populations.

A question from developmental biology that remains unanswered in our study is why the phenotypes of ADAM mutant mice described in literature do not show any of the classical Hh pathway mutant hallmarks such as holoprosencephaly, digit patterning abnormalities, or laterality defects [30]. Interpretation of these mouse mutants, however, is difficult and could be complicated by the compensatory activities of different sheddases with shared ligand specificity, resulting in very weak phenotypes. Furthermore, our data suggest that loss of ADAM function would result in an enhanced Hh signaling, and Hh pathway gain-of-function phenotypes are typically much harder to discern. It is conceivable that more targeted observations of for instance neural tube patterning in these animals

would demonstrate a role for Adams in modulating Hh levels *in vivo*. In the absence of such data at this moment, our results should be interpreted with care.

Although at first glance counterintuitive, our finding that retention of Hh protein on the surface of cancer cells leads to an increase in signaling range is perfectly compatible with a direct transport model of membrane-bound ligand. One such transport mechanism comes from the filopodia-like cell protrusions named cytonemes, initially described in the fruit fly [31]. These dynamic actin-based extensions span several cell diameters and can transport Hh molecules to target tissue [32]. In addition in the chick neural tube, Hh has been shown to be transported through long cytoplasmic extensions that span several cell diameters [33]. The exact location, inside or outside the cytoneme, as well as the actual form of the ligand transported, is still under debate.

Increasing the amount of Hh on cell protrusions, by knocking down ADAMs for instance, would allow this ligand to reach the threshold necessary to activate the pathway in receiving cells, therefore extending the range of signaling in our reporter cell system. Although we were able to detect actin based extensions from PANC-1 cells carrying a *LifeACT-mCherry* construct, these extensions only bridged over maximally two cell diameters towards reporter cells, which is not far enough to explain the activation observed up to eight cell diameters away as observed in the case of ADAM17 silenced PANC-1 cells. Reminiscent of a model proposed in *Drosophila* for Dpp signaling [31], it could also be the case that responsive fibroblasts reach out for ligand with their own cytonemes, therefore shortening the necessary reach of the cancer cells to activate the receiving cells, as they would 'meet in the middle'. In addition, we cannot exclude the trivial explanation that our current live imaging microscopic capacities are inadequate for visualizing finer structures that could potentially reach far enough.

An alternative explanation for the increased signaling range would be a model in which the block of release of Hh from the surface of producer cells leads to more Hh protein being accessible for transport from one reporter cell to the next through planar diffusion on heparan sulfate proteoglycan (HSPG) molecules. Studies in *Drosophila* have shown a crucial requirement of the glypicans *dally* and *dally-like*, the fruitfly equivalent of mammalian HSPG molecules, as well as the glycosaminoglycan transferase *tout-velu*, for activation of target cells at large distances from the source of Hh ligand [34, 35]. However, in vertebrate systems HSPGs have also been shown to affect the spread of Hh ligands [8, 11]. In agreement with this model is the previous observation that ADAM-mediated processing decreases the binding capacity of Hh to HSPGs by inactivating the heparin sulfate binding motif [36]. Decreasing ADAM-mediated processing would therefore enhance Hh binding to HSPG molecules on producer cells, fitting the results in our study from ADAM knockdown cells. As a consequence, more HSPG-sequestered ligand could be handed to reporter cells and the threshold needed for activation would be reached in reporter cells further away from the ligand source. The data shown in supplementary material Fig. S4 at least suggest that release of Hh by competing with HSPG binding is detrimental to transsignaling, but whether this is due to solubilization or masking of

Ptch1-binding residues cannot be concluded. Both the models of cytoneme transport and HSPG-bound lateral diffusion are not mutually exclusive, as Hh transported on cytonemes could be HSPG associated, allowing for enrichment of Hh on these structures.

In conclusion, our data show that ADAMs can act on Hh expressed in cancer cells, and that they generate soluble, biologically active forms of this protein. More importantly however, is the functional consequence of this solubilization: In our experimental setups, diffusion of Hh limits its concentration in the relevant compartment where it exerts its biological effect and, conversely, retention of Hh on the surface of cells increases the effective signaling range *in trans*. To exclude the possibility that our conclusions are based on models that possibly overestimate the contribution of cell-tethered Hh or otherwise skew the results in a non-physiological way, future studies will be needed to address the relevance of different ligand distribution mechanisms as well as effect of ADAM manipulation on Hh target genes in the context of pancreatic cancer. In addition, a better understanding of how the stroma in this disease is activated by tumor cell-derived Hh is especially relevant in light of recently published work that shows that outright ablation of Hh signaling in the stroma can have unexpected detrimental outcomes (Rhim et al., 2014; Lee et al., 2014).

## MATERIAL AND METHODS

### Cell Culture and Chemicals

PANC-1, Capan1, HEK293T (ATCC, Manassas, VA), and mouse embryonic fibroblasts [(a gift from Matthew P. Scott [(Stanford University, School of Medicine, Stanford, CA)[37]], were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 8% fetal bovine serum (FBS), L-glutamine, penicillin and streptomycin (all from Lonza, Basel, Switzerland) according to routine cell culture procedures. Shh-LIGHT II cells ([27], ATCC) were grown in the abovementioned medium supplemented with 400 µg/mL neomycin (Sigma, St Louis, MO) and 150 µg/mL zeocin (Invitrogen, Carlsbad, CA). 9E10 and 5E1 hybridoma cells (Developmental Studies Hybridoma Bank, Iowa City, IA) were maintained in RPMI containing 10% FBS, L-glutamine, penicillin and streptomycin (all from Lonza). Hybridoma cells were switched to serum free medium for antibody production. Treatment of pancreatic cancer cell lines with 100 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma), 1 mg/ml methyl-β-cyclodextrin (MβCD, sigma), or 10 µM lysophosphatidic acid (LPA, Sigma) was performed in serum-free DMEM for 1 h at 37°C. Subsequently, cells were washed twice with phosphate-buffered saline (PBS) and processed for flow cytometry analysis.

### Flow Cytometry

Cell were harvested with trypsin-EDTA solution (Lonza) and washed in FACS buffer (PBS containing 1% FBS). Antibodies were diluted in FACS buffer and incubated 30 min at 4°C. Concentrations of antibodies used were: ADAM10; 1:500 (MAB1427 from R&D, Minneapolis, MN), ADAM17; 1:100 (R&D, MAB9301), ADAM12; 1:500 (Sigma, SAB2100046); and conditioned hybridoma supernatant containing either anti-Hh antibody



5E1 (0.08 µg/ml) or anti-Myc antibody 9E10 (1 µg/ml); diluted 1:5 in FACS buffer before used for staining. Secondary APC-labeled anti-mouse-IgG (BD, 550826) or anti-rabbit-IgG antibodies (southern biotechnology, 4050-115) were diluted 1:500. After washing cells were resuspended in FACS buffer containing 1 µg/ml Propidium Iodide (PI) (Sigma) and acquired on a FACSCanto II (BD, Franklin Lakes, NJ). Data were analyzed with FlowJo 7 (Tree Star, Ashland, OR). From the PI negative fraction the geometric mean fluorescence intensity (gMFI) in the APC channel was calculated and the gMFI from the isotype control was subtracted from the respective staining yielding the change in gMFI.

### **Immunoprecipitation and detection of Hedgehog by Western blot**

Equal amount of cells were seeded in six-well plates and the next day, 1 mL serum-free medium was added and the cells were incubated for a further 48h. Supernatants were harvested and cleared of cells by centrifugation at 500 g for 5 minutes. Shh was precipitated from the supernatants by addition of 5E1 hybridoma supernatant 1:5 overnight, and 30 µL Protein A/G beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 2h. Elution was performed using Laemmli sample buffer (Biorad) with DTT. For detection of cell-bound Shh, cells were directly lysed. Samples were loaded on 4-15% gradient SDS PAGE gels. Following transfer and blocking with 5% milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T), membranes were incubated in anti-Shh antibody (H160, Santa Cruz Biotechnology) at 1:2000 overnight. All appropriate secondary antibodies were used at 1:5000. Proteins were visualized using a FujiFilm LAS 4000 imager (GE Life Sciences, Pittsburgh, PA). Band intensity was quantified in ImageJ using the Gel Analyzer plugin, and supernatant:lysate ratio was calculated. For determining the (input corrected) ratio between cell-bound and supernatant Shh, supernatant and lysate signals of control shRNA-transduced PANC-1 cells were quantified from the same blot and exposure, and corrected for fraction of sample loaded on gel. This ratio was used to normalize the ratios in control transduced PANC-1 cells in all other experiments.

### **Immunofluorescence staining**

Transduced PANC-1 cells were grown on coverslips, incubated with 5E1 supernatant (0.016 µg/ml) for 1h at 37 °C, and fixed using 4% formaldehyde. Following blocking and permeabilization in 5% goat serum in phosphate-buffered saline with 0.1% Triton X100 (PBS-T), Alexa-Fluor-488-conjugated anti-mouse-IgG secondary antibody (Invitrogen) was added at 1:2000 for 1h at room temperature and slides were mounted using ProLong Gold (Invitrogen) and images were taken on a Zeiss AxioVert microscope.

### **Constructs**

The 8x3'GLI binding site sequence (GBS) was isolated from the GBS-luciferase reporter construct [38] by PCR using the following primers (forward 5'-TATAACCGG TACAGATTCGCGATCGACC-3'; reverse: 5'-ACTTGCTAGCTGCAGGTCGACTCTAGAGGAT-3')

introducing Agel and NheI restriction sites. The digested PCR fragment was cloned into the lentiviral reporter vector pRRL TOP-d2GFP after excision of the TCF/LEF binding sites using XmaI and XbaI. The d2GFP was then replaced by eGFP from the pRRL TOP-GFP vector [39] using XhoI and EcoRI. All enzymes were purchased from New England Biolabs (Ipswich, MA). The H2B-mCherry expression construct was obtained by removing the TOP site from the Wnt reporter vector TOP-GFP-PGK-H2BmCherry (a gift from Harmen van Andel, Academic Medical Center, Amsterdam, The Netherlands). The expression construct for mouse DISP1 was a kind gift from Philip A. Beachy (Stanford University, School of Medicine, Stanford, CA). The D99Y proton channel mutant was made using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA), and the following primers (forward 5'-TCGGGATTGGCGCGTACGACGCTTTGTC-3'; reverse 5'-GACAAAAGCGTCGTACGCGCCAATCCCGA-3'). Silencing construct sequences (shRNA) in pLKO from the Sigma Mission library: DISP1, CATTTCAGAAGCATCTCGAA; ADAM12 #1, GCTGCCGGATTGTGGTTTAT; ADAM12 #2, CCTCGCAAATCCCATGACAAT; ADAM17 #1, CCTGGTTACAACCTCATGAATT; ADAM17 #2, CCTATGTGCATGCTGAACAAA; ADAM10 #1, GCAGTTCTATCTGTGAGAAA; ADAM10 #2, GCTGTGCAGATCATTAGAT; SHH, CTACGAGTCCAAGGCACATAT.

### Lentiviral transduction

Lentiviral particles were produced by transiently transfecting HEK293T cells with pLKO constructs from the Sigma Mission library, TurboGFP™-targeting control shRNA (shc004) or the GBS-GFP reporter and the packaging plasmids pMD2.G and psPAX2 with calcium phosphate. At 48 h and 72 h after transfection the supernatant was harvested, filtered through a 0.45 µm filter (Millipore, Germany) and subsequently used to transduce 60% confluent PANC-1 cells or MEFs in the presence of 7 µg/ml polybrene (Sigma) overnight. At two days after transduction, cells were selected for stable expression of shRNA with 2 µg/ml puromycin (Sigma) and knockdown efficiency was analyzed by qPCR and flow cytometry.

### GGM reporter cell line

MEFs transduced with the GBS-GFP reporter construct were sorted on a BD FACSAria for GFP expression after stimulation with ShhN conditioned supernatant from HEK293T cells for four days. Sorted GFP positive cells were grown under 8% FCS conditions, which resulted in loss of GFP activity. For reporter assays, GGM cells were seeded in 96 well plates (Greiner) and upon becoming confluent, were treated with 500 nM of the Smoothed agonist SAG (EMD Millipore, Billerica, MA), 1 µM purmorphamine (EMD Millipore), ShhN conditioned supernatant or 2000 cancer cells per well in DMEM containing 0.5% FCS. For blocking experiments 100nM KAAD-cyclopamine (Merck Millipore), or hybridoma supernatant containing 5E1 or 9E10 was added to the co-cultures. After 3-5 days, cells were imaged on a Zeiss fluorescence microscope or on an Operetta High Content Imaging System (PerkinElmer, Waltham, MA) and the percentage of GFP-positive cells was determined by flow cytometry on a FACSCanto II.

### Patient-derived xenografts and establishment of the primary cell line 53M

Collection of material from patients undergoing surgical resection for pancreatic adenocarcinoma in our institute was approved by the institute's ethical committee. Informed consent was obtained. Tumor pieces originating from primary tumor or liver metastasis were washed several times in PBS and grafted with Matrigel (BD) subcutaneously into the flank of immunocompromised NSG mice (JAX 005557). Animals were bred and maintained at the local animal facility according to the legislation and ethical approval was gained for the establishment of patient derived xenografts. Upon reaching a size of 500 mm<sup>3</sup>, PDX tumors were harvested and transplanted in a new animal. After the second passage of the liver metastasis derived 53M PDX, xenograft tissue was dissociated with collagenase IV (0,5 mg/ml, Sigma) and hyaluronidase (20 µg/ml, Sigma) and isolated cells were grown in 8% FBS containing DMEM. Purity of the epithelial culture was assessed by human specific EpCAM antibody staining (DAKO, F0860 at 1:100).

### Quantitative real-time PCR

PDX tissue pieces or cells grown in culture were homogenized and lysed in Trizol (Invitrogen) and RNA was isolated according to the manufacturer's protocol. cDNA was synthesized using Superscript III (Invitrogen) and random primers (Invitrogen). Real-time quantitative RT-PCR was performed with SYBR green (Roche, Basel, Switzerland) on a Lightcycler LC480 II (Roche). Relative expression of genes was calculated using the comparative threshold cycle (Cp) method and values were normalized to those for the reference gene *GAPDH*. Primer sequences used are; *GAPDH*, forward 5'-GAAGGTGAAGGTCGGAGTC-3'; reverse 5'-TGGAAGATGGTGATGGGATT-3'; *ADAM12*, forward 5'-TTCCACCACCCTCTCAGAC-3'; reverse 5'-GCCTCTGAAACTCTCGTTG-3'; *ADAM10*, forward 5'-TTCGATGCAAATCAACCAGA-3'; reverse 5'-TTCCTTCCCTTGACAGTCT-3'; *ADAM17*, forward 5'-GGGAACA TGAGGCAGTCTCT; reverse 5'-ACCGAATGCTGCTGGATAT-3'; *SHH*, forward 5'-CGGCT TCGACTGGGTGACT-3'; reverse 5'-GCAGCTCCCGATTGG-3'; *DISP1*, forward 5'-ACAG CTTTTCTGCGACGTT-3'; reverse 5'-TCCATAATGTCTCCCCTCCA-3'.

### Luciferase reporter assay

Shh-LIGHT II cells were grown to confluency in a 96 well plate (Greiner), starved overnight in 0.5% FBS containing DMEM and treated with conditioned supernatant or 50,000 cancer cells per well. After 24h of treatment, cells were lysed in 20 µl of lysis buffer, scraped, freeze-thaw cycled, and luciferase activity was assayed according to the Dual-Glo Luciferase Assay System (Promega, Madison, WI) protocol on a Biotek Synergy HT plate reader. Each firefly luciferase value was corrected for its CMV-driven *Renilla* luciferase standard to control for nonspecific effects. For production of conditioned supernatant, an equal amount of PANC-1 cells with different knockdown constructs were seeded in a 6 well plate, adhered overnight and supernatant containing 0.5% FCS was harvested after 24 h. Conditioned supernatant was spun at 500 g for 10 min in a conventional table top centrifuge and diluted 1:10 in 0.5% FCS medium for treatment of Shh-LIGHT II cells.

### **Transfection and ShhN production**

HEK293T cells were transfected with the expression plasmid for full length ShhN (in pRK5 (a kind gift from Henk Roelink; University of California at Berkeley, Berkeley, CA) using the calcium phosphate method. For production of ShhN conditioned medium used in signaling assays, DMEM containing 0.5% FCS was incubated on cells starting 24h after transfection for 2-3 days before harvest. PANC-1 cells were transfected in 12-well plates (Greiner) with 1 µg plasmid DNA per well (0.25 µg spectrin-GFP; 0.75 µg CMV-Renilla, mouse DISP1 or mouse DISP1 D99Y) using 3 µg polyethylenimine (Sigma).

### **Tritium labeling**

Cells were plated in a 10 cm dishes (Greiner), and upon reaching 70% confluency, medium was changed to serum free DMEM containing 55.5 kBq of [1,2(n)-<sup>3</sup>H] cholesterol or [<sup>3</sup>H] palmitate (GE Healthcare). After 72 hours, 300 µg/ml MbCD (Sigma) was added to the dishes and medium was collected 24h later. Supernatant was cleared by centrifugation for 5 min at 500 g, and immunoprecipitation was performed as described above. Washed agarose beads were resuspended in 4 ml Scintillation liquid (PerkinElmer). Samples were vigorously shaken and disintegrations per minute (DPM) were measured using the liquid scintillation analyser TRI-carb 2000CA (PerkinElmer).

### **Proliferation assay**

PANC-1 cells were seeded in 96 well plates at a density of 1000 cells per well. After adhesion overnight, cells were washed with PBS, medium containing 8%, 0.5% or 0% FCS was added and cell viability on the same day (= day 1) was measured by addition of 20 µl CellTiter-Blue reagent (Promega) to the respective wells. After 4 hours incubation at 37°C, the fluorescence signal was measured on a Biotek Synergy HT plate reader. Measurements were repeated on day 2 and day 3 after seeding.

### **Statistical analysis**

Data were analyzed by Students *t*-test using Graph Pad Prism 5 (GraphPad Software, La Jolla, CA). The mean ± s.d. was determined with a significance level of  $p > 0.05$ .

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### **Competing interests**

The authors declare no competing interests.

### **Author contributions**

H.D., V.L.V., and M.F.B. designed and performed experiments, analyzed data and wrote the manuscript. J.P.M. and H.W.M.v.L. helped with manuscript preparation

and experimental design. J.A.M.G.T. performed the analysis of data from patients and helped obtaining patient-derived xenografts.

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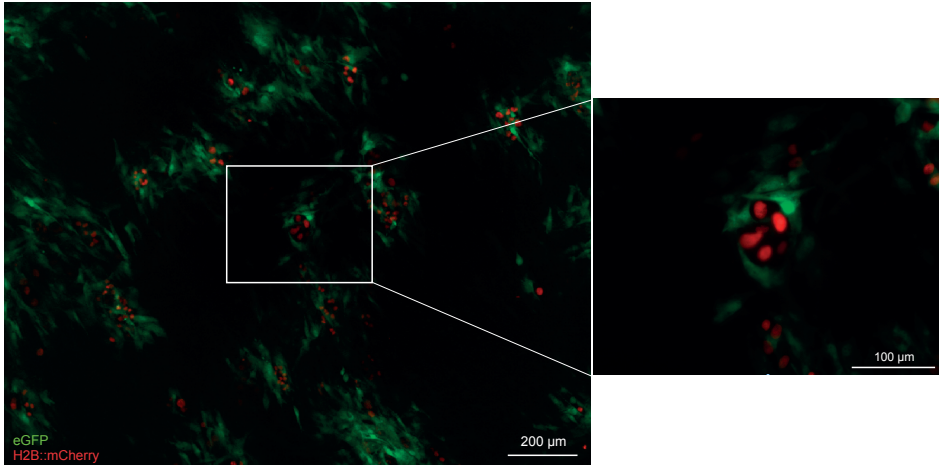
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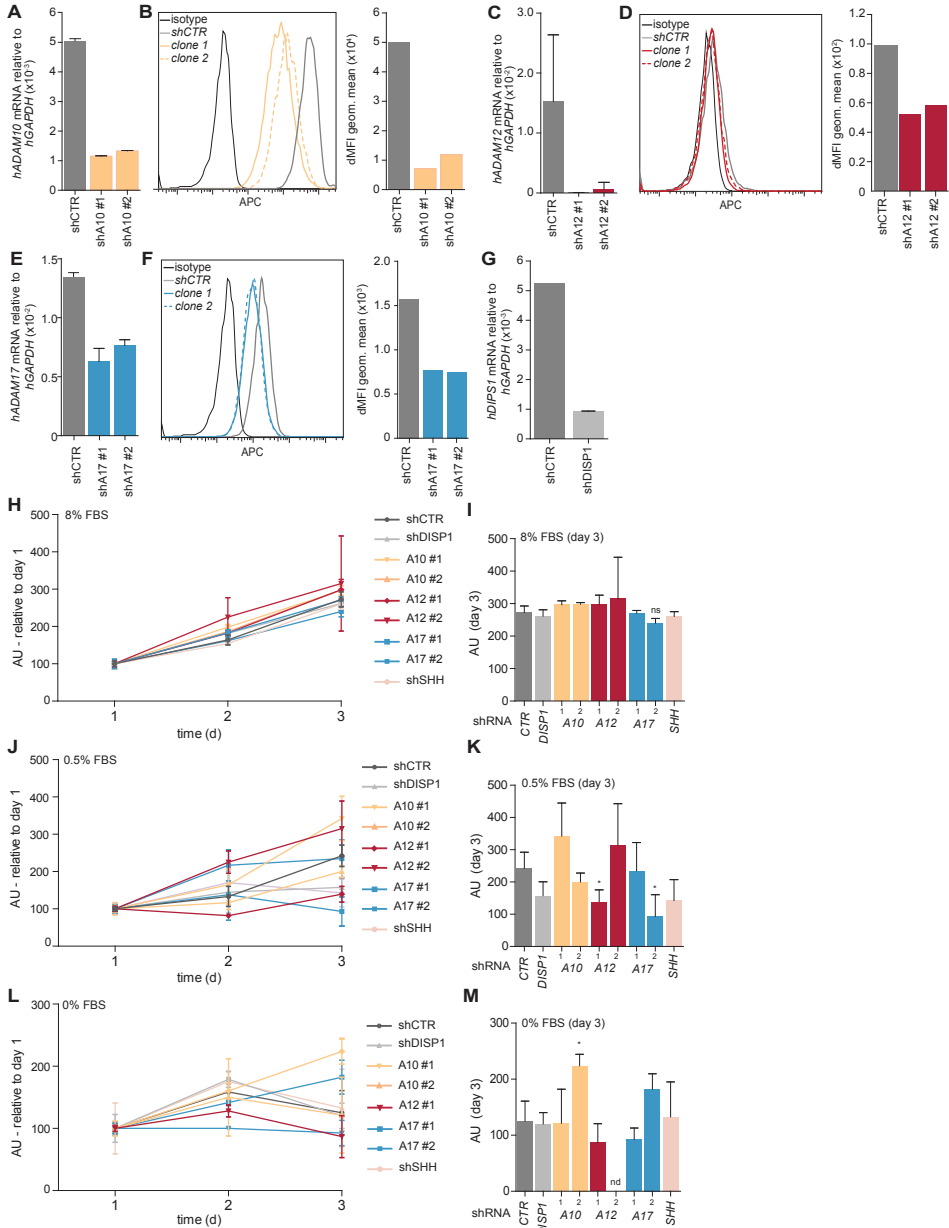
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## SUPPLEMENTARY INFORMATION

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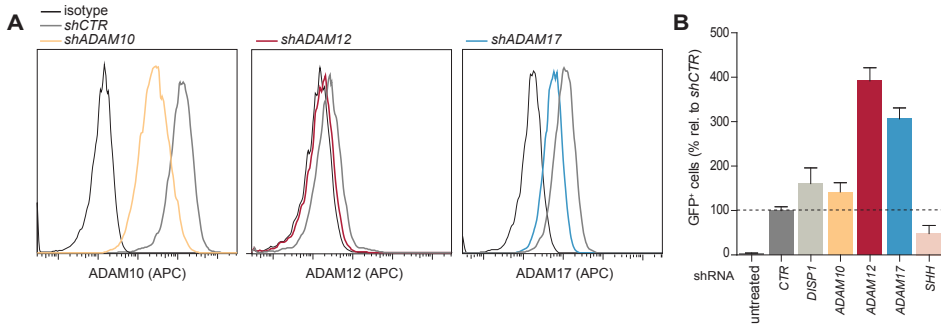


**Figure S1.** Representative fluorescence image showing a low and high magnification field of GGMs on which 2000 H2B-mCherry labeled PANC-1 cells were grown for 4 days. Cells were imaged live on a Zeiss fluorescence microscope. Scale bar size indicated in panels. Experiment was performed in 0.5% FCS.

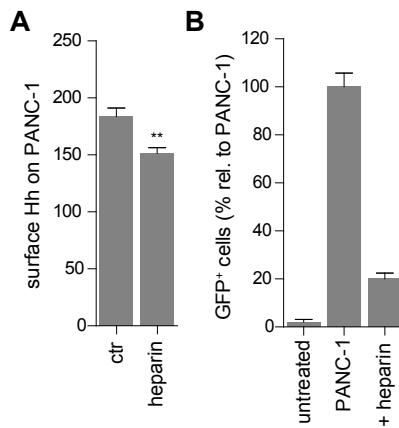


**Figure S2.** Knockdown efficiency of ADAMs and DISP1 in PANC-1 cells. qPCR showing efficient knockdown on transcript level for ADAM10 (A), ADAM12 (C), ADAM17 (E), and DISP1 (G). Data show the mean  $\pm$  s.d. relative to GAPDH, n=3. Downregulation of surface ADAM protein level was confirmed with flow cytometry staining using antibodies directed against the ectodomain of ADAM10 (B), ADAM12 (D), and ADAM17 (F). Representative histogram overlays are shown. Right panel shows isotype corrected geometric mean fluorescence intensity (dMFI) of respective ADAM staining. Proliferation assay of all knockdown cell lines over the period of 3 days in 8% (H), 0.5% (J) and 0% FCS (L) and bar graph depicting results on day 3 relative to day 1 in the respective serum concentrations (I,K,M). Data show mean  $\pm$  s.d, n=3. \*P<0.05, ns. not significant; nd. not determined.





**Figure S3.** Knockdown of ADAMs in 53M leads to increased transsignaling capacity. (A) shRNA-mediated knockdown of ADAMs in 53M cells was confirmed by flow cytometry. Representative histogram overlays are shown. Cells were grown in 8% FCS prior to analysis. (B) Indicated 53M knockdown cell lines were seeded on top of confluent GGM reporter cells and after 4 days, GFP percentage was determined. Data show the means  $\pm$  s.d.,  $n \geq 8$ . Experiments were performed in 0.5% FCS.



**Figure S4.** Effects of heparin on Hh surface levels and transsignaling capacity. (A) PANC-1 cells were treated with 400  $\mu\text{g}/\text{mL}$  heparin (Sigma) for 4h in serum free medium, and Hh surface levels were measured by flow cytometry. Shown is the geometric mean fluorescence intensity (gMFI) of the HH surface staining after subtraction of isotype gMFI as mean  $\pm$  s.d.,  $n=3$  (B) GGMs and PANC-1 were cocultured as for Figure 5A for 3 days in serum free medium, with or without the addition of 400  $\mu\text{g}/\text{mL}$  heparin. Data are normalized to GFP<sup>+</sup> cells in PANC-1 co-culture and show the means  $\pm$  s.d.,  $n \geq 7$ .