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EXTENDED EXPERIMENTAL PROCEDURES

Chemicals

Photocholesterol (6-azi-5α-cholestan-3β-ol, here referred to as 6-azicholestanol) was synthesized as previously described (Thiele et al., 2000). To synthesize 25-azi-27-norcholesterol (here referred to as 25-azicholesterol), 100 mg 25-oxo-27-norcholesterol (Steraloids) dissolved in 20 ml anhydrous methanol was stirred on ice, under inert gas. Ammonia gas was bubbled for 1 hr, after which 100 mg hydroxylamine-O-sulfonic acid in anhydrous methanol was added, and the reaction was allowed to warm to room temperature. After another 2 hr, the crude reaction was filtered, supplemented with 0.5 ml triethylamine, and concentrated under reduced pressure. The residue was dissolved in methanol and 1 ml triethylamine, and 10% iodine in methanol was added drop-wise until the yellow color persisted. After excess iodine was removed by addition of sodium thiosulfate, the solution was evaporated under reduced pressure, and the crude product was purified by flash chromatography using an automated system (Biotage Isolera One, linear gradient of 10%–60% ethyl acetate in hexanes). TLC solvent system: 40% ethyl acetate/hexanes, stained with 2% H_2SO_4 in ethanol, starting material R_f = 0.32, product R_f = 0.41. Final yield = 25 mg (24%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.35, 3.52, 2.26, 1.56, 0.99, 0.89, 0.67; ^{13}C NMR (400 MHz, $CDCl_3$): δ 140.75, 121.67, 71.79, 56.72, 55.86, 50.08, 42.27, 39.73, 37.23, 35.61, 35.49, 35.46, 34.71, 31.88, 31.63, 28.20, 28.19, 24.24, 21.05, 20.54, 19.38, 18.56, 11.82; HR-ESI-MS m/z $[M\text{-}H_2O]^+$ Found: 380.2145, calculated for $C_{26}H_{40}N_2^+$: 380.3192.

DNA Constructs

The cDNA for full-length *Xenopus tropicalis* Scube2 was obtained from ATCC and the open reading frame was subcloned by PCR into the mammalian expression vector, pCS2+. The inactive Scube2ΔCUB mutant has a stop codon after amino acid 635, as in the ty97 mutation of zebrafish Scube2 (Woods and Talbot, 2005). Amino-terminally tagged Scube2 constructs were generated in the pCS2+ vector and contain the signal sequence of human calreticulin, an HA tag or five myc tags, followed by the Scube2 sequence beginning with amino acid 24 (the first residue after the predicted signal sequence cleavage site). Untagged and tagged Scube2 constructs behaved identically in activity assays. Constructs encoding secreted Halotag (Los and Wood, 2007) were assembled in pCS2+ and contained the calreticulin signal sequence followed by an HA tag and the Halotag sequence. This sequence was fused to the sequence of hShh encoding amino acids 190–462 (to express secreted, cholesterol-modified Halotag) or to the sequence of hShh encoding amino acids 190–198 (to express secreted Halotag without cholesterol). Mouse DispA constructs were C-terminally tagged with either five myc epitopes or mCherry, and were subcloned in pCS2+ and in a vector for lentiviral production. The DispA constructs used were the WT, the inactive NNN and AAA mutants (Ma et al., 2002), and the inactive DispA-Δloop1 mutant (missing amino acids 220–483). hShh (untagged or C-terminally tagged with an HA epitope), hShh-C24S (the hShh mutant that cannot be palmitylated), and hShhN (the N-terminal fragment of hShh, amino acids 1–198) were cloned into pCS2+ and into the retroviral vector pLHCX (Clontech).

Cell Culture

Human 293T cells, MEFs, and mouse 3T3 cells were cultured by standard methods. Stable cell lines were generated by retroviral and/or lentiviral transduction, followed by selection with hygromycin and/or blasticidin, as previously described (Chen et al., 2011). For immunofluorescence microscopy, cultured cells were fixed with formaldehyde and stained with anti-myc (9E10, mouse monoclonal) and anti-ShhN (rabbit monoclonal; Cell Signaling) antibodies, followed by detection with Alexa-594- and Alexa-488-conjugated secondary antibodies (Invitrogen). The cells were imaged on an inverted Nikon TE2000E microscope equipped with an OrcaER camera (Hamamatsu) and 100x PlanApo 1.4NA or 40x PlanApo 0.95NA objectives. Images were collected using Metamorph software (Applied Precision).

Radioactive pulse-chase assays were performed as previously described (Chen et al., 2011). To detect DispA binding to hShh by immunoprecipitation, 293T cells expressing myc-tagged DispA (WT, DispA-Δloop1, DispNNN, or DispAAA) and full-length hShh or hShhN were lysed on ice in 20 mM HEPES, pH 7.5, 150 mM NaCl, 0.5% dodecyl-β-maltoside (DDM; Anatrace), supplemented with protease inhibitors (Roche). The lysate was clarified by centrifugation at 20,000 *g* and the supernatant was subjected to immunoprecipitation with anti-myc antibodies (9E10; Roche). The precipitated proteins were separated by SDS-PAGE, and hShh was detected by western blotting with rabbit anti-hShhN antibodies (Cell Signaling).

For production of Scube2 and Scube2ΔCUB, 293T cells transiently or stably expressing Scube2 or Scube2ΔCUB constructs were washed with DMEM to remove serum and then grown in DMEM for 24 hr. The conditioned media were harvested, centrifuged to remove debris, and concentrated using a 10 kDa cutoff centrifugal filter device (Millipore).

To detect Scube2 binding to hShh, serum-free conditioned media from 293T cells coexpressing HA-tagged Scube2 or Scube2ΔCUB, and hShh or hShhN were subjected to immunoprecipitation with the anti-hShh monoclonal antibody 5E1. The precipitated proteins were immunoblotted with anti-HA antibodies to detect Scube2 and Scube2ΔCUB.

To determine whether photoreactive sterols modify hShh in vivo, 293T cells stably expressing hShh-HA (Chen et al., 2011) were sterol-depleted by incubation with 1% MCD in DMEM for 45 min. Sterols were then added back as soluble MCD complexes in DMEM supplemented with the proteasome inhibitor bortezomib (1 μM, to block degradation of hShh and the C-terminal fragment, hShhC) and the HMG-CoA reductase inhibitor pravastatin (20 μM). Sterol-MCD complexes were prepared as previously described

(Klein et al., 1995), and contained sterol and MCD at a molar ratio of 1:10. After 3 hr, the cells were harvested and hShh processing was assayed by immunoblotting with anti-HA antibodies (3F10; Roche), to detect hShh and hShhC, and with anti-ShhN antibodies.

Analyzing Hh and Halotag Secretion

293T cells stably expressing hShh-HA or hShhN were washed several times with DMEM to remove serum, and then incubated in DMEM for the desired amount of time in the absence or presence of indicated additives. In some experiments, DispA constructs were stably coexpressed with the hShh constructs. Scube2 and Scube2ΔCUB were added as conditioned serum-free media or were coexpressed with hShh and DispA constructs. The cells were harvested and lysed, and the protein in the culture medium was precipitated with TCA. hShhN was analyzed by SDS-PAGE followed by immunoblotting, and tubulin or GSK3 served as the loading control. 293T cells expressing HA-tagged Halotag, with or without the cholesterol modification, were assayed similarly to determine Halotag secretion.

Reporter Assays

Hh activity assays were performed using Shh Light II cells (Taipale et al., 2000), a stable NIH 3T3 line that expresses firefly luciferase under the control of an Hh-responsive promoter and Renilla luciferase under the control of a constitutive promoter. Hh stimulation was performed as previously described (Tukachinsky et al., 2010). After 30 hr, Renilla and firefly luciferase activity was measured using the Dual-Glo kit (Promega). Hh pathway activity was calculated as the firefly/Renilla luciferase ratio, normalized to one for unstimulated cells. Each experiment was performed in triplicate, and error bars represent the SD of the mean. For coculture assays, 293T cells or DispA−/− MEFs expressing various hShh, DispA, and/or Scube2 constructs were plated together with Shh Light II cells in the indicated ratios. After 12 hr, serum was washed off and the cells were incubated for 30 hr in serum-free media, followed by luciferase reporter assays as described above.

Photocrosslinking in Cells

Human 293T cells stably expressing myc-tagged DispA or the inactive control DispA-Δloop1, and full-length hShh or hShhN were sterol-depleted as described above, followed by incubation with sterol-MCD complexes in OptiMEM (Invitrogen) supplemented with 20 μM pravastatin for 2 hr at 37°C. The final concentration of MCD complexes was 75 μM for cholesterol and 25-azicholesterol, and 25 μM for 6-azicholestanol. After washing with OptiMEM, cells were incubated for 6 hr in OptiMEM with 20 μM pravastatin, followed by UV irradiation for 10 min on ice. The cells were harvested and DispA was subjected to denaturing immunoprecipitation with anti-myc antibodies (9E10 monoclonal) followed by SDS-PAGE and immunoblotting with rabbit anti-hShhN antibodies. A similar protocol was used to detect interaction of hShh with HA-tagged Scube2 by photocrosslinking in cells.

Photocrosslinking of Azi-Cholesterol-Modified MBP to Scube2 In Vitro

A fragment of *Drosophila* Hh encompassing amino acids 244-471 was expressed and purified from bacteria as a soluble MBP fusion (MBP-DHh), as described (Chen et al., 2011). Sterol-modified MBP was generated from MBP-DHh by in vitro processing with the appropriate sterol for 6 hr at room temperature. The processing reactions contained 0.5 mg/mL MBP-DHh in 20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% DDM, 1 mM dithiothreitol, and with or without 200 μM cholesterol, 6-azicholestanol, or 25-azicholesterol, added from 10 mM stocks in DMSO. Sterol-modified MBP proteins were then incubated for 1 hr at room temperature with concentrated conditioned media containing HA-tagged Scube2 or Scube2ΔCUB, followed by UV irradiation for 10 min on ice. The samples were then analyzed by SDS-PAGE and immunoblotting with HA antibodies.

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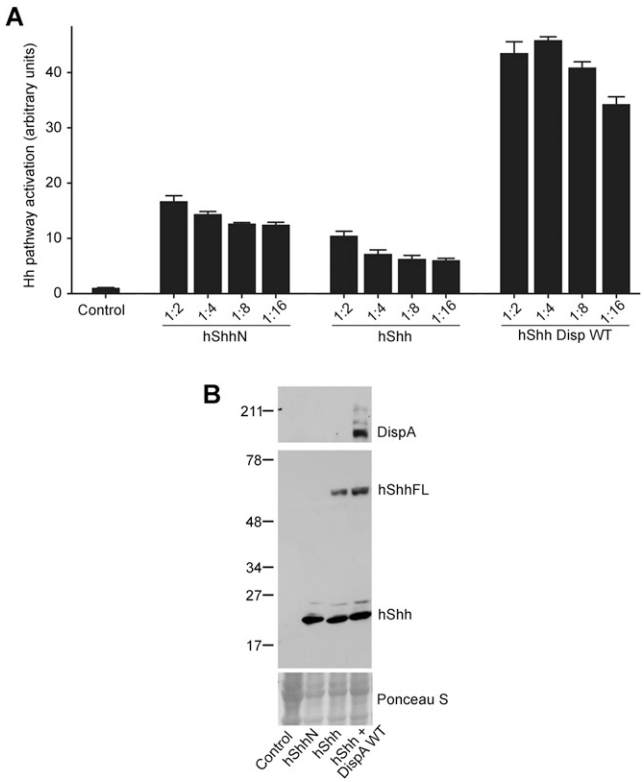

Figure S1. DispA Activity Measured in NIH 3T3 Cells, Related to Figure 1

(A) NIH 3T3 cells stably expressing hShh or hShhN, transduced with a lentivirus expressing myc-tagged DispA-WT or not, were cocultured at the indicated dilution ratios with Hh-responsive Shh Light II cells. Twelve hours after plating, serum was washed off and the cells were incubated for 30 hr in serum-free media, followed by luciferase reporter assays. Luciferase measurements were normalized to Shh Light II cells grown alone (control). All experiments were performed in triplicate. Error bars represent SD of the mean. DispA expression increased the response of reporter cells to hShh but not to hShhN.

(B) The cells used in (A) were analyzed by SDS-PAGE followed by immunblotting with anti-hShh and anti-myc antibodies. Shh Light II cells were used as negative control. HShhFL denotes the full-length hShh precursor. Ponceau S staining of the blotting membrane is shown for loading control.

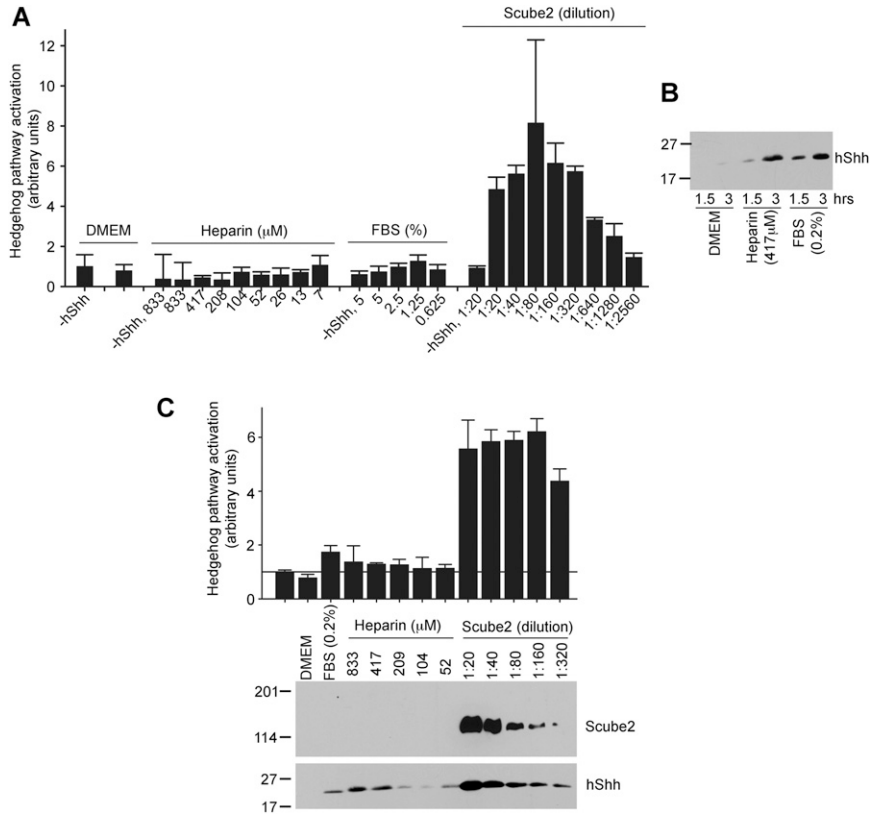

Figure S2. HShh Released by Serum or Heparin Is Inactive, in Contrast to hShh Released by Scube2, Related to Figure 3

(A) 293T cells stably expressing hShh were incubated with heparin, fetal bovine serum (FBS), or HA-tagged Scube2 added at the indicated concentrations in serum-free media (DMEM). Secreted hShh was collected for 3 hr and then analyzed by luciferase assay in Shh Light II reporter cells. As negative controls (−hShh), reporter cells were incubated with DMEM alone, DMEM with heparin (883 μM), DMEM with serum (5%), or DMEM with Scube2 (1:20 dilution).

(B) 293T cells stably expressing hShh were incubated with serum-free media, heparin, or FBS for 1.5 or 3 hr. Supernatants were precipitated with TCA, and were analyzed by SDS-PAGE and immunoblotting with anti-hShh antibodies.

(C) Collection of supernatants and analysis by luciferase reporter assays was performed as in (A). Aliquots of the supernatants were precipitated and analyzed as in (B), with additional immunoblotting for Scube2 with anti-HA antibodies.