Supplementary Information

Transit-Amplifying Cells Orchestrate Stem Cell Activity and Tissue Regeneration

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I. Supplemental Figure Legends:

Figure S1. Early Anagen Markers, Bu-SC Activity across Strains and Sex, Signaling Activities within the Niche and *Gli1-LacZ* Activity, Related to Figure 1.

(A) Marker expression during early anagen. In AnaII, matrix cells (LEF1 and Pcad double positive) start to form at the bottom of the hair bulb, right on top of DP (LEF1 single positive). In AnaIII, differentiated IRS cells (AE15+) begin to emerge. (B) EdU incorporation in AnaIII HFs from male and female samples. (C) Quantifications of EdU+ Bu-SCs per bulge comparing outbred CD1 mice to inbred C57BL/6 mice at AnaIII (n=3 for CD1; n=2 for C57BL/6; ≥15 HFs per mice). n.s., not significant. (D) RT-PCR results of *Axin2* (WNT signaling) and *Tmeff1* (TGFβ signaling) from purified Bu-SCs at different hair cycle substages. (E) RT-PCR examining *Bmp6* and *Fgf18* levels in purified Bu-SCs and K6+ bulge, *Bmp4* in dermal fibroblast (DF), and *Id1* (BMP signaling) in Bu-SCs. (F) RT-PCR of *Dhh and Ihh* levels from dissected DRGs, FACS-purified DP, HG, or matrix at either telogen→anagen transition or at AnaII-III. *Ihh* was not detected (ND) in any of these cell types. (G) β-gal activity (blue) of AnaV, *Gli1-LacZ* HF (Top) and AnaV, *Gli1-LacZ* HF with SHH overexpression (OE, bottom). (H) Examining HFs,

DRGs, and cutaneous nerve fibers for expression of YFP+ cells 2 days after induction of *ShhCreER/ Rosa*$^{flox\text{-}stop\text{-}flox\text{-}YFP}$ with one injection of Tamoxifen (150 μg/g tamoxifen in corn oil). Bulge, yellow dashed lines; the rest of the HF, white dashed lines. DP, solid line. Box-and-whisker plots: mid-line, median; Box, 25th and 75th percentiles; whiskers, minimum and maximum. Scale bars: 30μm. Data are mean±SD. n.s., not significant. *:p<0.05.

Figure S2. *Shh*, *Shh* signaling levels, and apoptosis analysis in control and *Shh*-cKO HFs, and the Design of the Denervation Scheme, Related to Figure 2.

(A) RT-PCR of *Shh* in purified hair bulb from control and *Shh*-cKO HFs at AnaII. (B) *In situ* hybridization of *Shh* in control and *Shh*-cKO HFs at AnaII. (C) Immunolocalization of active Caspase 3 (Cp3), CD34 and Pcad in control and *Shh*-cKO HFs and quantifications. (D) Schematic and low power view to demonstrate the design and efficiency of the denervation surgery. An incision was made at the midline and nerve fibers were manually removed by a spring scissor only at the right side of the skin (Denervation Side). The connective tissues were also cleaned by a scissor but the nerves were not cut at the left side (Sham Control Side). Arrowheads mark the Tuj1+ cutaneous nerve fibers, which are intact at the control side but are absent at the denervation side. Asterisks mark the Tuj1+ IRS cells, which are present at both sides. The HFs on both sides are in AnaIII. (E) *In situ* hybridization detecting *Shh* expression in HFs at different time-points after plucking during telogen. HFs are plucked at D0. *Shh* expression is absent at D1 after plucking, but becomes detectable starting from D2. (F) β-gal activity of *Gli1-LacZ* HFs at D1 and D3 post plucking. Bulge: thick dashed lines or yellow dashed lines. Hair bulbs: thin dashed lines or white dashed lines. Scale bars: 30μm.

Figure S3. *Gli1 In Situ* Hybridization and Gene Expression Analysis in *Smo-cko*, Related to Figure 3

(A) *In situ* hybridization of *Gli1* in control, *Shh-cKO*, and *Gli1* KO HFs. Bulge, yellow dashed lines; the rest of the HF, white dashed lines. DP, solid lines. (B) RT-PCR results of WNT signaling target *Axin2* and TGFβ signaling target *Tmeff* in FACS-purified WT or *Smo-cko* Bu-SCs in AnaII-III. Both pathways operate normally in Bu-SCs when they are defective of SHH pathway activity. Data are mean±SD. n.s., not significant.

Figure S4. Isolation Strategies and Gene Expression Analysis for Various Dermal Cell Types, Related to Figure 4

(A) Isolation strategies employed to enrich for dermal cell types including DP (Dermal papillae), DF (dermal fibroblasts), APC (adipocyte precursor cells), and Endo (endothelial cells). (Top) Schematic representation of dermal cell types that are in close proximity to the HFs and surface markers used to enrich for various dermal cell types. (Bottom Left) Immunolocalization of markers for different dermal cell types revealing their relative positions to a HF. HF, white dashed lines. DP, solid line. (Bottom Right) RT-PCR of signature genes verifies the successful enrichment of DP from DF. *Lef1*, *Alx4*, and *Nog* are known DP signatures, while *Vimentin* (*Vim*) is expressed by both the DP and DF. (B) RT-PCR examining gene expression in purified dermal cell types from WT and *Shh-cKO* skin enriched for AnaII and AnaIII HFs. (C) *In situ* hybridization of *Shh* in *Smo-cko* and *Gli2-cKO* HFs. Bulge: thick dashed lines. Hair bulbs: thin dashed lines. (D) *In situ* hybridization of *Gli1* in control, *Gli2-cKO*, and *Smo-cKO* HFs. Bulge, yellow dashed lines; the rest of the HF, white dashed lines. DP, solid lines. Scale bars: 30μm. Data are mean±SD. *:p<0.05. n.s., not significant.

Figure S5. Efficiency of *Smo* Inducible Knockdown, Related to Figure 5

(A) Schematic summarizes the design of Doxycycline (Doxy) induction scheme for testing hairpin efficiency *in vivo*. Mice are fed with Doxy chow starting at D0, and knockdown efficiency was examined at D5 and D8 in FACS-purified Bu-SCs. (B) RT-PCR examining *Smo* expression levels in FACS-

purified Bu-SCs from control, *Smo*^{het}, and skins infected with *Smo* hairpins (SmoBri: RFP^{Bright} *Smo* knockdown cells; SmoDim: RFP^{Dim} *Smo* knockdown cells). RFP^{Dim} *Smo* knockdown cells have comparable *Smo* expression levels to *Smo*^{het} cells, which resemble wild-type phenotypically. Data are mean±SD. *:p<0.05; **:p<0.01. n.s., not significant. (C) Model summarizing direct and indirect requirement of SHH in Bu-SCs and hair bulb proliferation during early anagen.

Figure S6. *Gas1* Expression in Bu-SCs and Matrix, and *Gli1* levels in *Gas1-/-*, Related to Figure 6

(A) ChIP-seq signal tracks around the *Gas1* locus. Chromatin is from anagen Bu-SCs and TAC matrix. Antibodies bind to H3 modifications of different transcriptional events including initiation (lysine 4 trimethylation, H3K4me3), elongation (lysine 79 dimethylation, H3K79me2), and dominant repression (lysine 27 trimethylation, H3K27me3). *Gas1* is highly and actively transcribed in the anagen Bu-SCs, but is repressed in the TAC matrix. All tracks are shown on the same scale. (B) RT-PCR testing *Gas1* levels in purified Bu-SCs and matrix from HFs enriched at AnaII-AnaIII. Data are mean±SD. ***:p<0.001.) (C) *In situ* hybridization of *Gli1* in control and *Gas1-/-* HFs. Bulge, yellow dashed lines; the rest of the HF, white dashed lines. Scale bars: 30μm.

Figure S7. *Shh* Pathway Mutants in Full Anagen and *Gli2-cKO* phenotypes, Related to Figure 7

(A) (Top) Control, *Gli2-cKO*, *Smo-cKO*, and *Shh-cKO* HFs five days after AnaII and *Shh-cKO* HFs 18 days after AnaII. (Middle) Quantifications of regenerated HF length below bulge, proliferation status (measured by pH3, phospho-Histone H3), and matrix size. Note that proliferation status of matrix and ORS was measured by pH3 staining instead of EdU, as EdU incorporation becomes saturated rapidly in highly proliferative populations like matrix and lower ORS, which often leads to an underestimation of proliferation differences. (Bottom) Representative pictures of hair coat recovery of different SHH pathway mutants. The mice were shaved in P21 and photos were taken between P55-P62, when all littermate controls had recovered their hair coat completely.(B) 2nd telogen HFs with *Gli2* knocked-out at

different timepoints and quantifications of their HG sizes compared to those of the control. *Gli2* is knocked-out either in 1st AnaIV, when Bu-SCs return to quiescence (red bars) or 2nd telogen (blue bars). (C) Representative images of control and *Gli2-cKO* mice with one round of hair plucking. *Gli2* is knocked-out either in 1st telogen or 2nd telogen. (D) Close up pictures of skin surface in control and *Gli2-cKO* animals after 1 round or 5 rounds of hair depilation. *Gli2* is knocked-out since 1st telogen. Bulge, yellow dashed lines; the rest of the HF, white dashed lines. Box-and-whisker plots: mid-line, median; Box, 25th and 75th percentiles; whiskers, minimum and maximum. Scale bars: 30μm. n.s., not significant. ***:p<0.001

II. Supplementary Experimental Procedures

Mice

K14-rtTA, Shh-CreER, Sox9-CreER, K15-CrePGR, Smo^{flox}, Gli2^{flox}, Shh^{flox}, Shh^{neo}, Gas1^{Lacz}, Gli1^{Lacz}, and *Rosa26$^{Flox\text{-}Stop\text{-}Flox\text{-}YFP}$* (Bai et al., 2002; Chiang et al., 1999; Corrales et al., 2006; Dassule et al., 2000; Harfe et al., 2004; Long et al., 2001; Mao et al., 1999; Martinelli and Fan, 2007; Morris et al., 2004; Nguyen et al., 2006; Soeda et al., 2010; Srinivas et al., 2001) were described previously. *Lhx2-EGFP* mice were from The Gene Expression Nervous System Atlas (GENSAT) Project, NINDS Contracts N01NS02331 & HHSN271200723701C to The Rockefeller University (New York, NY). *K6-RFP* mice were generated by cloning a 7KB promoter sequence upstream of the mouse *Krt6a* gene and assembled with β-globin intron, mRFP, and polyA sequences. Transgenic mice were generated with standard pronuclear injections. To overexpress SHH in the hair follicle, the first 594 bp of mouse *Shh* is cloned after the Tet-responsive element (TRE) to produce an N-terminus fragment of SHH same as the autocatalytic cleaved product. For intradermal injections of growth factors, recombinant NOGGIN (250ng, R&D systems), FGF7 (250 ng, R&D systems), or BSA control was intradermally injected with FluoSpheres (Life Technologies).

Immunohistochemistry

The following antibodies and dilutions were used: CD34 (rat, 1:100, eBioscience), BrdU (rat, 1:100, Abcam), active-Caspases3 (rabbit, 1:500, R&D), GFP (rabbit, 1:500, Life Technologies or chicken, 1:2000, Abcam), P-cadherin (goat, 1:200, R&D or rat, 1:100, Fuchs lab), LEF1 (rabbit, 1:200 Fuchs Lab), AE15 (mouse, 1:300, Santa Cruz), CD140a (rat, 1:100, eBioscience), ALPL (goat, 1:200, R&D), CD31 (rat, 1:100, eBioscience), RFP (rabbit, 1:5000, MBL). Phospho-Histone H3 (rabbit, 1:500 Cell signaling). Nuclei were stained using 4'6'-diamidino-2-phenylindole (DAPI). EdU click-it reaction was performed according to manufacturer's directions (Life Technologies).

Histology and Immunofluorescence

For immunofluorescence microscopy of sagittal sections, backskins were embedded in OCT, frozen, cryosectioned (14-30 μm) and fixed for 10 min in 4% paraformaldehyde in PBS. Sections were permeabilized for 10 min in PBS + 0.1-0.3%Triton (PBST) and blocked for 1 h in 2.5% fish gelatin, 5% normal donkey serum, 0.5% BSA, 0.1-0.3% Triton in PBS. Primary antibodies (Abs) were incubated overnight 4°C and secondary Abs were incubated 1-2 hrs at RT. Mouse Abs were incubated with M.O.M. block according to manufacturer's directions.

Detection of β-galactosidase Activity and *In situ* Hybridization

Frozen sections (20μm) were fixed with 0.5% glutaraldehyde in PBS for 2 mins, washed with PBS, and then incubated with 1mg/ml Xgal substrates in PBS with 1.3 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, and 3mM $K_4Fe(CN)_6$ for 1hr at 37°C. *In situ* hybridization for *Shh* was performed essentially as described previously (DasGupta and Fuchs, 1999). The construct used to generate *Shh in situ* probe was a gift from Andrew McMahon (Echelard et al., 1993). *In situ* hybridization for *Gli1* (region: 25-1205) was performed using RNAscope assays according to the manufacturer's instructions (Advanced Cell Diagnostics).

FACS

Purification of K6+ bulge was performed using K6-RFP, Lhx2-EGFP double transgenic mice. Purification of ORS was performed using Lgr5-EGFP mice. The upper and lower ORS cells were differentiated based on differences in their α6 levels. For cell isolation, the backskin was placed dermis side down in Collagenase (Sigma) for 30mins-1hr. The dermal fractions were collected by scraping the dermal side using a scalpel. The remaining epidermal side was then transferred to Trypsin (GIBCO) at 37°C for 20-30mins. Single cell suspensions were obtained by scraping the skin gently. The cells were then filtered with strainers (70μM, followed by 40μM). Cell suspensions were incubated with the appropriate antibodies for 30 minutes at RT. The following antibodies were used: CD34-eFluor660 (1:100, eBioscience), α6-PE (1:500, eBioscience), β1-PE_Cy7 (1:250, eBioscience), Sca1-PerCP_Cy5.5 (1:1000, eBioscience), CD140a-PE (1:100, eBioscience), CD31-eFluor450 (1:100, eBioscience). CD45-eFluor450 (1:100, eBioscience), CD24-FITC (1:100, eBioscience), Pcad-Biotin (1:25, R&D), EphrinB1-Biotin (1:50 R&D), CD200-eFluor660 (1:100, eBioscience). DAPI was used to exclude dead cells. Cell isolations were performed on FACSAria sorters equipped with Diva software (BD Biosciences). FACS analyses were performed using LSRII FACS Analyzers and then analyzed with FlowJo program.

Real-Time PCR

Total RNAs were purified from FACS-sorted cells by directly sorting into Trizol^{LS} (Life Technologies) followed by extraction using Direct-Zol RNA mini-prep kit (Zymo Research). Equal amounts of RNA were reverse-transcribed by Oligo-dT (Superscript III, Life Technologies). cDNAs were normalized to equal amounts using primers against Ppib2 or β-Actin.

Confocal Microscopy and Image Processing

Images were acquired with a Zeiss LSM510 or a Zeiss LSM780 laser-scanning microscope (Carl Zeiss MicroImaging) through a 40X water objective or a 25X objective. Representative single Z-planes are presented and co-localizations were interpreted only in single Z-stacks. Z-stacks were projected using ImageJ software. RGB images were assembled in Adobe Photoshop CS5 and panels were labeled in Adobe Illustrator CS5.

III. Supplementary References:

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