Supplemental Materials & Methods

In vivo experimentation

Six-week-old male mice were allowed to adapt to their new environment for 1 week and were used to confirm the synergistic effect of PTD-DBM and VPA on hair re-growth. The 7-week-old mice, whose hair follicles were in telogen phase, were anesthetized and their backs were shaved with a hair clipper. Next, 100 μl of each drug at a following concentration was applied topically daily for 28 days: PTD-DBM alone (2 mM), VPA alone (500 mM; Acros), PTD-DBM and VPA in combination, or MNX alone (100 mM; Daejung) (*n*= 12 per group). As a control, one group of mice was treated with vehicle composed of 50% (vol/vol) ethanol, 30% water, and 20% propylene glycol. In the WIHN model, 1 cm^2 full-thickness incision wounds were created on the backs of 3-week-old male mice (*n*= 12 per group). Fifteen microliters of each drug at the same concentration as the hair re-growth experiments was applied to the wounds daily for 13 days. In the depilation model, the hair cycle was induced in the backs of 7-week-old male C3H mice (*n* = 3 per group), leading to synchronized induction of anagen hair follicles. To monitor the expression of β-catenin and CXXC5 during the hair cycle of mice, tissues were obtained at days 0, 1, 6, 10, 13, 16, 18, 20, 22, 25, and 30 after depilation.

Cell culture

HFDPCs (*n* = 3) were purchased from Cell Applications. The cells were transferred to dishes

coated with collagen coating solution (Cell Applications), cultured in papilla cell growth medium (Cell Applications) and incubated in 5% (v/v) CO_2 at 37°C. For transient transfection, the cells were grown for 1 day, transfected with the required plasmids or siRNAs using the Fugene-6 transfection reagent according to the manufacturer's instructions (Roche Diagnostics), and then further cultured for 2 days before harvesting. Human-specific *CXXC5* siRNAs (5′-CUCAGUGGCAGAUGACACATT-3′ and 5′-GCACCCGUCUUUAGAACCATT-3′) and*β-catenin* siRNAs (5′-GAAACGGCTTTCAGTTGAG-3′ and 5′-AAACTACTGTGGACCACAAGC-3′) were synthesized (Bioneer) for knockdown of CXXC5 and β-catenin, respectively.

Wnt reporter assay

HFDPCs were plated in 24-well plates at a density of 4×10^4 cells per well. The cells were transfected using the Fugene-6 transfection reagent with 0.4 μg of the TOPflash reporter construct, whereas the FOPflash luciferase vector was used as a control for transfection efficiency. Luciferase activities were measured using a FLUOstar OPTIMA luminometer (BMG LABTECH). Wnt reporter activities are presented as TOPflash/FOPflash.

ALPL promoter reporter assay

A luciferase reporter construct under control of the −1838-bp to +81-bp *ALPL* promoter was obtained from Dr. Hyun-Mo Ryoo (Seoul National University, Seoul, Korea). HFDPCs were

plated in 24-well plates at a density of 4×10^4 cells per well. The cells were transfected with 0.4 μg of an ALPL-luciferase construct using the Fugene-6 transfection reagent and then further cultured for 24 hours before harvesting. A β-galactosidase reporter vector was also co-transfected as a control for transfection efficiency. Luciferase and β-galactosidase activities were measured using a FLUOstar OPTIMA luminometer (BMG LABTECH).

ALP activity quantification

HFDPCs were plated in 24-well plates at a density of 4×10^4 cells per well. After culturing for 3 days, the cells were washed and lysed in reporter lysis buffer (Promega). The ALP activity in the lysis was determined using a p-nitrophenyl phosphate (pNPP) liquid substrate (Sigma). Thirty microliters of each cell lysate solution was added to 30 μl of pNPP substrate and incubated in the dark at 37°C for 30 minutes. The absorbance was measured using a FLUOstar OPTIMA luminometer (BMG LABTECH) at 405 nm. The intracellular total protein content was determined using a Bradford reagent (Bio Rad), and ALP activity was normalized to the amount of total protein.

MTT colormetric assay

Cell proliferation was assessed using MTT assay. HFDPCs were plated in 24-well plates at a density of 2×10^4 cells per well. After 72 hours of culture, the medium was removed by aspiration and replaced with 300 μl of fresh medium. Fifteen microliters of MTT solution (3-

(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, AMRESCO) was added to each well, and the plates were incubated in the dark at 37°C for 3 hours. At the end of each experiment, the medium was removed and 500 μl of dimethyl sulfoxide (DMSO) was added to immediately dissolve the formazan crystals. Absorbance at 595 nm was read on a FLUOstar OPTIMA luminometer (BMG LABTECH).

Hematoxylin and eosin (H&E) staining

Skin tissues were excised, fixed overnight in 4% (w/v) paraformaldehyde in PBS, dehydrated and paraffinized. Following complete paraffinization, the tissues were embedded in paraffin, and then sliced into 4-μm sections. The slides were deparaffinized in three changes of xylene and rehydrated through a graded ethanol series. The sections were stained with hematoxylin for 5 minutes and with eosin for 1 minute. The H&E-stained slides were photographed using a bright-field optical microscope (ECLIPSE TE2000-U, Nikon).

Immunohistochemistry

Immunohistochemical staining was performed on 4-μm paraffin sections. Deparaffinization and rehydration were performed with three changes of xylene and a graded ethanol series. For antigen retrieval, the slides were autoclaved in buffer (10 mM sodium citrate buffer, Ph 6.0, Sigma). After cooling, sections were pre-incubated in PBS and then blocked in PBS containing 10% bovine serum albumin (BSA) at room temperature for 30 minutes. The slides were

incubated overnight at 4°C with the following dilutions of primary antibodies: anti-β-catenin (BD Transduction Laboratory, 1:100), anti-CXXC5 (Santa Cruz Biotechnology, 1:50), anti-PCNA (Santa Cruz Biotechnology, 1:500), anti-human ALPL (R&D, 1:30), anti-mouse ALPL (R&D, 1:30), anti-keratin 15 (Abcam, 1:200), anti-CD34 (Abcam, 1:200), anti-CD200 (R&D, 1:30), anti-α-SMA (Abcam, 1:100), anti-keratin 14 (Covance,1:1000), anti-Fgf9 (Abcam,1:1000), and anti-keratin17 (Abcam, 1:500). Subsequently, the sections were rinsed with PBS and incubated with Alexa Fluor 488- or Alexa Fluor 555-conjugated IgG secondary antibody (Molecular Probes,1:400) in the dark at room temperature for 1 hour, and counterstained with 4',6-diamidino-2-phenylindole (DAPI, Boehringer Mannheim,1:5000). The images were taken on a LSM510 META confocal microscope (Carl Zeiss) using 405, 488, or 543 nm laser excitation lines.

Immunocytochemistry

HFDPCs were plated in 12-well culture plates at a density of 8×10^4 cells per well. The cells were washed with PBS before being fixed in 4% (w/v) paraformaldehyde in PBS for 15 minutes at room temperature. Then the cells were washed with PBS and permeabilized with 0.1% (v/v) Triton X-100 in PBS for 15 minutes. After washing with PBS three times, the cells were incubated with 10% BSA in PBS for 30 minutes and then with antibodies specific for β-catenin (BD Transduction Laboratory, 1:100) or CXXC5 (Santa Cruz Biotechnology, 1:50) at 4°C overnight. The cells were washed in PBS three times, followed by incubation with Alexa Fluor

488- or Alexa Fluor 555-conjugated IgG secondary antibody (Molecular Probes, 1:400) in the dark at room temperature for 1 hour. Cell nuclei were counterstained with DAPI for 10 minutes and the images were taken with a LSM510 META confocal microscope (Carl Zeiss) after excitation with 405, 488, or 543 nm laser lines.

Western blot analysis

For western blot analysis, cells or tissue that was ground in mortars were lysed in RIPA buffer (150 mM NaCl; 10 mM Tris, pH 7.2; 0.1% sodium dodecyl sulfate (SDS); 1.0% Triton X-100; 1% sodium deoxycholate; 5 mM ethylene diamine tetra acetic acid (EDTA)). Equal amounts of protein were separated on 6 to 12% SDS polyacrylamide gels and transferred onto PROTRAN® nitrocellulose membranes (Schleicher and Schuell Co.). After blocking for 1 hour with PBS containing 5% nonfat dry skim milk and 0.07% (v/v) Tween 20, the membranes were incubated with antibody specific for β-catenin (Santa Cruz Biotechnology, 1:1000), CXXC5 (Santa Cruz Biotechnology, 1:500), Erk (Santa Cruz Biotechnology, 1:5000), ALPL (Abcam, 1:500), PCNA (Santa Cruz Biotechnology, 1:500), Myc (Cell Signaling Technology, 1:5000), α-tubulin (Oncogene Research Products, 1:5000), Dvl-1 (Santa Cruz Biotechnology, 1:500), keratin 14 (Covance, 1:1000), Fgf9 (Abcam,1:1000), or keratin17 (Abcam, 1:1000) at 4°C overnight. The membranes were washed three times and then incubated with horseradish peroxidase-conjugated anti-rabbit (Bio-Rad Laboratories,1:5000) or anti-mouse (Cell Signaling Technology, 1:5000) IgG secondary antibody. Immuno reactive bands were

visualized with enhanced chemiluminescence (Amersham Bioscience) using a luminescent image analyzer, LAS-3000 (Fujifilm).

Immunoprecipitation

Cells were harvested and the proteins were extracted in RIPA buffer. The extracts were centrifuged at 14,000 × *g* for 30 minutes at 4°C and the resulting supernatants were transferred to new tubes. The supernatants were then subjected to immunoprecipitation with anti-Myc (Abcam, 1:250) or anti-Dvl-1 (Santa Cruz Biotechnology, 1:250) antibody and protein A or G beads (GenDEPOT) overnight at 4°C with constant agitation. The immunoprecipitated complexes were washed with RIPA buffer, and the complex samples were resuspended in SDS sample buffer and boiled for 5 minutes at 100°C. Proteins were subjected to SDS-PAGE on 10% poly acrylamide gels for western blot analysis.

ALP staining

HFDPCs were plated in 12-well plates at a density of 8×10^4 cells per well. After growth for 24 hours, cells were treated with each drug for 72 hours, and then fixed in 4% (w/v) paraformaldehyde for 10 minutes and washed with PBS three times. The cells were rinsed in TN buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) for optimal staining, followed by incubation with nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate (NBT/BCIP, Roche) in TN buffer for 30 minutes. The reaction was stopped by washing with PBS, and the cells were

examined under a bright field microscope. Dark blue staining represents positive signal for ALP. For ALP staining of tissues, 20-μmcryosections were incubated with NBT/BCIP and analyzed as above. Whole mount ALP staining was performed as previously described (Ito et al., 2007).

X-gal staining

Skin tissues from *Axin2*$^{LacZ/+}$ mice were fixed with 0.4% (w/v) paraformaldehyde in PBS for 3 hours at room temperature. Twenty-micrometer sections were fixed again with 0.2% (v/v) glutaraldehyde in distilled water for 15 minutes at room temperature and stained with 1 mg/ml X-gal solution (Promega) overnight at 37°C. Cell nuclei were counterstained with nuclear fast red (Sigma) for 5 minutes at room temperature.

Quantification of immunostaining

Immunohistochemical and immunocytochemical staining was analyzed with NIS Elements V3.2 software (Nikon). The threshold was defined for the red, green, and blue channels, and the blue channel was used to visualize the nuclei. Mean intensity was calculated in the red and green channels separately, and mean values were estimated from analyses of three independent experimental results. Quantitative analyses were performed in at least three random, representative fields.

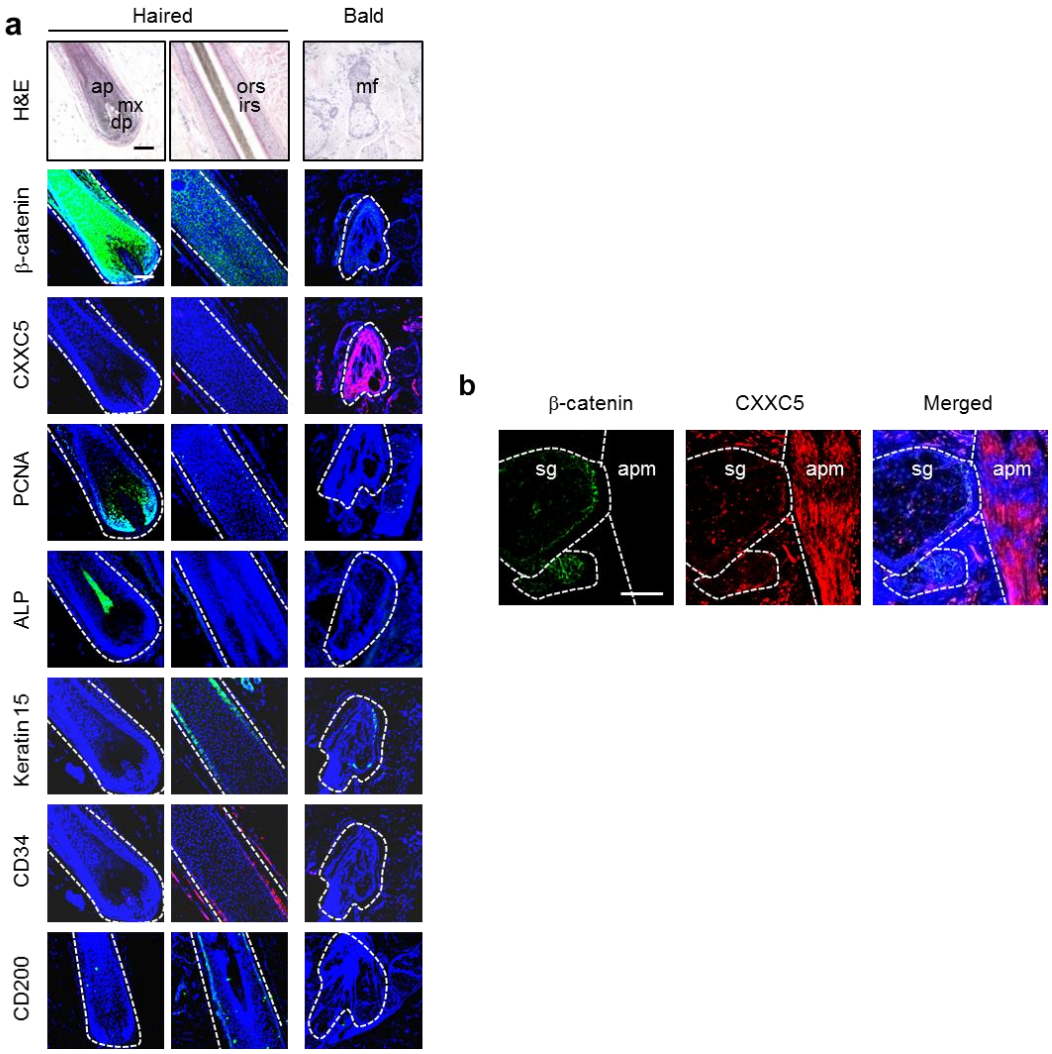
Statistics

Data are presented as means ± SD. Statistical analyses were performed using an unpaired two-tailed Student's *t*-test. Statistical significance is indicated in the figures as follows: * $P<0.05$, ** $P<0.005$, and *** $P<0.0005$.

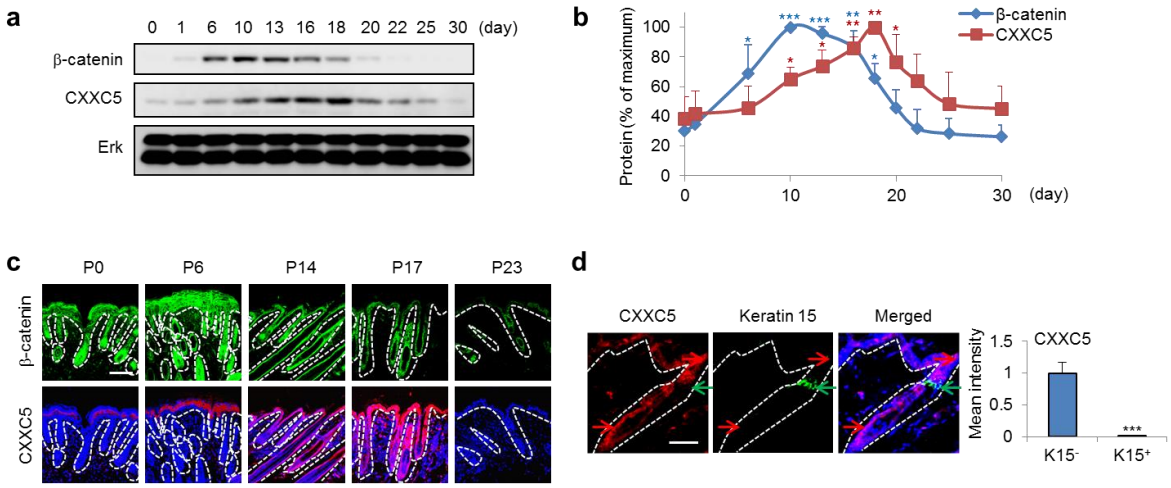
References

Ito M, Yang ZX, Andl T, Cui C, Kim N, Millar SE, et al. Wnt-dependent de novo hair follicle regeneration in adult mouse skin after wounding. Nature 2007; 447:316-20.

Supplemental Figures

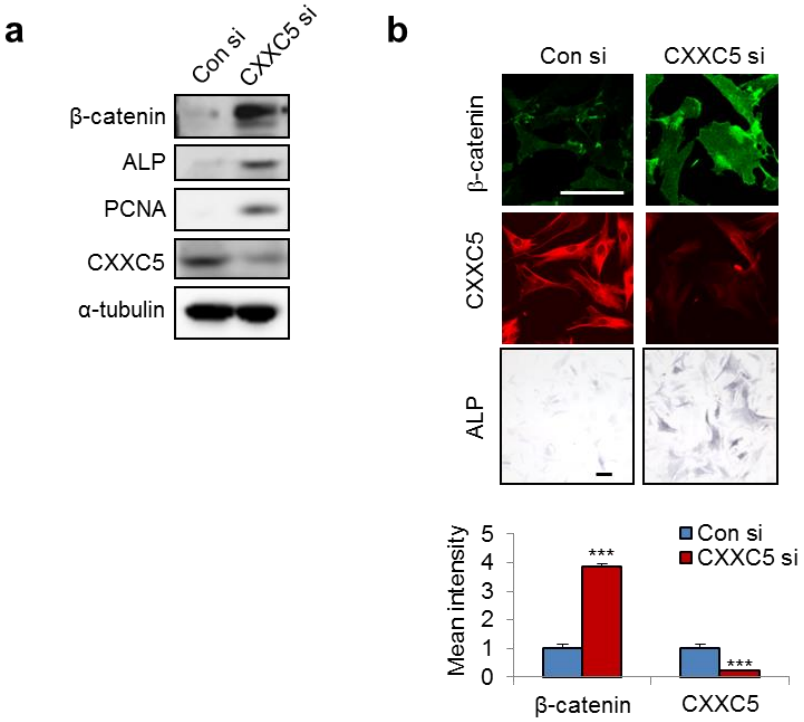


Supplementary Figure S1. CXXC5 levels are inversely associated with β-catenin levels in balding human scalp. (a) Immunohistochemical staining for β-catenin, CXXC5, PCNA, ALP, keratin 15, CD34, or CD200 with DAPI nuclear counterstaining (blue) in the human haired and bald scalps. ap: apex of papilla; mx: matrix; dp: dermal papilla; ors: outer root sheath; irs: inner root sheath; mf: miniaturized follicle. Dashed lines indicate the outlines of hair follicles. (b) Immunohistochemical staining for β-catenin and CXXC5 was performed in the sebaceous gland (SG) and arrector pili muscle (APM) of balding human scalp. sg: sebaceous gland; apm: arrector pili muscle. Dashed lines outline sebaceous gland and arrector pili muscle. Data are representative of the results of at least three independent experiments. Scale bar, 100 μm.

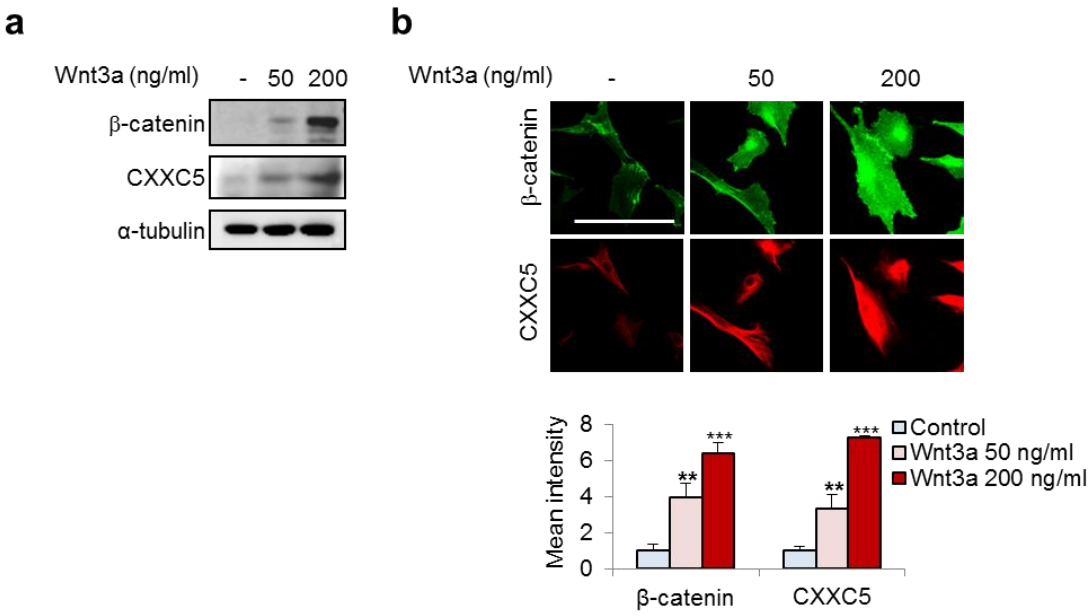


Supplementary Figure S2. CXXC5 is highly expressed in the catagen phase during the murine hair cycle. (a-c) Back skins from C3H mice (*n* = 3 for each time point) representing each phase of the hair cycle (telogen, days 0, 22, 25, 30 post-depilation; anagen, days 1, 6, 10, 13 post-depilation; and catagen, days 16, 18, 20 post-depilation) were processed for immunohistochemical and western blot analyses. (a) Western blot analyses to detect the protein levels of β-catenin, CXXC5 and Erk in mice skin during the depilation-induced hair cycle. (b) Expression profiles of β-catenin (blue) and CXXC5 (red) at different phases of the murine hair cycle. Individual bands from western blots (*n* = 3) were measured using Multi-Gauge V3.0 software and normalized to Erk. (c) Mice skin tissues (*n* = 3 for each time point) were obtained at different time points (anagen, P0, P6, P14; catagen, P17; telogen, P23) during the physiological first postnatal hair cycle. Immunohistochemical staining of mice skin during the first postnatal hair cycle with antibodies against β-catenin (green) or CXXC5 (red) were performed, followed by DAPI staining for visualization of nuclei (blue). Dashed lines delineate epidermis and hair follicles. (d) Immunohistochemical staining was performed with antibodies against CXXC5 (red) or keratin 15 (green) in mice skin on day 18 after depilation (left panels) and quantitative analyses (*n* = 3) of immunohistochemical staining for CXXC5 in keratin 15-positive or keratin 15-negative regions of mice skin tissues (right graph). Red and green arrows represent that CXXC5 is expressed in the keratin 15-negative cells of follicles. Dashed lines outline epidermis and hair follicles. All data are representative of at least three independent experiments. Scale bars, 100 μm for panels (c) and (d). Data are presented as means ± SD. *P <

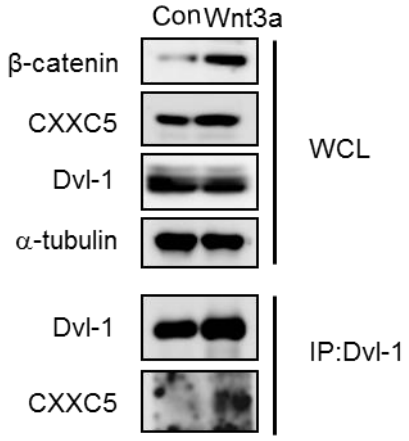
0.05, **P < 0.005, ***P < 0.0005 for panels (b) and (d).



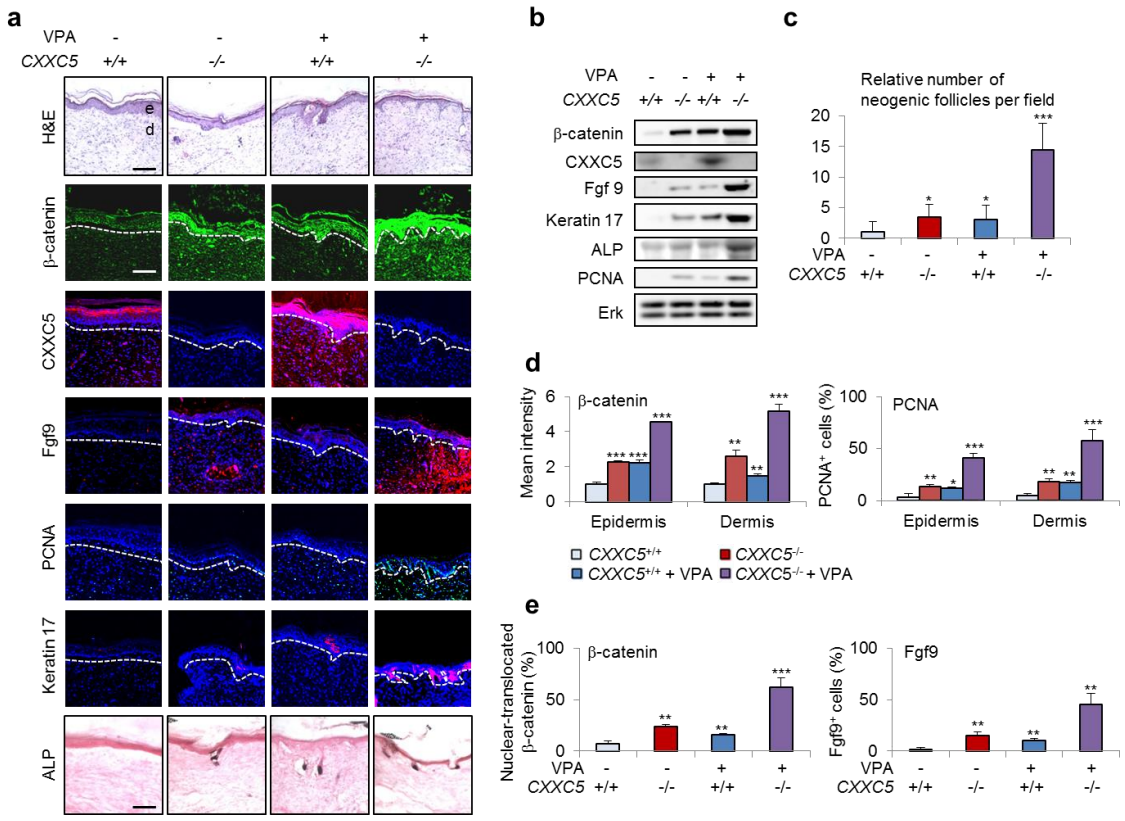
Supplementary Figure S3. CXXC5 knockdown in HFDPCs results in increased ALP activity and proliferation. HFDPCs were transfected with 100 nM control siRNA (con si) or CXXC5 siRNA (CXXC5 si) for 3 days. (a) Western blot analyses of WCLs from cells transfected with the indicated siRNA to detect the protein levels of β-catenin, ALP, PCNA, CXXC5, and α-tubulin. (b) Immunocytochemical staining for β-catenin (green) and CXXC5 (red), and ALP staining (dark blue) in cells transfected with the indicated siRNA, and mean intensity quantitation (*n* = 3). All data are representative of the results of at least three independent experiments. Scale bars, 100 μm for panel (b).



Supplementary Figure S4. Wnt3a treatment simultaneously induces levels of β-catenin and CXXC5. HFDPCs were treated with recombinant Wnt3a at the indicated concentration for 3 days. (a) Western blot analyses of WCLs from cells treated with or without Wnt3a using antibodies against β-catenin, CXXC5, or α-tubulin. (b) Immunocytochemical staining for β-catenin (green) and CXXC5 (red) in cells treated with or without Wnt3a, and mean intensity quantitation (*n* = 3). All data are representative of the results of at least three independent experiments. Scale bar, 100 μm for panel (b).

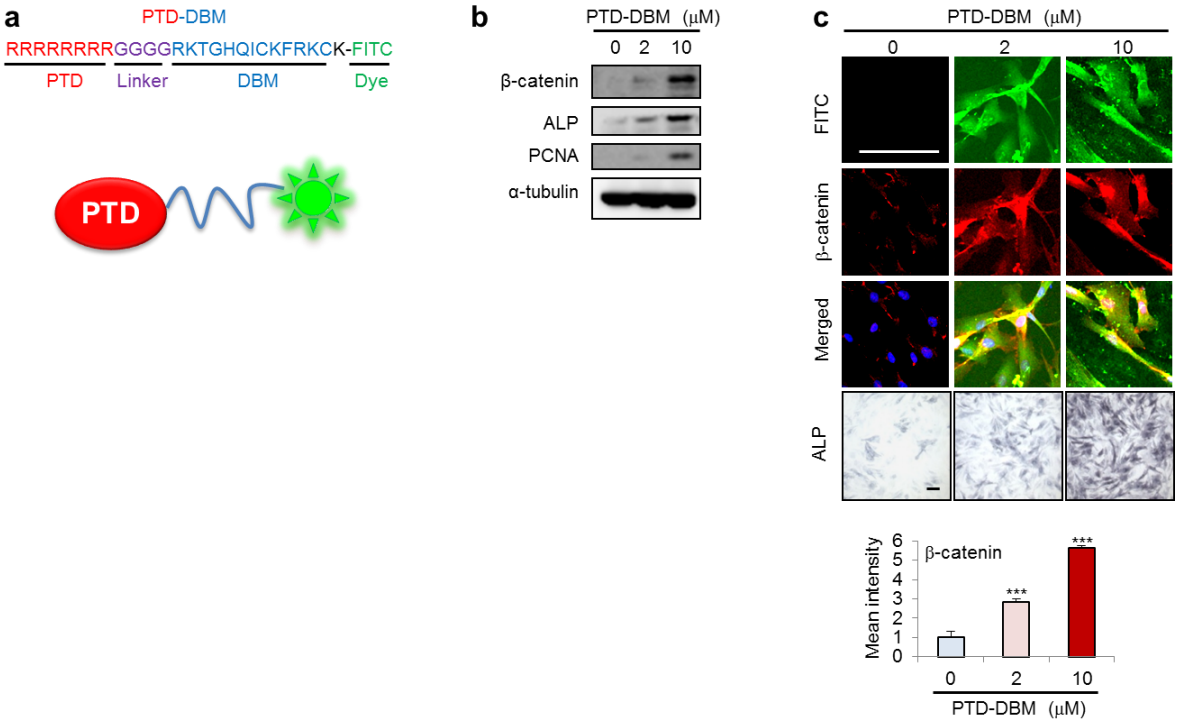


Supplementary Figure S5. Endogenous CXXC5 interacts with Dvl-1. WCLs from HFDPCs were subjected to immunoprecipitation with anti-Dvl-1 antibody followed by western blotting with antibody against β-catenin, CXXC5, Dvl-1, or α-tubulin. Data are representative of the results of at least three independent experiments.

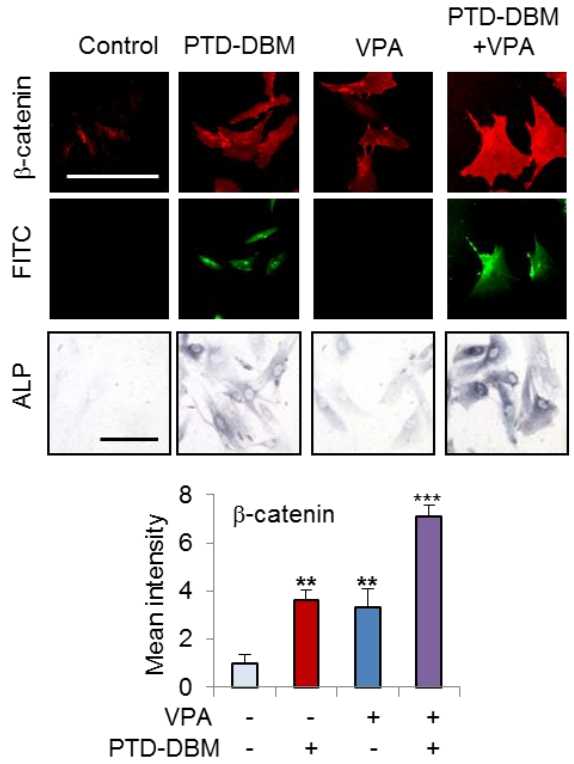


Supplementary Figure S6. Treatment of CXXC5-deficient mice with VPA significantly enhances WIHN. After generation of full-thickness wounds (diameter = 1 cm) on the backs of *CXXC5*$^{+/+}$ and *CXXC5*$^{-/-}$ mice, vehicle or 500 mM VPA was applied topically to the wounds daily (*n* = 7 per group). (a) H&E, immunohistochemical staining for β-catenin, CXXC5, Fgf9, PCNA, or keratin 17 with DAPI staining (blue), and ALP staining (dark blue) in the wounds of *CXXC5*$^{+/+}$ and *CXXC5*$^{-/-}$ mice treated with or without VPA treatment 14 days post-wounding. e: epidermis; d: dermis. Dashed lines indicate border between epidermis and dermis. (b) Western blot analyses of β-catenin, CXXC5, Fgf9, keratin 17, ALP, PCNA, and Erk in vehicle-treated or VPA-treated wound tissues from *CXXC5*$^{+/+}$ and *CXXC5*$^{-/-}$ mice. (c) Measurements (*n* = 7) of neogenic hair follicle number per field in H&E-stained sections for each group of mice. (d) Quantitative analyses of immunohistochemical staining shown in Supplementary Figure S6a for β-catenin (left graph) or PCNA (right graph) in the epidermis and dermis of *CXXC5*$^{+/+}$ and *CXXC5*$^{-/-}$ mice wounds treated with or without VPA treatment 14 days post-wounding. (e) Quantitative analyses of immunohistochemical staining shown in Supplementary Figure S6a for nuclear β-

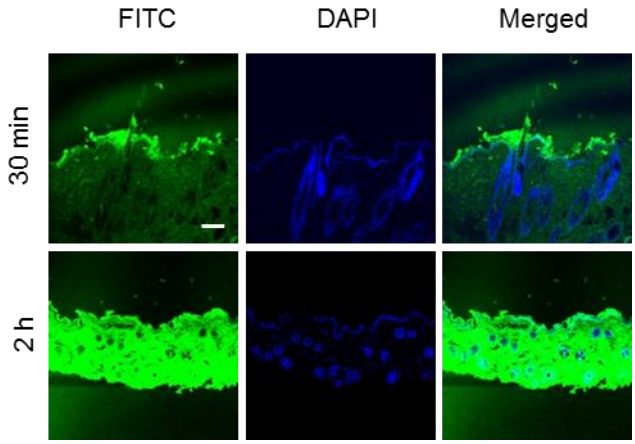
catenin (left graph) or Fgf9 (right graph) in the dermis of *CXXC5*$^{+/+}$ and *CXXC5*$^{-/-}$ mice wounds treated with or without VPA treatment 14 days post-wounding. All results presented are representative of at least three independent experiments. Scale bars, 100 μm for panel (a). Data are expressed as means ± SD. **P* < 0.05, ***P* < 0.005, ****P* < 0.0005 for panels (c)-(e).



Supplementary Figure S7. PTD-DBM induces ALP activity and proliferation in HFDPCs. (a) PTD-DBM includes a PTD for enhanced protein delivery, a poly glycine linker for flexibility, DBM, and lysine conjugated with FITC for visualization. (b-c) HFDPCs were treated with PTD-DBM at the indicated concentration for 3 days. Western blot analyses of WCLs were performed with antibodies against β-catenin, ALP, PCNA, and α-tubulin (b). Immunocytochemicalstaining for β-catenin (green) or CXXC5 (red) and ALP staining (dark blue), and mean intensity quantitation (*n* = 3) were also performed (c). All results presented are representative of at least three independent experiments. Scale bars, 100 μm for panel (c).



Supplementary Figure S8. Combination treatment with PTD-DBM and VPA significantly induces the expression of β-catenin and ALP. Immunocytochemicalstaining for β-catenin (red), FITC (green) for PTD-DBM peptide detection, and ALP staining (dark blue) in cells treated with 2 μM PTD-DBM and/or 1 mM VPA, and mean intensity quantitation (*n* = 3) were performed. Results presented are representative of at least three independent experiments. Scale bars, 100 μm.



Supplementary Figure S9. PTD-DBM is efficiently delivered to murine skin. Skin biopsies were collected to determine the penetration efficiency of PTD-DBM either 30 minutes or 2 hours after treatment of shaved mice skin with 2 μM FITC-conjugated PTD-DBM. The FITC signal was detected using a LSM510 META confocal microscope. Results presented are representative of at least three independent experiments. Scale bar, 100 μm.