Supplemental Information

Methods

Mouse strains

The Gpr177Fx, K5-Cre, Dermo1-Cre, R26R, β-catΔEx3Fx and Axin2^{lacZ} mouse strains, and genotyping methods were reported previously (Fu et al., 2011; Harada et al., 1999; Soriano, 1999; Sosic et al., 2003; Tarutani et al., 1997; Yu et al., 2005a; Yu et al., 2003). For generating Gpr177^{K5} mouse strain, mice carrying the K5-Cre transgene was first crossed with the Gpr177Fx/Fx mice to obtain the K5-Cre; Gpr177Fx/+ strain. The K5-Cre; Gpr177Fx/+ mice were then crossed with the Gpr177Fx/Fx mice to obtain mice carrying the Gpr177^{K5} (genotype: K5-Cre; Gpr177Fx/Fx). A similar breeding strategy was used to generate the Gpr177^{Dermo1} (genotype: Dermo1-Cre; Gpr177Fx/Fx) mutant strain. To examine the Cre activity, K5-Cre and Dermo1-Cre mice were bred into the R26R heterozygous background to obtain the K5-Cre; R26R and Dermo1-Cre; R26R mice, respectively. To simultaneously delete Gpr177 and monitor the Cre-mediated recombination, Gpr177^{Dermo1} mice were crossed into the R26R background to obtain the Gpr177^{Dermo1}; R26R mutants. The deletion of Gpr177 and expression of lacZ reporter occurred when Cre was expressed. For detecting the Axin2 expression, K5-Cre; Gpr177Fx/+ mice was crossed with mice homozygous for Gpr177Fx and Axin2^{lacZ} to create the Gpr177^{K5}; Axin2^{lacZ} (genotype: K5-Cre; Gpr177Fx/Fx; Axin2^{lacZ}+/-) mice. To express the stabilized β-catenin mutant protein in the Gpr177^{K5} mice, the K5-Cre; Gpr177Fx/+ mice were crossed with mice carrying Gpr177Fx/Fx and β-catΔEx3Fx/+ to generate the Gpr177^{K5}; sβcat^{K5} strain. Care and use of experimental animals described in this work comply with guidelines and policies of the University Committee on Animal Resources at the University of Rochester.

Cells

To isolate epidermal and dermal cells, the dorsolateral skins were dissected from the E14.5 embryos. The skin tissues were then incubated in 0.5% Dispase (STEMCELL Technologies Inc., Vancouver, BC, Canada) at 4^{o}C for 2 hours. The epidermal and dermal layers were separated from each other, followed by dissociating into single cells. Primary epidermal and dermal cells were cultured in the CnT-PCT (CellnTEC, Switzerland) and DMEM media, respectively, with 10% FBS in a humidified chamber with 5% CO2 at 37^{o}C. For detection of Wnt secretion, 293T cells were transfected with TOPFLASH and RL-TK plasmids and co-cultured with the dermal cells for 24 hours. Relative luciferase activity was determined using a dual reporter luciferase kit (Promega, Madison, WI, USA).

Histology, β-gal staining, immunostaining, immunoblot and TUNEL analysis

Samples were fixed in formaldehyde or paraformaldehyde and then embedded to obtain paraffin sections which were stained with hematoxylin and eosin for histology or antibodies for immunological staining with avidin:biotinlylated enzyme complex as described (Chiu et al., 2008; Fu et al., 2011; Fu et al., 2009; Maruyama et al., 2010; Yu et al., 2005a; Yu et al., 2010; Yu et al., 2005b). The immunological staining was visualized by enzymatic color reaction or fluorescence according to the manufacture's specification (Vector Laboratories, Burlingame, CA, USA). Images were taken using Zeiss Axio Observer microscope (Carl Zeiss, Thornwood, NY, USA). Immunoblot analysis was performed as described (Fu et al., 2009). Bound primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies, followed by enhanced chemical luminescence-mediated visualization (GE Healthcare Biosciences, Pittsburgh, USA) and autoradiography. Mouse monoclonal antibodies ABC (Millipore, Billerica, MA, USA), AE3 (Millipore), β-catenin (BD Biosciences, San Jose, CA, USA) BrdU (Thermo Scientific, Fremont, CA, USA), Collagen I (Abcam, Cambridge, MA, USA), Keratin 14

(Thermo Scientific) and SMA (Thermo Scientific); rabbit polyclonal antibodies Adiponectin (ProSci, Poway, CA, USA), CD133 (Novus, Littleton, CO, USA), Dvl2 (Cell Signaling Technology, Danvers, MA), Keratin 17 (Abcam), phosphor-GSK3 (Cell Signaling Technology) and Gpr177 (Fu et al., 2011; Fu et al., 2009; Yu et al., 2010); rabbit monoclonal antibody active caspase-3 (BD Biosciences) were used in these analyses. Details for β-gal staining in whole mounts or sections were described previously (Fu et al., 2009; Maruyama et al., 2010; Yu et al., 2005b). TUNEL staining was performed using ApopTag (Millipore) as described (Maruyama et al., 2010; Yu et al., 2007).

RT-PCR analysis

Total RNA isolated from E14.5 mouse skins was subject to the first strand cDNA synthesis using oligoT primers in 20 μl for 1 hour at 50^{o}C. The cDNA was then amplified by PCR (35 cycles, 94^{o}C for 15 seconds, 58^{o}C for 30 seconds and 72^{o}C for 60 seconds) in 50 μl buffered solution containing 1 μl of the diluted reverse transcription product in the presence of 20 pmoles each of the sense and antisense primers specific for the various target sequences as listed.

Wnt 1 Forward	ATGAACCTTCACAACAACGAG
Wnt 1 Reverse	GGTTGCTGCCTCGGTTG
Wnt 2 Forward	CTGGCTCTGGCTCCCTCTG
Wnt 2 Reverse	GGAACTGGTGTTGGCACTCTG
Wnt 2b Forward	CGTTCGTCTATGCTATCTCGTCAG
Wnt 2b Reverse	ACACCGTAATGGATGTTGTCACTAC
Wnt 3 Forward	CAAGCACAACAATGAAGCAGGC
Wnt 3 Reverse	TCGGGACTCACGGTGTTTCTC
Wnt 3a Forward	CACCACCGTCAGCAACAGCC
Wnt 3a Reverse	AGGAGCGTGTCACTGCGAAAG
Wnt 4 Forward	GAGAAGTGTGGCTGTGACCGG
Wnt 4 Reverse	ATGTTGTCCGAGCATCCTGACC
Wnt 5a Forward	CTCCTTCGCCCAGGTTGTTATAG
Wnt 5a Reverse	TGTCTTCGCACCTTCTCCAATG
Wnt 5b Forward	ATGCCCGAGAGCGTGAGAAG

Wnt 5b Reverse	ACATTTGCAGGCGACATCAGC
Wnt 6 Forward	TGCCCGAGGCGCAAGACTG
Wnt 6 Reverse	ATTGCAAACACGAAAGCTGTCTCTC
Wnt 7a Forward	CGACTGTGGCTGCGACAAG
Wnt 7a Reverse	CTTCATGTTCTCCTCCAGGATCTTC
Wnt 7b Forward	TCTCTGCTTTGGCGTCCTCTAC
Wnt 7b Reverse	GCCAGGCCAGGAATCTTGTTG
Wnt 8a Forward	ACGGTGGAATTGTCCTGAGCATG
Wnt 8a Reverse	GATGGCAGCAGAGCGGATGG
Wnt 8b Forward	TTGGGACCGTTGGAATTGCC
Wnt 8b Reverse	AGTCATCACAGCCACAGTTGTC
Wnt 9a Forward	GCAGCAAGTTTGTCAAGGAGTTCC
Wnt 9a Reverse	GCAGGAGCCAGACACACCATG
Wnt 9b Forward	AAGTACAGCACCAAGTTCCTCAGC
Wnt 9b Reverse	GAACAGCACAGGAGCCTGACAC
Wnt 10a Forward	CCTGTTCTTCCTACTGCTGCTGG
Wnt 10a Reverse	CGATCTGGATGCCCTGGATAGC
Wnt 10b Forward	TTCTCTCGGGATTTCTTGGATTC
Wnt 10b Reverse	TGCACTTCCGCTTCAGGTTTTC
Wnt 11 Forward	CTGAATCAGACGCAACACTGTAAAC
Wnt 11 Reverse	CTCTCTCCAGGTCAAGCAGGTAG
Wnt 16 Forward	AGTAGCGGCACCAAGGAGAC
Wnt 16 Reverse	GAAACTTTCTGCTGAACCACATGC
Shh Forward	GGAACTCACCCCCAATTACA
Shh Reverse	GAAGGTGAGGAAGTCGCTGT
Edar Forward	GCCCTACATGTCCTGTGGAT
Edar Reverse	GGCCTGAGAGCTCTTTGTGA
BMP2 Forward	AGGCGAAGAAAAGCAACAGA
BMP2 Reverse	GTCTCTGCTTCAGGCCAAAC
BMP4 Forward	TGAGAGACCCCAGCCTAAGA
BMP4 Reverse	AAACTTGCTGGAAAGGCTCA
Lef1 Forward	CACACATCCCGTCAGATGTC
Lef1 Reverse	TGAGGCTTCACGTGCATTAG
Dkk4 Forward	GTGGAAGACACAAGGCCAGT
Dkk4 Reverse	TGGAGCAGACTTGTCCCTCT

In situ hybridization

In situ hybridization analysis was performed as described (Chiu et al., 2008; Fu et al., 2009; Yu et al., 2010). In brief, embryos were incubated with digoxygenin labeled probes, followed by recognition with an alkaline phosphatase conjugated anti-digoxygenin antibody. To visualize the bound signals, samples were incubated with BM-purple for 4-5 hours. The RNA

probes were generated using a PCR based method described previously (David and Wedlich, 2001). Briefly, T3 or T7 promoter sequences were introduced to the 5-prime end of the reverse and forward primers, enabling the synthesis of antisense and sense transcripts, respectively. PCR fragments were then amplified using gene-specific primers and purified with Quick-spin columns (Qiagen Inc., Valencia, CA, USA), followed by generation of the digoxigenin labeled probes using T3 or T7 RNA polymerase. Quantification of the positively stained domains was performed by measuring areas with J-image software (n=3 individual experiments, mean ± SD).

Fig. S1. Gpr177 is expressed in hair follicle development. Double labeling of Gpr177 with AE3, a marker for the entire epidermis, or K14, a marker for the epidermal basal layer, identifies the Gpr177-expressing cells at E14.5 and E17.5. Sections are counterstained by DAPI. Panels show the individually stained and superimposed images. Scale bars, 50 μm.

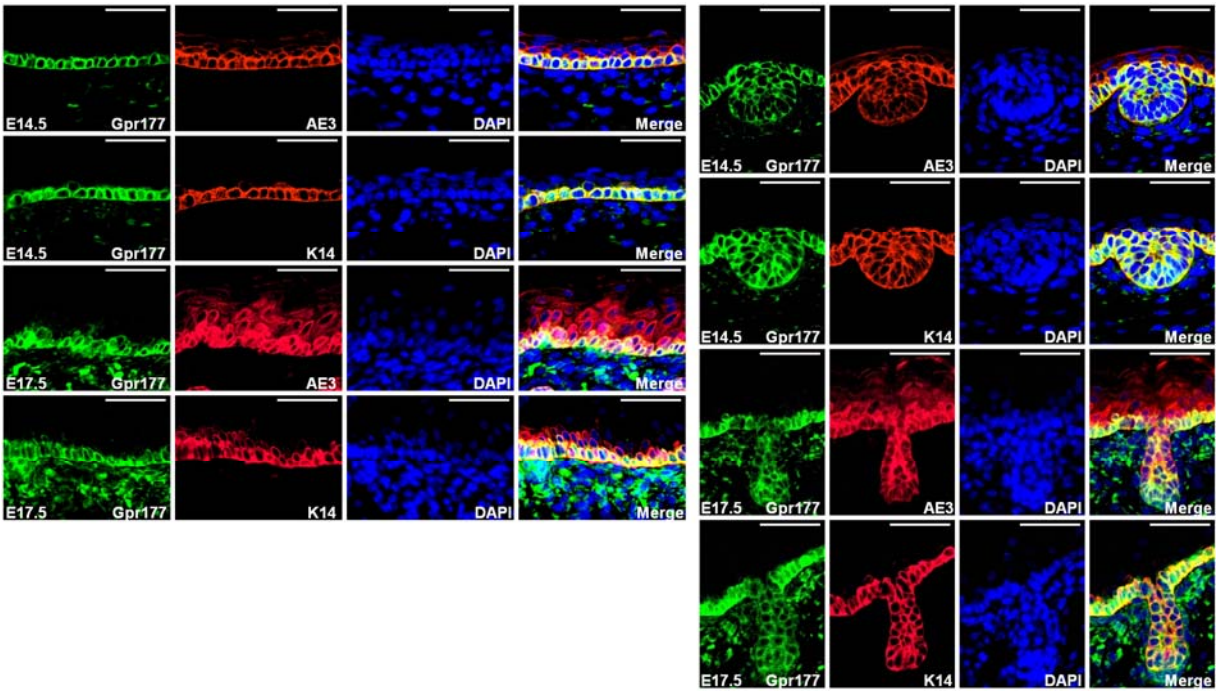

Fig. S2. Wnt production and signaling is impaired in the Gpr177^{K5} mutants. (a) Primary epidermal cells isolated from the E14.5 control and Gpr177^{K5} skins as the signal-producing cells were co-cultured with the signal-receiving cells harboring the TOPFLASH reporter. Relative luciferase activity (RLA) determined activation of the β-catenin and Lef/Tcf-dependent transcription. The addition of canonical Wnt3a and noncanonical Wnt5a in the media was used as the positive and negative controls, respectively. Asterisk indicates the statistical significance of reduction (*p* value <0.01, n=3). (b) Immunoblot analysis of Dvl2, phosphorylated Dvl2 (pDvl2), phosphorylated GSK3, activated β-catenin (ABC), β-catenin (β-cat) determined the canonical Wnt signaling activity in the E14.5 epidermis of control and Gpr177^{K5}. The level of pDvl2, pGSK3 and ABC is elevated upon stimulation of Wnt signaling (Logan and Nusse, Annu Rev Cell Dev Biol, 2004). The epidermal deletion of Gpr177 reduces activation of Dvl2, GSK3 and β-catenin in the epidermis. Actin level is used as a loading control.

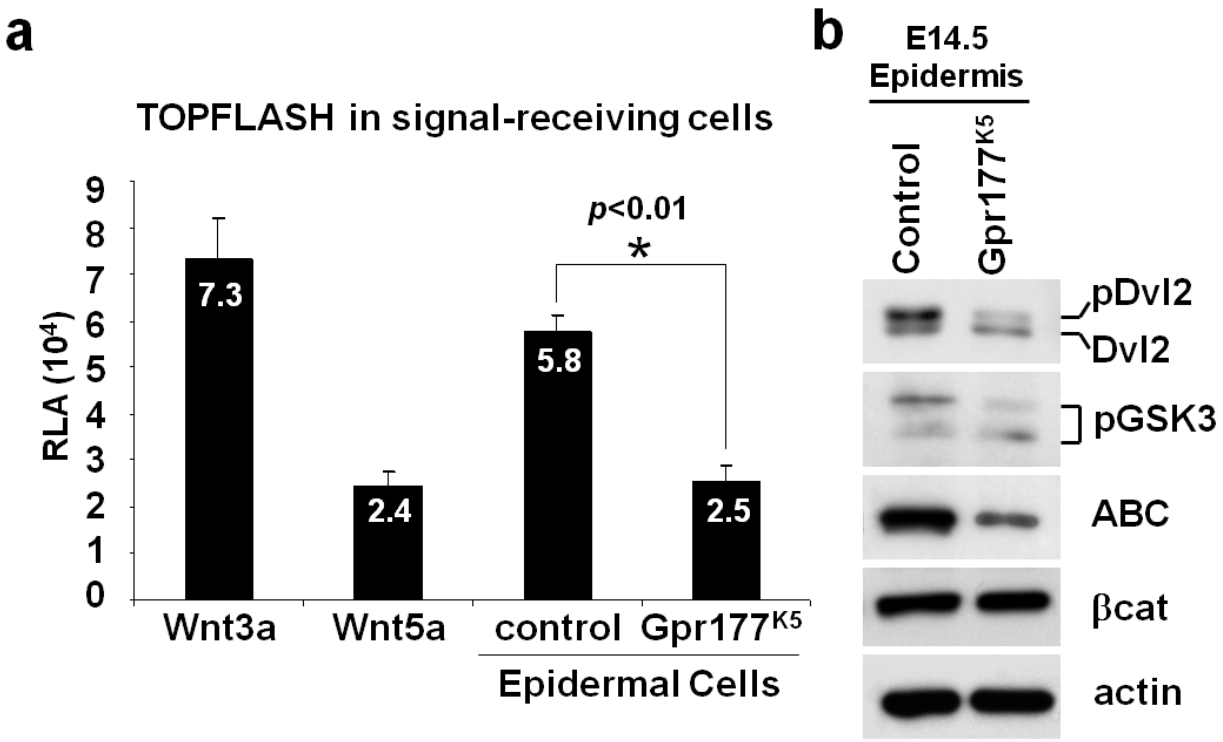

Fig. S3. Multiple members of the Wnt family are expressed in developing embryonic skin. RT-PCR analysis detects the transcript of *Wnts 2*, *3*, *4*, *5a*, *6*, *7a*, *7b*, *10a*, *10b*, *11* and *16*, but not *Wnts 1*, *2b*, *3a*, *5b*, *8a*, *8b*, *9a* and *9b* in the E14.5 skins.

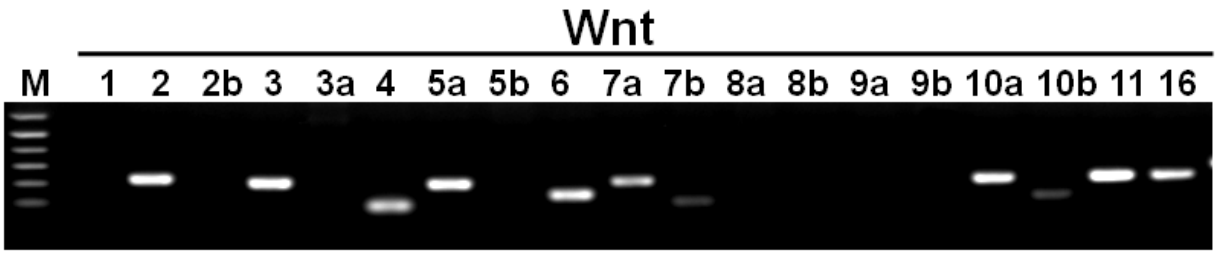

Fig. S4. Epidermal cell proliferation is affected by Gpr177 deficiency. Using BrdU incorporation assay, cells undergoing mitotic division are identified by immunostaining of BrdU in the E14.5 control (a) and Gpr177^{K5} (b) skins. Graph shows the percentage of proliferating cells positive for BrdU in the epidermis and dermis (c). Asterisk indicates that the reduction in the epidermis caused by the epidermal deletion of Gpr177 is statistically significant (Gpr177^{K5}: 23.91±0.01% and control: 34.03±0.03%; *p* value <0.01, n=3). TUNEL staining (d-f) and immunostaining of activated caspase3 (g-i) detect apoptotic cells in the skins (d, e, g, h) and dorsal root ganglia (DRG; f, i) of control (d, f, g, i) and Gpr177^{K5} (e, h) at E14.5. Broken lines indicate the epidermal-dermal junction and the DRG. Genotype – Control: Gpr177Fx/Fx or K5-Cre; Gpr177Fx/+ and Gpr177^{K5}: K5-Cre; Gpr177Fx/Fx. Scale bars, 50 μm (a, b, d-i).

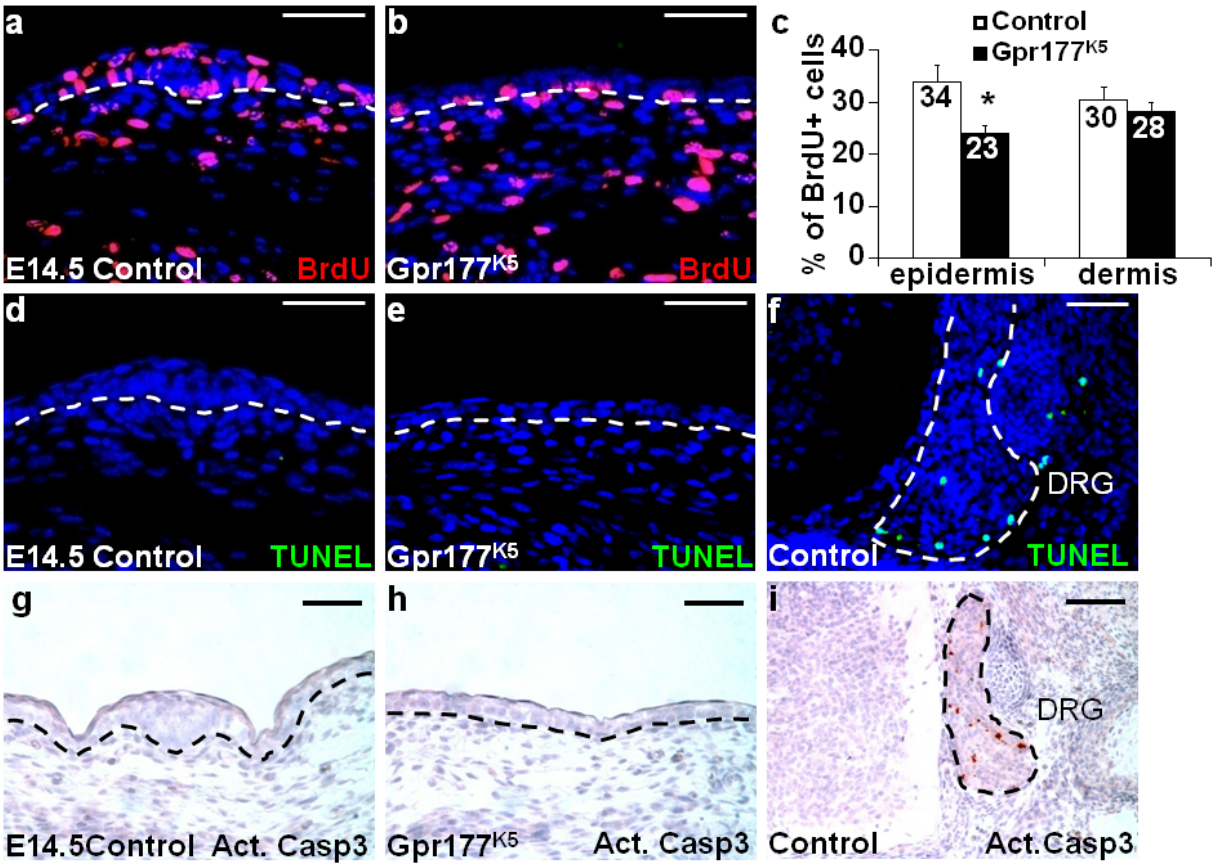

Fig. S5. Deletion of Gpr177 in the dermis causes dermal abnormalities. Using BrdU incorporation assay, cells undergoing mitotic division are identified by immunostaining of BrdU in the E14.5 control (a) and $Gpr177^{Dermo1}$ (b) skins. Graph shows the percentage of proliferating cells positive for BrdU in the epidermis and dermis (c). Asterisk indicates that the reduction in the dermis caused by the dermal deletion of Gpr177 is statistically significant ($Gpr177^{Dermo1}$: 19.01±0.03% and control: 32.21±0.03%; *p* value <0.01, n=3). TUNEL staining (d-f) and immunostaining of activated caspase3 (g-i) detect apoptotic cells in the skins (d, e, g, h) and DRG (f, i) of control (d, f, g, i) and $Gpr177^{Dermo1}$ (e, h) skins at E14.5. Sections of the E15.5 control (j, l, n) and $Gpr177^{Dermo1}$ (k, m, o) skins are analyzed by immunostaining of Collagen I (Col 1; j, k), smooth muscle actin (SMA; l, m) and adiponectin (AdipoNT; n, o). Graph shows the percentage of cells positive for the differentiation markers in the dermis (p). Asterisks indicates that the alterations of differentiation caused by the dermal deletion of Gpr177 are statistically significant (*, *p* value <0.01; **, *p* value <0.05; n=3). Broken lines indicate the epidermal-dermal junction and the DRG. Genotype – Control: Gpr177Fx/Fx or Dermo1-Cre; Gpr177Fx/+ and $Gpr177^{Dermo1}$: Dermo1-Cre; Gpr177Fx/Fx. Scale bars, 50 μm (a, b, d-o).

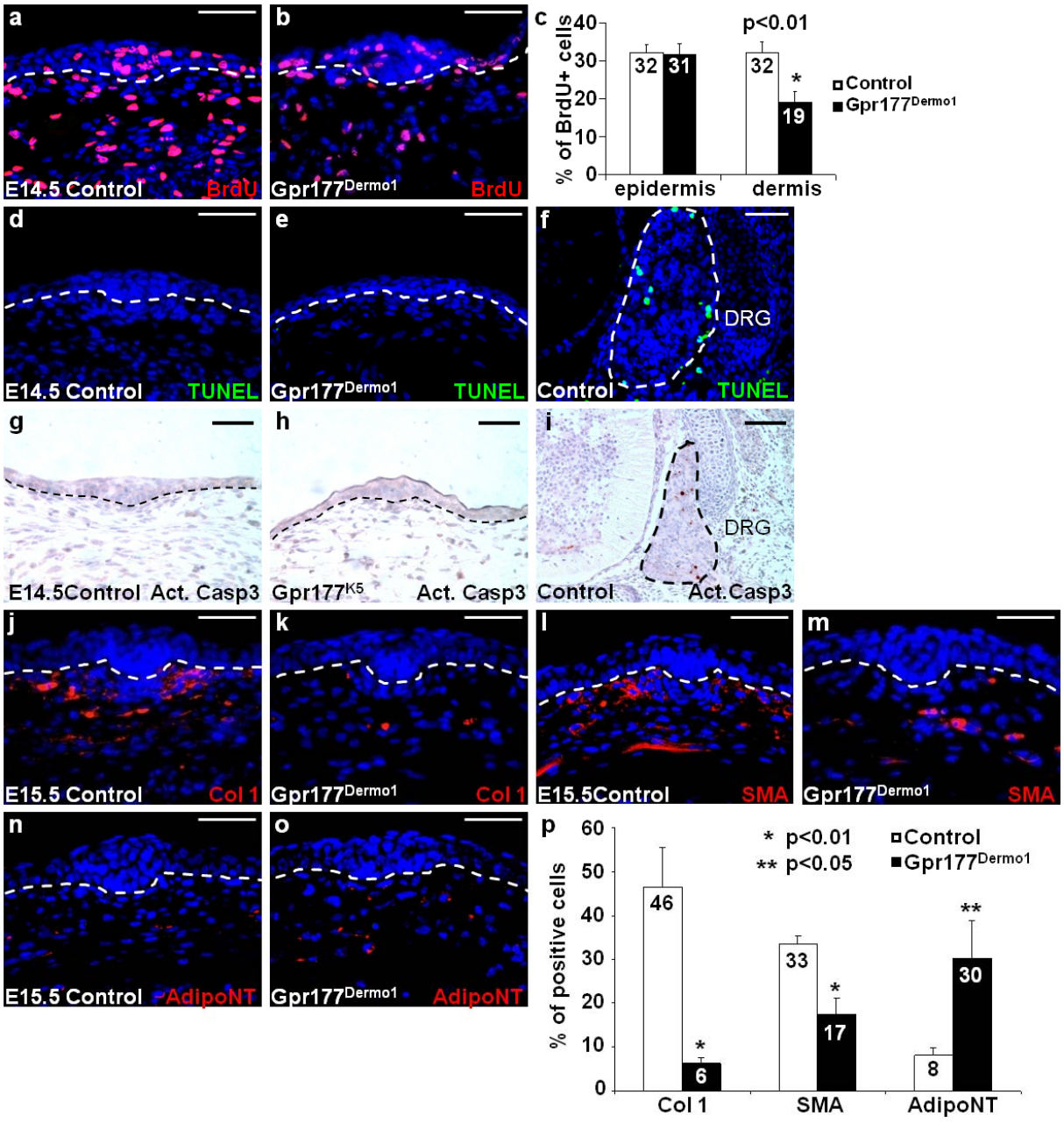
a
E14.5 Control
BrdU
b
Gpr177Dermo1
BrdU
c
% of BrdU+ cells
p<0.01
32
31
32
*
19
epidermis
dermis
Control
Gpr177Dermo1
d
E14.5 Control
TUNEL
e
Gpr177Dermo1
TUNEL
f
DRG
Control
TUNEL
g
E14.5Control Act. Casp3
h
Gpr177K5
Act. Casp3
i
DRG
Control
Act.Casp3
j
E15.5 Control
Col 1
k
Gpr177Dermo1
Col 1
l
E15.5Control
SMA
m
Gpr177Dermo1
SMA
n
E15.5 Control
AdipoNT
o
Gpr177Dermo1
AdipoNT
p
* p<0.01
** p<0.05
Control
Gpr177Dermo1
% of positive cells
46
*
6
33
*
17
8
**
30
Col 1
SMA
AdipoNT

Fig. S6. Wnt secretion is impaired in the Gpr177^{Dermo1} mutants. (a) Primary dermal cells isolated from the E14.5 control and Gpr177^{Dermo1} skins as the signal-producing cells were co-cultured with the signal-receiving cells harboring the TOPFLASH reporter. Relative luciferase activity (RLA) determined activation of the β-catenin and Lef/Tcf-dependent transcription. The addition of canonical Wnt3a and noncanonical Wnt5a in the media was used as the positive and negative controls, respectively. Asterisk indicates the statistical significance of reduction (*p* value <0.013, n=3). (b) Immunoblot analysis of Dvl2, phosphorylated Dvl2 (pDvl2), phosphorylated GSK3, activated β-catenin (ABC), β-catenin (β-cat) determined the canonical Wnt signaling activity in the E14.5 epidermis and dermis, and the primary dermal cells of control and Gpr177^{Dermo1} in vivo and ex vivo, respectively. The level of pDvl2, pGSK3 and ABC is elevated upon stimulation of Wnt signaling. The dermal deletion of Gpr177 causes either a slight reduction or no obvious effects on activation of Dvl2, GSK3 and β-catenin most likely due to the presence of epidermal Wnt in vivo. However, activation of canonical Wnt signaling was significantly reduced in the Gpr177^{Dermo1} dermal cells free of epidermal cells in the ex vivo culture. Actin level is used as a loading control.