# Supporting Information

## Enshell-Seijffers et al. 10.1073/pnas.1007326107

### SI Materials and Methods

**Mice.** *ROSA26 YFP* reporter (*r26YFP*), β-*actin cre*, and conditional floxed alleles of β-*catenin* (*Ctnnb1*$^{Flox/Flox}$*and Ctnnb1*$^{Flox3/Flox3}$) strains were obtained from F. Costantini (Columbia University, New York), Gail Martin (University of California, San Francisco), The Jackson Laboratory, and M.M.T., respectively. The DP-specific Cre (Cor-cre) mouse was generated by inserting sequences of Cre recombinase into the Corin locus (1). A knockout allele of β-catenin (Ctnnb1$^{Del/+}$) was generated by crossing the floxed allele *Ctnnb1*$^{Flox/Flox}$ to the *β-actin cre* line. For the loss of function analysis, mice of the genotype *Ctnnb1*$^{Del/+}$*; Cor-cre/Cor-cre;A/A* or *Ctnnb1*$^{Del/+}$*;Cor-cre/Cor-cre;a/a* were crossed with mice of the genotype *Ctnnb1*$^{Flox/Flox}$*;r26YFP/r26YFP;a/a* to obtain mice deleted for β-catenin and expressing YFP specifically in the DP with or without a functional allele of *Agouti (A/a or a/a)*. The genotype of mutant progeny was Ctnnb1$^{Del/Flox}$;Cor-cre/+;r26YFP/+, and littermates of the genotype Ctnnb1$^{Flox/+}$;Cor-cre/+;r26YFP/+ were used as controls and designated "wild type" because these mice were phenotypically indistinguishable from wild-type or Ctnnb1$^{Del/Flox;+/+}$; r26YFP/+ mice. For the real-time PCR analysis of Corin mice, mice of the genotype *Cor-cre/Cor-cre;Ctnnb1*$^{+/+}$*;a/a* were crossed with mice of the genotype *Cor-cre/+; Ctnnb1*$^{Flox/Flox}$*;r26YFP/r26YFP;a/a* to generate progeny of the genotype *Cor-cre/+; Ctnnb1*$^{Flox/+}$*;r26YFP+;a/a* (Corin$^{+/-}$) and *Cor-cre/Cor-cre; Ctnnb1*$^{Flox/+}$*;r26YFP+;a/a* (Corin$^{-/-}$). Note that the Cor-cre allele is a knockout allele of *Corin*, and heterozygous progeny of this cross represent a control group genotypically identical to the wild-type control group of the β-catenin loss of function experiment. For the gain of function analysis, mice of the genotype *Cor-cre/Cor-cre;A/A* were crossed with mice of the genotype *Ctnnb1*$^{Flox3/+}$*;r26YFP/r26YFP;A/A* to generate mice expressing constitutively activated β-catenin and YFP specifically in the DP (*Cor-cre/+;Ctnnb1*$^{Flox3/+}$*;r26YFP/+; A/A)* and littermate controls (*Cor-cre/+;Ctnnb1*$^{+/+}$*;r26YFP/+;A/A).*

**In Situ Hybridization and Immunostaining.** Middorsal skins were harvested, fixed in 4% paraformaldehyde, dehydrated with sucrose, and embedded in OCT. For in situ hybridization, frozen sections were hybridized with dig-labeled RNA probe corresponding to nt 126–613 of Agouti (NM_015770). Detection of Agouti transcript was performed with AP-conjugated anti-dig antibody (1:1,000) and BM purple solution (both from Roche). For immunostaining, fixed-skin sections were incubated for 1 h at room temperature with rabbit polyclonal anti-corin antibody (2) diluted 1:800 or 1:200, and detection was carried out with TRITC-conjugated donkey anti-rabbit antibodies (1:250) (The Jackson Laboratory) in the presence of TO-PRO-3 (1:40,000) to label nuclei. For Mitf and Tyr double immunostaining, fixed-frozen skin sections were used. Antigen retrieval in citrate buffer was followed by staining Mitf and Tyr at 4 °C overnight with mouse monoclonal anti-Mitf (1:10) and rabbit polyclonal anti-Tyr (1:500). The MOM kit (Vector Laboratories) was used to detect Mitf, and TRITC-conjugated donkey anti-rabbit antibodies (1:1,000) (The Jackson Laboratory) in the presence of TO-PRO-3 (1:40,000) were used to detect Tyr and label nuclei, respectively. Confocal imaging was carried out with a Lieca imaging system.

**Hair Shaft Analysis.** Hairs were plucked at the end of the first cycle at P20 and mounted on slides with a thin layer of Gelvatol. To collect hairs formed in the second cycle, the hair coat was shaved at P20 to get rid off hairs generated in the first cycle and newly formed hairs were plucked at P50 after the end of the second cycle. At least 200 hairs per mouse were scored for pigment deposition by using at least nine wild-type and nine mutant mice. Hair shafts were photographed on Nikon Eclipse E800 microscope at 100× magnification with a Spot RT Slider digital camera. Bright field and green fluorescent images were taken for each hair sample. Lack of black pigment in the hair shaft results in autofluorescence that corresponds with the deposition of yellow pigment. The green channel of the fluorescent image was duplicated in the red channel to generate a yellow color. The modified fluorescent image was overlaid on the bright field image in Adobe Photoshop and reduced to 45% opacity.

**Real-Time PCR.** Middorsal skins of wild-type and mutant mice from P1–P10 were collected and used to prepare RNA with TRIzol solution (Invitrogen). The total RNA was further purified by using RNeasy mini kit (Qiagen), and a DNase I digestion step was included to eliminate genomic DNA. Normalized RNA quantities were reverse transcribed by using random hexamer primers and SuperScript First-Strand synthesis system III (Invitrogen). For real-time PCR, primer pairs (SuperArray) for β-*actin*, *Agouti*, *Prss12*, *Corin*, *Pomc*, *Defb14 (*β*-defensin)*, *Mitf*, *Dct*, *Tyrp1*, and *Silver* were used by using iCycler thermal cycler (Bio-Rad), MyiQ Single-color Detection system, MyiQ Optical System Software and CYBR Green/Fluroescein PCR Master Mix (SuperArray). Differences between samples were quantified based on the ΔΔCt method.


1. Enshell-Seijffers D, Lindon C, Kashiwagi M, Morgan BA (2010) β-catenin activity in the dermal papilla regulates morphogenesis and regeneration of hair. *Dev Cell* 18:633–642.
2. Enshell-Seijffers D, Lindon C, Morgan BA (2008) The serine protease Corin is a novel modifier of the Agouti pathway. *Development* 135:217–225.


**Fig. S1.** Ablation of β-catenin in the DP in nonagouti mice results in distinct coat color. In *A*, 2-mo-old wild-type and mutant mice are shown. The wild-type hair is a deeper black than the mutant. In *B*, hairs plucked from a 3-wk-old mutant after the first hair cycle are shown and compared with hairs plucked from 1-y-old wild type. In *C*, 15-month-old wild-type and mutant mice are shown. Because most follicles do not regenerate in the mutant, its hair coat is essentially that produced in the first hair cycle, whereas the wild-type hair coat is a composite derived from multiple hair cycles because most hairs are not shed. However, the change to brown pigment over time reveals a change in the properties of the hair pigment. In *D*, black wild-type hairs plucked after the first hair cycle and aged for a similar period ex vivo remain black, whereas mutant hairs aged in vivo are red-brown.

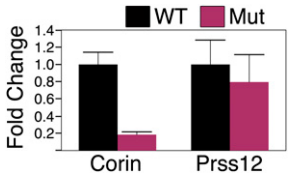


**Fig. S2.** Corin expression is reduced in DP cells. FACS-sorting was used to purify DP cells from P9 wild-type and mutant mice (1). Real-time PCR analysis was performed to measure the RNA levels of *Corin* and *Prss12*. The *y* axis represents fold change in expression with wild-type levels set to 1. Fivefold reduction in Corin levels is observed.

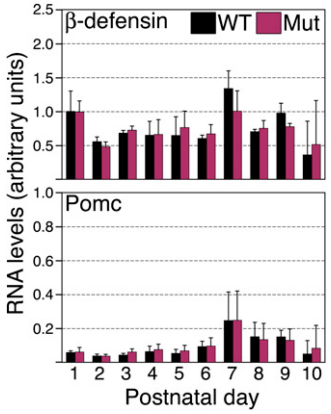


**Fig. S3.** β-Catenin in the DP does not regulate β-*defensin* (*Defb14*) or *Pomc* expression. Real-time PCR analysis of β-*defensin* and *Pomc* in whole-skin preparations from P1 to P10 was performed. The expression levels of these genes in mice lacking β-*catenin* in the DP (Mut) are comparable with those in littermate controls (WT). Note that, although RNA levels in both experiments were normalized to the same unit, different scales were used to accommodate the large difference in expression observed between β-*defensin* and *Pomc*.

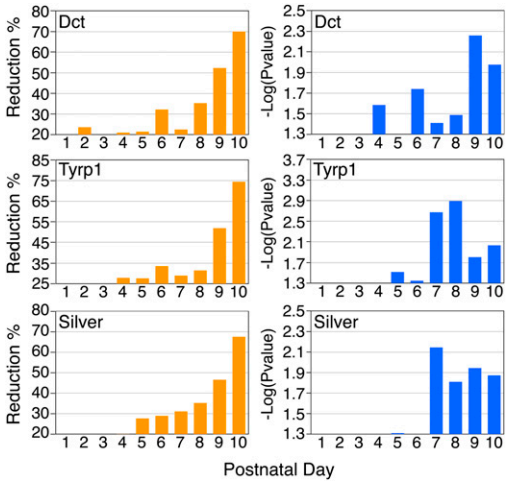


**Fig. S4.** Statistical analysis of the change in eumelanogenic gene expression on nonagouti background. (*Left*) Reduction in gene expression above 20% or 25% is shown. (*Right*) The statistical significance is shown as –Log(*P* value). Note that –Log(*P* value) = 1.3 represents $P = 0.05$.

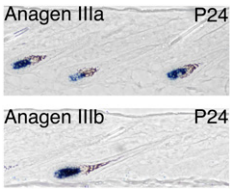


**Fig. S5.** *Agouti* expression during early anagen of the second hair cycle in wild-type mice. In situ hybridization for Agouti at P24 reveals that *Agouti* transcripts are detected through a wide range of early anagen stages in wild-type mice. (*Upper*) *Agouti* transcripts are detected in the DP, whereas black pigment is deposited in the extreme tip of the newly formed hair shaft. (*Lower*) A slightly more advanced follicle is shown in which the black apical tip is displaced away from the DP, whereas pheomelanin production in close proximity to the DP is accompanied by detectable levels of *Agouti* transcripts. This ■■■ demonstrates that the lack of *Agouti* expression in early anagen follicle of littermate ΔEX3 mice shown in Fig. 5*G* is a result of *Agouti* suppression and not different developmental stages in follicle regeneration.