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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Antibodies

Antigen	Vendor	Clone # or Product #	Dilution	Usage Notes
AE15	Santa Cruz	M-215	1:500	
Gata3	Santa Cruz	HG3-31	1:200	
Gata6	Cell Signaling	D61E4	1:300	
GFP/YFP	Aves Labs	GFP-1020	1:1,000	Used in most figures.
GFP/YFP	Abcam	ab6673	1:500	Used in Fig. S2C only.
K6	BioLegend	PRB-169P	1:500	
K14	Santa Cruz	C-14	1:500	
K75	American Research Products	03-GP-CK6HF	1:400	
K79	Santa Cruz	Y-17	1:250	Lot #J2412. Subsequent lots may not be effective.
K79	Abcam	Ab7195	1:500	This antibody, originally generated against mouse Gli2, is known to cross-react with K79 (Veniaminova et al., 2013).
Lef1	Cell Signaling	C12A5	1:200	
Lrig1	R&D Systems	AF3688	1:300	
Msx2	Santa Cruz	M-70	1:200	
pSmad1/5	Cell Signaling	D5B10	1:100	
Shh	R&D Systems	AF464	1:1,000	Antigen retrieval required (see below).
Shh	DSHB	5E1	1:1,000	Acetone fixation required (see below); used only in Fig. S2B.
Sox9	Santa Cruz	H-90	1:150	

Quantitating Shh$^+$/EGFP$^+$ matrix cell contribution to differentiated cell layers
Shh-EGFP-Cre mice were depilated at 8 weeks of age, and dorsal biopsies were harvested between 6-10 days afterwards. Non-consecutive sections were scored for potential overlap between EGFP and differentiation markers. The CL was identified by K79 expression in Anagen II follicles, and by K75 expression at more advanced stages. The IRS was identified by AE15 expression at all stages. For each stage (Anagen II, IIIa-b or IIIc-IV) and marker combination, at least 25 hair follicles were scored.

For Anagen II follicles, sections were initially examined in the EGFP channel, and hair follicles were analyzed only if there were at least 3 EGFP$^+$ cells within the secondary hair germ. Hair follicles were subsequently scored as positive if there was at least 1 EGFP$^+$ cell that overlapped with the differentiation marker (**Figure 6F**). For Anagen IIIa-b and IIIc-IV, sections were initially examined in the EGFP channel, and only hair follicles that were longitudinally cut, harbored at least 10 EGFP$^+$ cells in the hair bulb, and displayed expression of the differentiated marker, were used for analysis. Hair follicles were scored as positive only if there were cells that displayed overlap between EGFP and the differentiation marker, and appeared as a continuous stream originating directly from the Shh$^+$ matrix cell pool (similar to **Figure 6E**). Occasionally, hair follicles contained isolated EGFP$^+$ cells that possessed marker expression but were not continuous with the Shh$^+$ matrix cell pool. Since these cells may have arisen during earlier stages, they were not scored as positive. All follicles that met the above scoring criteria were evaluated, independent of hair subtype.

Lineage tracing and quantitating *Shh-Cre^{ERT2};R26R-YFP* matrix cell contribution to differentiated cell layers

Shh-Cre^{ERT2};R26R-YFP mice were depilated at 8 weeks of age and induced with a single dose of tamoxifen (1 mg per 40 grams body weight, dissolved in corn oil), 8 days post-depilation. Dorsal skin biopsies were harvested 3 days after induction and non-consecutive sections were scored for potential overlap between YFP and the IRS marker, AE15. Sections were initially examined in the YFP channel and hair follicles were analyzed only if there were at least 10 YFP$^+$ cells present. The contribution of Shh$^+$ matrix cells to each differentiation layer (CL, IRS, HS) was evaluated based on the spatial relationship between YFP$^+$ and AE15$^+$ cells. If YFP$^+$ cells were present to the outside of the AE15$^+$ cell domain, they was scored as CL. If YFP$^+$ cells were observed to the interior of the AE15$^+$ cell domain, they were scored as HS. Instances of YFP/AE15 overlay were scored as IRS. These categories were not mutually exclusive, as a given follicle could display Shh$^+$ matrix cell contributions to multiple differentiated cell layers. For this analysis, a total of 45 hair follicles were scored from 3 independent mice (**Figure S2C**). All follicles that met the above scoring criteria were evaluated, independent of hair subtype.

Immunohistochemistry

In most cases, skin biopsies were fixed for 1 hour in cold 3.7% paraformaldehyde, incubated overnight in 30% sucrose at 4°C, before embedding in OCT. Frozen sections were rehydrated with PBS, blocked and stained using standard conditions. For the Shh antibody from R&D, frozen sections prepared similarly to above were exposed to heat-mediated antigen retrieval at 98°C in citrate buffer (Biogenex Laboratories) (pH 6.0) for 1 minute. Following this treatment, standard staining protocols were followed. For the 5E1 Shh antibody, skin biopsies were harvested and directly embedded into OCT without fixation. Frozen sections were incubated in 100% acetone for 15 minutes at room temperature, rinsed with PBS and air dried for 30 minutes. Following this treatment, standard staining protocols were followed. For images containing GFP/EGFP/YFP, fluorescence was typically visualized by antibody staining, except for the epifluorescent whole mount images shown in **Figure 1E**. In *K79-Cre;R26R-YFP* mice, which harbor both GFP and YFP alleles, we did not distinguish between these signals for our analyses. Image processing was performed using Adobe Photoshop CS6, with the Auto-Blend feature applied to maximize image sharpness across focal planes in the same microscopic field.

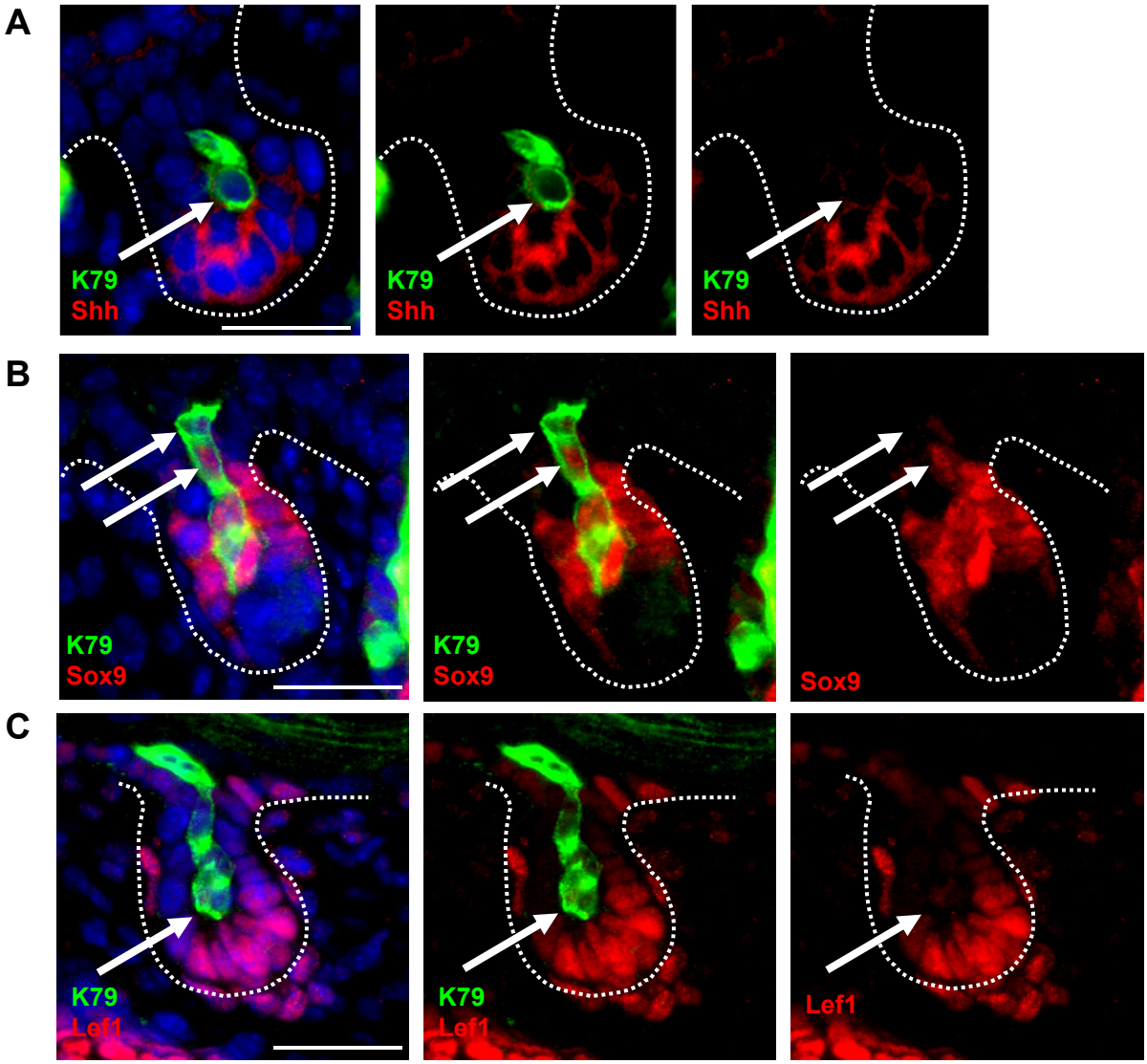

Figure S1. Differentiated K79$^+$ cells in P2.5 hair germs initially express Sox9, but not Shh or Lef1. Related to Figure 3. A. K79$^+$ cells (green, arrow) do not express Shh (red). **B.** K79$^+$ cell columns overlay with Sox9 proximally (lower arrows), but lose Sox9 distally (upper arrows). **C.** K79$^+$ cell columns do not express Lef1 (arrow). Middle and right boxes are separated channel views of the left boxes. The middle image in (B) is also depicted in Figure 3E. Scale bars, 50 μm.

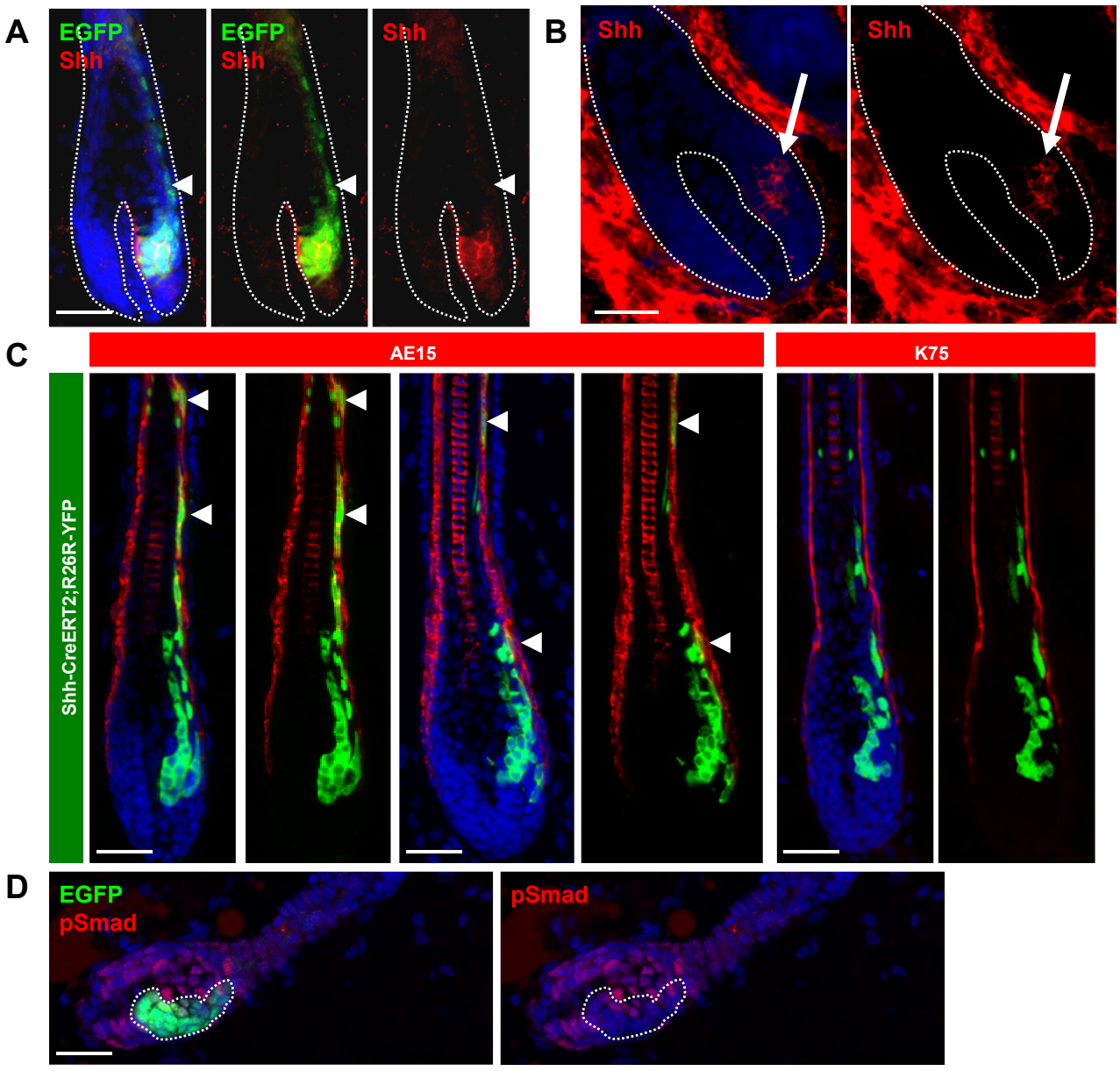

Figure S2. The CL is completed prior to other differentiated layers during hair regeneration.
Related to Figure 6. A. In *Shh-EGFP-Cre* anagen follicles, 8 days post-depilation, EGFP+ cells overlap with, and extend beyond (arrowhead), the domain of Shh protein expression, as assessed by IHC using an anti-Shh antibody (R&D Systems). **B.** A similar domain of Shh expression (arrow) is seen using an independent anti-Shh antibody (clone 5E1). **C.** In *Shh-CreERT2;R26R-YFP* mice, Shh^+ matrix progenitors and their progeny were permanently labeled upon treatment with tamoxifen, administered 8 days post-depilation. In biopsies harvested 3 days subsequently, labeled matrix cells formed $AE15^+$ IRS (left, arrowheads; 2 examples depicted), but not $K75^+$ CL (right). No matrix contribution to the CL was observed in a total of 45 follicles from 3 independent *Shh-CreERT2;R26R-YFP* mice treated in this manner. **D.** In *Shh-EGFP-Cre* anagen follicles, 8 days post-depilation, Shh+ matrix progenitors (dotted lines) sometimes continued to display reduced pSmad, similar to what is seen during earlier stages of anagen. In all panels, separated channel views are also depicted. Scale bars, 50 μm.

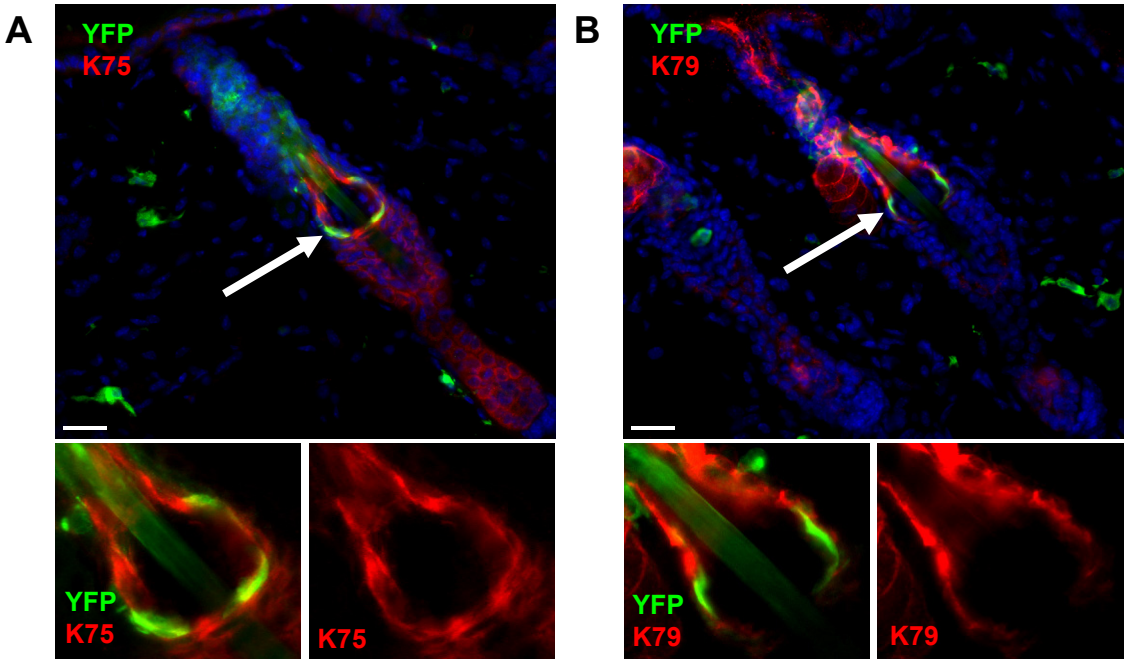

Figure S3. CL cells are shed into the hair canal during catagen.
Related to Figure 7. A. In *K79-Cre;R26R-YFP* catagen skin, labeled CL cells (green) continue to express K75 (red) as these cells move up and are shed into the hair canal. **B.** These same labeled cells do not express K79 (red), and are located to the inside of $K79^{+}$ suprabasal cells lining the infundibulum. Lower panels are separated channel views of the regions indicated by the arrows. Scale bars, 50 μm.

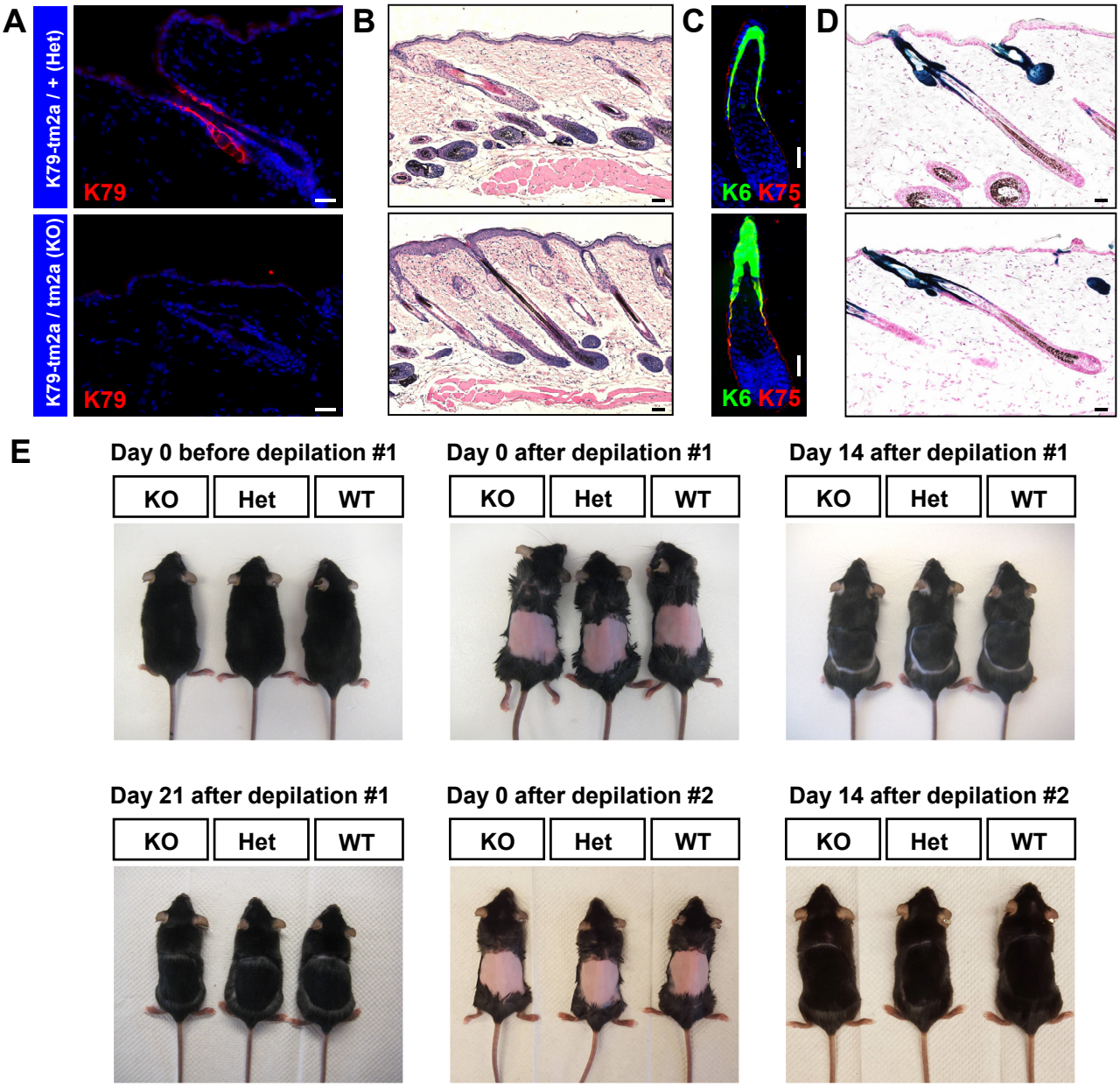

Figure S4. Functional testing of K79.
Related to Figure 7. A. Complete loss of K79 (red) was validated in *K79*$^{tm2a/tm2a}$ mice (KO) compared to *K79*$^{tm2a/+}$ control mice (Het) in adult telogen follicles from 8 week old mice. **B.** Hematoxylin and eosin staining of anagen skin from *K79-Het* and *K79-KO* skin, both from adult mice biopsied 10 days after depilation. **C.** Loss of K79 does not affect canonical expression of K75/K6 in the CL of hair follicles, 8 days after depilation. **D.** LacZ activity, which identifies cells which would normally express *K79*, is unchanged between *K79-Het* and *K79-KO* hair follicles from adult mice biopsied 8 days after depilation. **E.** Loss of K79 does not affect hair regeneration in depilated adult mice. Scale bars, 50 μm.

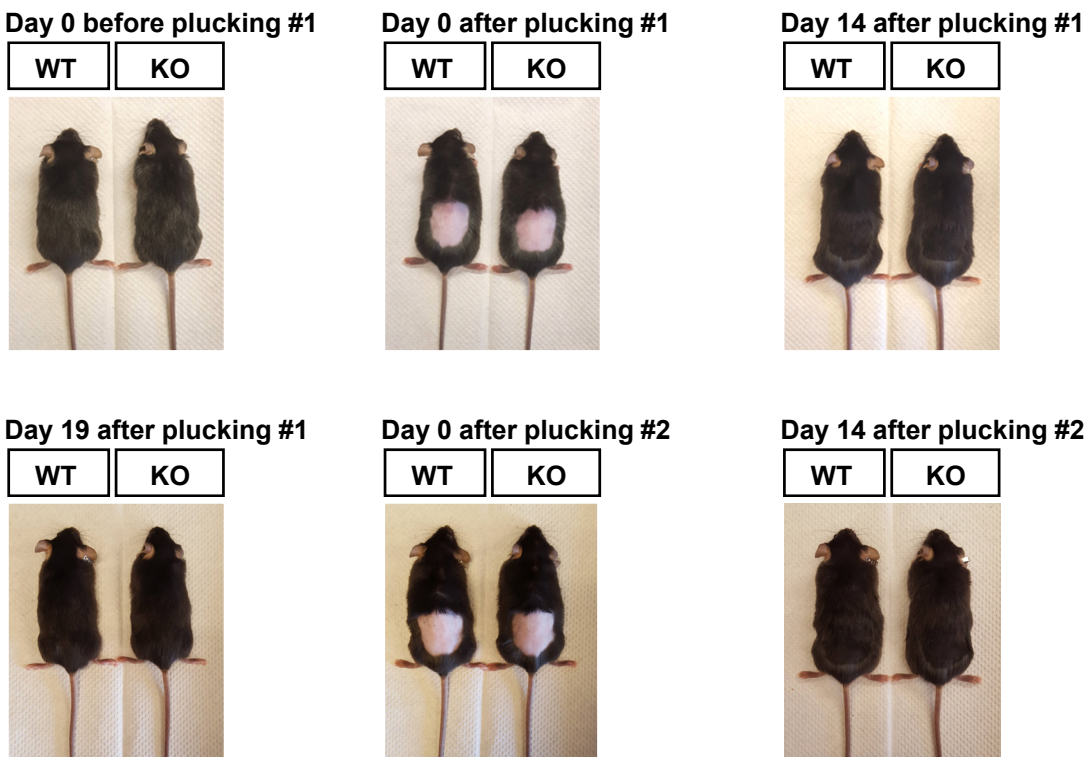

Figure S5. Loss of K79 does not affect plucking-induced hair regeneration.
Related to Figure 7. Similar to in Figure S4E, K79 is not required for hair regeneration induced by 2 consecutive cycles of hair plucking.

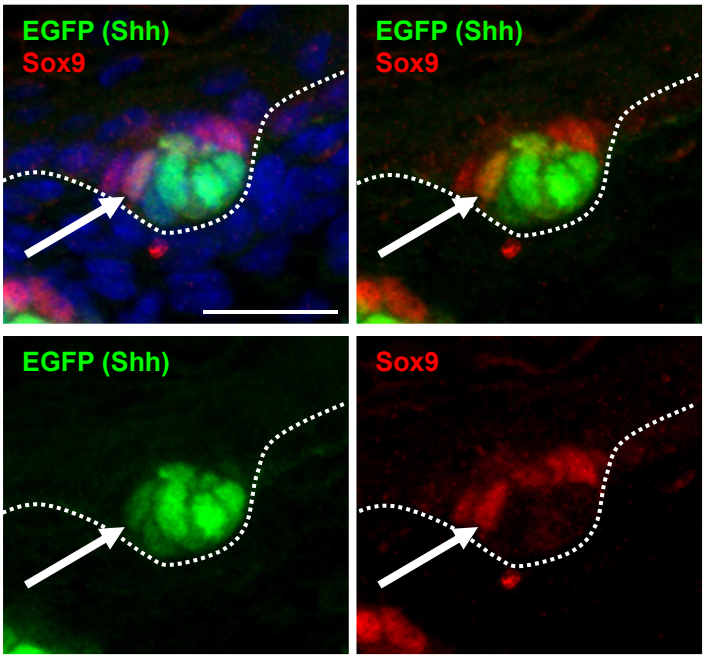

Figure S6. Early formation of basal layer future bulge cells. Related to Figure 3.
Hair follicle placodes from newborn *Shh-EGFP-Cre* mice display strong EGFP at the base of the developing follicle and weak EGFP in overlying basal layer future bulge cells ($Sox9^{+}$, arrow), suggesting a direct lineage relationship. Individual panels represent separated channels of the same image, as indicated. Scale bars, 50 μm.