# SUPPLEMENTAL MATERIALS AND METHODS

## Generation of *Tcf3-2A-eGFP-2A-CreER$^{T2}$* knock-in mice

To generate *Tcf3* knock-in mice, the targeting construct (Figure S1A) was generated by two-step gap repair recombineering (Lee et al., 2009). First, the *2A-eGFP-2A-CreER$^{T2}$* fragment was generated by overlap extension PCR in two steps using the primers GFP_T2A_fw, GFP_F2A_rv, Cre_F2A_fw, and Cre_Nhe_Eco_rv with the plasmids pMSCV-IRES-GFP (Addgene #20672, Tannishtha Reya lab) and pCAG-CreER$^{T2}$ (Addgene #14797, Connie Cepko lab) as templates. The resulting fusion product was cloned into plasmid PL451 (Liu et al., 2003) containing a *PGKneo* selection cassette. A *2A-eGFP-2A-CreER$^{T2}$-PGKneo* fragment was then PCR-amplified with appropriate genomic homology using primers T3KI_cass_fw/rv and recombined into a 129Sv-derived bacterial artificial chromosome (BAC) (Adams et al., 2005) containing the desired portion of the *Tcf3* gene (clone bMQ275e20; obtained from Source BioScience, Nottingham, UK). Finally, the targeting construct and appropriate homology arms were recombined by gap repair into the plasmid PL253, which had been PCR-amplified using primers PL253_genom_L2/R2. The targeting vector was electroporated into AB2.2 mouse embryonic stem cells (ESCs) by the Mouse Embryonic Stem Cell Core Facility at BCM. ESC clones were initially screened by long-range PCR using Crimson LongAmp *Taq* polymerase (New England Biolabs, Ipswich, MA, USA) with primers T3KI_5LR2_fw/rv and T3KI_3LR_fw/rv (data not shown). Targeting was confirmed in selected clones by Southern blot of *Spe*I-digested DNA using probes outside the 3’ and 5’ homology arms (Figure S1B). Southern blots were performed using the DIG nonisotopic

detection system (Roche Applied Sciences, Indianapolis, IN, USA) according to the manufacturer's instructions; probes were generated using PCR primers T3KI_5SB_fw/rv and T3KI_3SB_fw/rv. Germline transmission in agouti-colored progeny of chimeric mice was verified by Southern blot (data not shown) and subsequent genotyping was performed by PCR using primers Tcf3KI_fw, Tcf3KI_GC_rv, and Tcf3KI_WT_rv (Figure S1C). Mice with germline transmission of the knock-in allele were crossed with *ROSA26-FLPe* mice (Farley et al., 2000) in order to delete the *FRT*-flanked *PGKneo* drug selection cassette (Figure S1A). Primers used in cloning and genotyping are listed in Table S1.

## SUPPLEMENTAL REFERENCES


**Adams, D. J., Quail, M. A., Cox, T., van der Weyden, L., Gorick, B. D., Su, Q., Chan, W.-I., Davies, R., Bonfield, J. K., Law, F., et al.** (2005). A genome-wide, end-sequenced 129Sv BAC library resource for targeting vector construction. *Genomics* **86**, 753–758.

**Farley, F. W., Soriano, P., Steffen, L. S. and Dymecki, S. M.** (2000). Widespread recombinase expression using FLPeR (flipper) mice. *Genesis* **28**, 106–110.

**Lee, S.-C., Wang, W. and Liu, P.** (2009). Construction of gene-targeting vectors by recombineering. *Methods Mol. Biol.* **530**, 15–27.

**Liu, P., Jenkins, N. A. and Copeland, N. G.** (2003). A highly efficient recombineering-based method for generating conditional knockout mutations. *Genome Res.* **13**, 476–484.

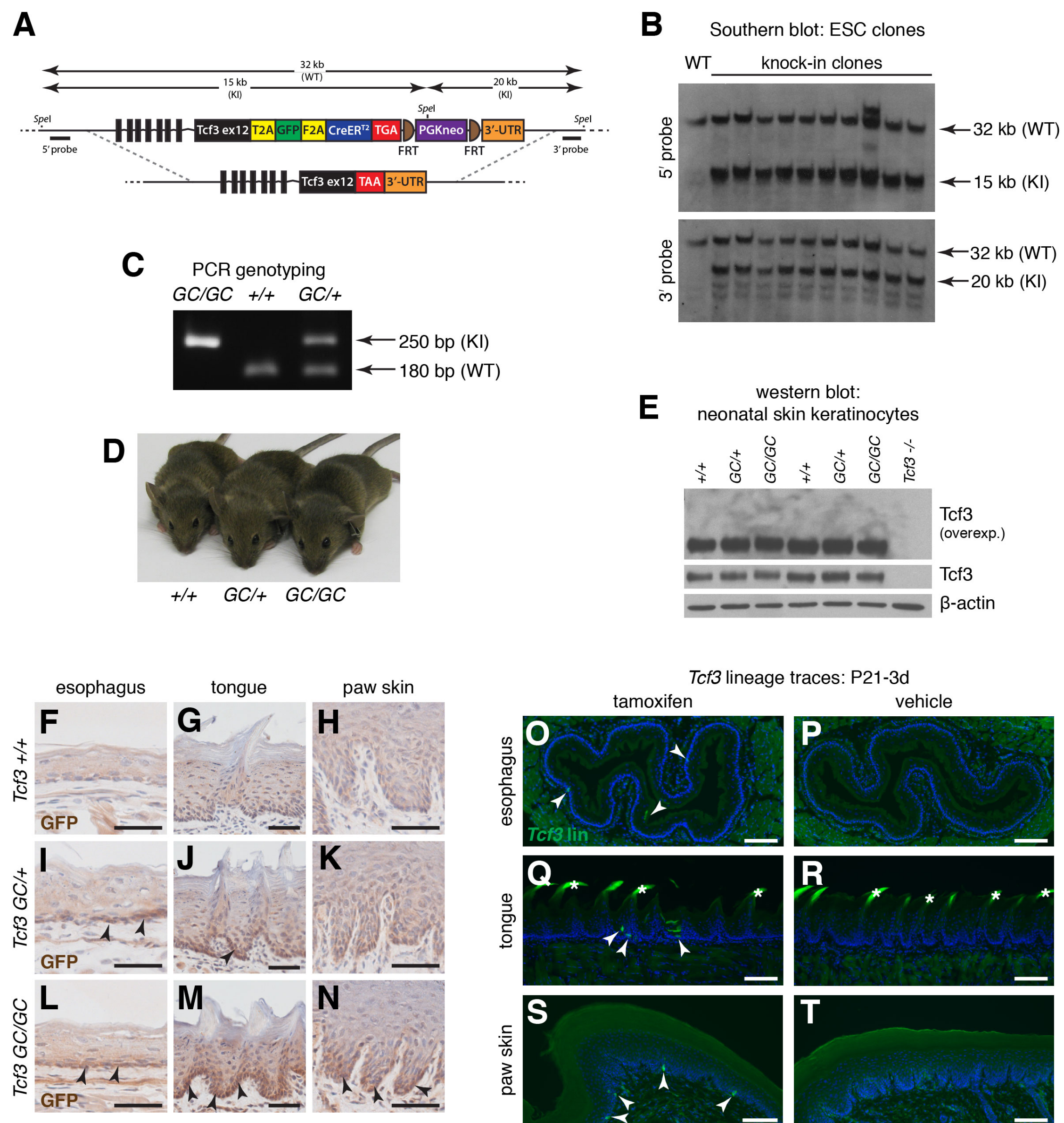


**Figure S1. Generation and validation of *Tcf3* knock-in mice.**

(**A**) Schematic of *Tcf3* knock-in targeting strategy, showing targeting construct (top) and the targeted region of the wild-type genome (bottom). *Spe*I restriction sites and location of 5’ and 3’ probes for Southern blotting are shown.

(**B**) Representative example of Southern blot to confirm targeting in ESC clones. *Spe*I-digested genomic DNA was separated on an agarose gel and probed first with the 5' probe, then stripped and re-probed with the 3' probe. One clone (third from right) shows an abnormal double insertion; the others yield the expected bands. WT: control gDNA from wild-type ES cells.

(**C**) Representative results from routine PCR genotyping of *Tcf3* knock-in mice.

(**D**) Representative image of young adult (P50) *Tcf3*$^{+/+}$, $^{GC/+}$, and $^{GC/GC}$ littermates. Knock-in mice are viable, fertile, and manifest no obvious phenotype.

(**E**) Western blot of Tcf3 expression in neonatal skin keratinocytes from *Tcf3*$^{+/+}$, $^{GC/+}$, and $^{GC/GC}$ mice. Knock-in mice express Tcf3 at wild-type levels (middle panel). No Tcf3-GFP fusion products are detected even with prolonged exposure (top panel), suggesting minimal "read-through" of the 2A peptides *in vivo*. Far right lane contains protein from *Tcf3*$^{-/-}$ keratinocytes as a negative control.

(**F-N**) Immunohistochemical staining for GFP in esophagus, tongue, and plantar paw skin of *Tcf3*$^{+/+}$, $^{GC/+}$, and $^{GC/GC}$ mice. In all three tissues, *Tcf3*-GFP expression is detected in the epithelial basal layer (arrowheads) in a pattern mirroring that of endogenous Tcf3 (compare Figures 3A-C, 4A-D).

(**O-T**) Three days after tamoxifen treatment of *Tcf3*$^{GC/+}$*; ROSA26*$^{mTmG/+}$ mice, mGFP(+) *Tcf3*-lineage (*Tcf3* lin) cells are detected in the epithelia of the esophagus (O), tongue (Q), and plantar paw skin (S) (arrowheads) of tamoxifen-treated mice, but never in vehicle-treated control mice (P, R, T). Asterisks: autofluorescence of lingual filliform papillae. Scale bars: 50 μm (F-N); 100 μm (O-T).

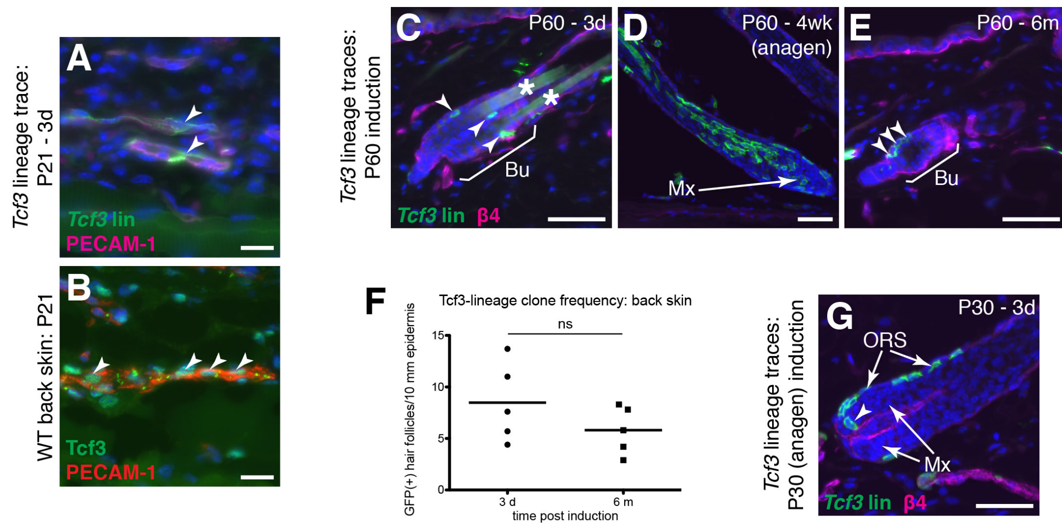


**Figure S2. *Tcf3* labels hair follicle stem cells in adult skin.**

(**A**, **B**) *Tcf3* is expressed in dermal blood vessels. (**A**) Colocalization of mGFP reporter (arrowheads) and endothelial marker PECAM-1 in *Tcf3*-lineage (*Tcf3* lin) dermal cells. (**B**) Antibody costaining for Tcf3 (arrowheads) and PECAM-1 verifies expression of *Tcf3* in dermal blood vessels.

(**C-E**) Lineage traces in $Tcf3^{GC/+}$; $ROSA26^{mTmG/+}$ mice with induction during the second postnatal telogen (~P60). Results are similar to those obtained with induction at P21. (**C**) Three days after induction, individual mGFP(+) cells (arrowheads) are observed within the hair follicle bulge (Bu). (**D**) In the subsequent anagen phase, mGFP(+) clones expand and incorporate into the growing hair shaft via the hair matrix (Mx). (**E**) Persistent clones (arrowheads), often multicellular, are still observed in the HF bulge (Bu) six months post induction.

(**F**) Quantitation of clone frequency in back skins three days and six months after induction of lineage tracing at P21/22. The majority (68.4 ± 3.7%, mean ± s.d.) of clones persist, with a moderate, non-significant decline in the frequency of labeled follicles ($p$ =

0.42, Mann-Whitney *U* test). Dots represent number of labeled follicles per 10 mm dorsal skin in individual mice; bars indicate mean.

(**G**) Three days after lineage-tracing induction in anagen, *Tcf3*-lineage clones (arrowhead) are observed in transition between the outer root sheath (ORS) and the matrix (Mx) (compare Figure 1D, E).

Scale bars: 50 µm (all). Asterisks: hair shaft autofluorescence.

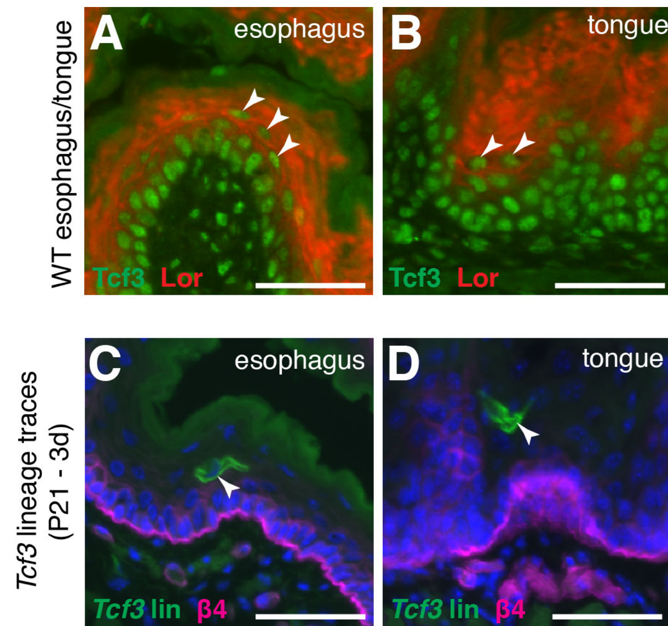


**Figure S3. *Tcf3* expression is observed in differentiating suprabasal cells in esophageal and lingual mucosa.**

(**A**, **B**) Immunofluorescent costaining for Tcf3 and granular layer marker loricrin (Lor) in esophageal (A) and lingual (B) mucosa. *Tcf3*-expressing cells (arrowheads) are observed within the differentiating granular layer.

(**C**, **D**) Three days after lineage-tracing induction, clones of solitary suprabasal *Tcf3*-lineage (*Tcf3* lin) cells (arrowheads) are seen in the esophagus (C) and tongue (D), confirming the specificity of Tcf3 antibody staining. β4: β4 integrin.

Scale bars: 50 µm.

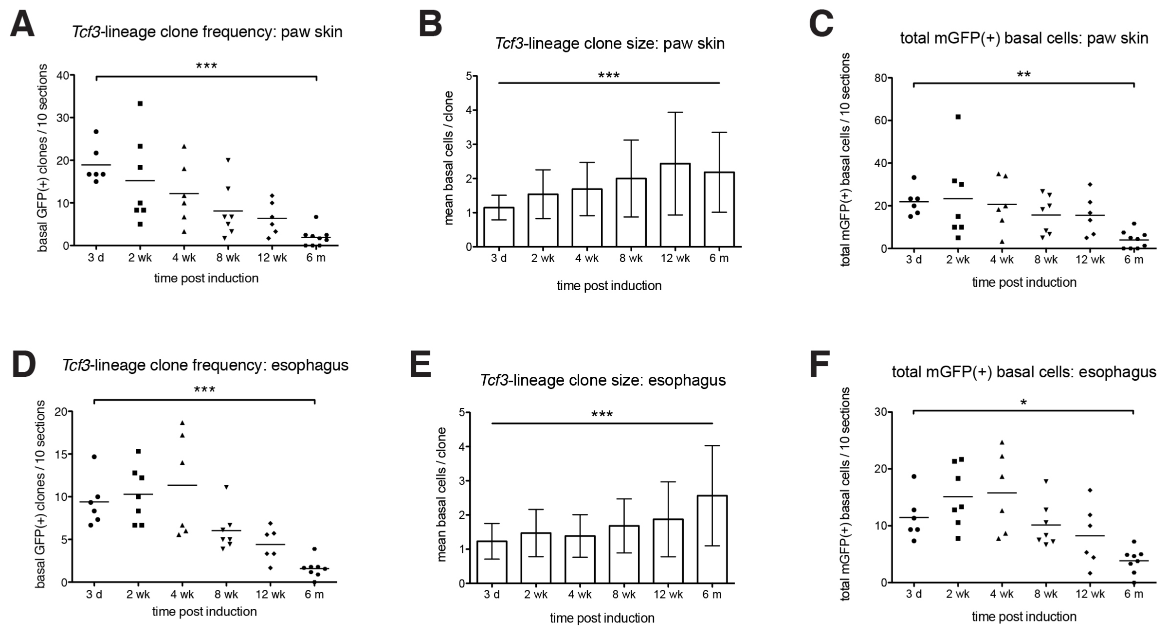


**Figure S4. *Tcf3*-lineage clones are progressively lost over time in both paw skin and esophageal mucosa, and surviving clones expand laterally through the basal layer.** $Tcf3^{GC/+};R26^{mTmG/+}$ mice were treated with tamoxifen at P21/22, and specimens of paw skin and esophageal mucosa were collected at various timepoints thereafter. The number of mGFP(+) clones (**A**, **D**), number of mGFP(+) basal cells per clone (**B**, **E**), and total number of mGFP(+) basal cells (**C**, **F**) were quantitated in random cryosections from both tissues. Counts were limited to those clones containing ≥ 1 basal cell in any given section.

(**A**, **D**) In both tissues, a progressive loss of clones was observed over time. Individual dots represent the number of mGFP(+) clones in individual mice, bars represent mean at each timepoint. Six months after induction, 9.9% ± 8.7% of initially labeled clones persisted in the paw skin; 17.0% ± 6.6% of clones persisted in esophagus (mean ± s.d., $p < 0.001$ for both comparisons, Dunn's multiple comparison test).

(**B**, **E**) Clone loss over time was accompanied by lateral expansion of the surviving clones through the basal layer. Columns represent the average number of basal cells per clone in all mice at each timepoint; error bars represent s.d. In both cases, the clone expansion over time was significant ($p < 0.0001$, Kruskal-Wallis test).

(**C**, **F**) The total number of labeled basal cells within both tissues declined over time, contrary to the predictions of the neutral competition model. However, the decrease only reached significance when comparing the 6-month and 3-day timepoints ($p < 0.01$ for both comparisons, Dunn's multiple comparison test).

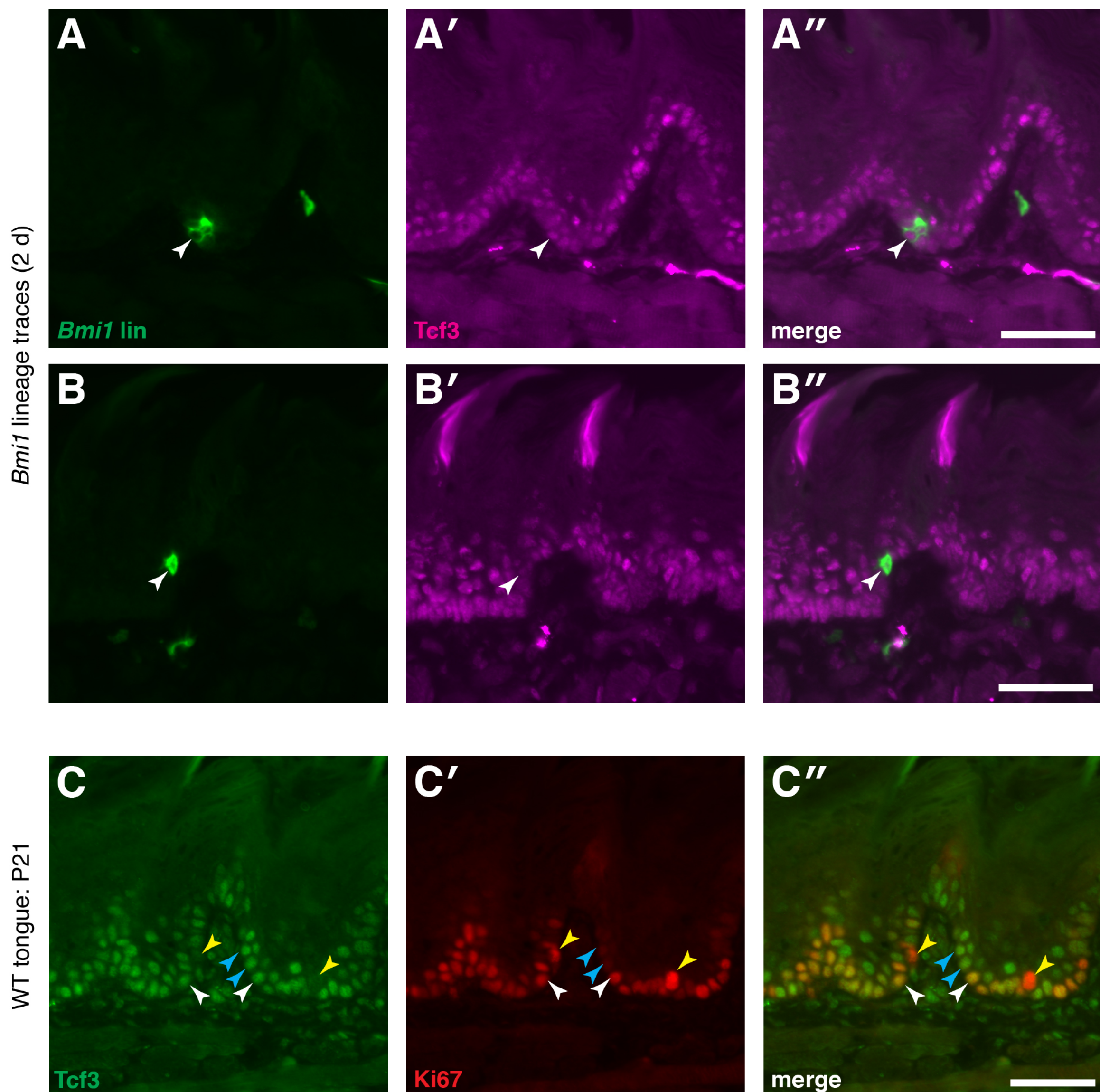


**Figure S5.** ***Tcf3*** **expression does not correlate with** ***Bmi1*** **expression or proliferative status in the lingual epithelium.**

(**A**, **B**) *Bmi1*$^{CreER/+}$;*R26*$^{mTmG/+}$ mice were treated with a single dose of tamoxifen and sacrificed two days later; specimens of tongue epithelium were collected and stained for Tcf3 protein. Clones of 1-2 *Bmi1*-lineage (*Bmi1* lin) basal cells (arrowheads) were observed at a frequency of ~1 cell/IPP, consistent with the previous report of Tanaka *et al*.

In some cases (**A**), the labeled cells expressed *Tcf3* at a level similar to that of neighboring cells; in others (**B**), they possessed little to no *Tcf3* expression. These observations suggest that expression of the two factors is not directly linked.

(**C**) Wild-type tongue specimens were costained for Tcf3 (green) and Ki67 (red) proteins. There was little correlation between expression of the two factors, and populations of cells were identified which were variously $Tcf3^{+}$ $Ki67^{+}$ (white arrowheads); $Tcf3^{+}$ $Ki67^{-}$ (blue arrowheads); and $Tcf3^{-}$ $Ki67^{+}$ (yellow arrowheads). These observations suggest that Tcf3 expression is not directly tied to proliferative status.

Scale bars: 50 µm (all).

**Table S1. PCR primers for cloning, blotting and genotyping**

| Primer | Sequence (5' → 3') |
|---|---|
| GFP_T2A_fw | GGCTCTGGA GAGGGCAGAGGAAGTCTGCTAACATGCGGTGACGTCGAG GAGAATCCTGGCCCA ATGGTGAGCAAGGGCGAGG |
| GFP_F2A_rv | CCGCCAACTTGAGAAGGTCAAAATTCAAAGTCTGTTTCAC TCCGGATCC CTTGTACAGCTCGTCCATGCC |
| Cre_F2A_fw | CTTTGAATTTTGACCTTCTCAAGTTGGCGGGAGACGTGGA GTCCAACCCAGGGCCC ATGTCCAATTTACTGACCGTACAC |
| Cre_Nhe_Eco_rv | CCGAA GCTAGC GAATTC TCAAGCTGTGGCAGGGAAACC |
| T3KI_cass_fw | TCCCCGTGCTACAGGCCCAGCCTCTTTCCTTGGTCACCAA GTCTGCCCAC GGCTCTGGA GAGGGCAG |
| T3KI_cass_rv | ATCATTACTACTTGGAGTCCCGAGACAGCCTGCATAGAAC CGGGGGCAGC CGAAGTTATATTATGTACCTGACTGATG |
| PL253_genom_L2 | ACCGAGTAGCTTCCTAGTTAGTCCTCTGTACTTGTTAGCT GTAGCAGCATAAGGTGAAAC GGCGCGCC GCGTCAGGTGGCACTTTTC |
| PL253_genom_R2 | ACAAACAGATTCATTTACCAGTTCACTTCCTCTAGCAAGG CAGAGTCAACATTATAAAAT CCTGCAGG CTCTAGAGTCGAGCAGTGTGG |
| T3KI_5LR2_fw | AGCGAACGGGGTTGACCAGAGTGA |
| T3KI_5LR2_rv | AGCTCCTCGCCCTTGCTCACCATT |
| T3KI_3LR_fw | ATGCTTCTGTCCGTTTGCCGGTC |
| T3KI_3LR_rv | TCTAGTCCTGCACGGCTGGAAGTT |
| T3KI_5SB_fw | AGCGGAGCCCAGCTAAGTCGC |
| T3KI_5SB_rv | GGCTGCCCAGGAAATCACCCAA |
| T3KI_3SB_fw | GTATCACAGCTTTTCGGGGTTGCTC |
| T3KI_3SB_rv | CCTTCACATGTCTGTCCACTCACTG |
| Tcf3KI_fw | TCTGCTGACCTCTCCCCCTACT |
| Tcf3KI_GC_rv | AACTTGTGGCCGTTTACGTCGC |
| Tcf3KI_WT_rv | TGTGTTTCCCCCTTCCTCTCCT |

**Table S2. Antibodies used for immunofluorescence, immunohistochemistry and western blotting**

| Antibody | Manufacturer | Prod. No. | Dilution |
|---|---|---|---|
| rat anti-β4 integrin (CD104), clone 346-11A | BD Biosciences | 553745 | 1:200 (IF) |
| rabbit anti-filaggrin | Covance | PRB-417P | 1:500 (IF) |
| chicken anti-GFP | Abcam | ab13970 | 1:1,000 (IF) |
| rabbit anti-GFP | Life Technologies | A6455 | 1:500 (IF); 1:10,000 (IHC) |
| rabbit anti-keratin 1 | Elaine Fuchs lab | | 1:500 (IF) |
| rabbit anti-keratin 5 | Covance | PRB-160P | 1:2,000 (IF) |
| rabbit anti-keratin 6 | Elaine Fuchs lab | | 1:250 (IF) |
| rabbit anti-keratin 13, clone EPR3671 | Abcam | ab92551 | 1:1,000 (IF) |
| mouse AE13 (anti-hair cortex cytokeratin) | Abcam | ab16113 | 1:100 (IF) |
| rabbit anti-Lef1, clone C12A5 | Cell Signaling | 2230 | 1:200 (IF) |
| rabbit anti-loricrin | Covance | PRB-145P | 1:500 (IF) |
| rat anti-PECAM-1 (CD31), clone MEC 13.3 | BD Biosciences | 550274 | 1:100 (IF) |
| guinea pig anti-Tcf3 | Nguyen lab | | 1:200 (IF; IHC w/ HIER); 1:1,000 (WB) |
| mouse AE15 (anti-trichohyalin) | Santa Cruz | sc-80607 | 1:100 (IF) |
| mouse anti-β-actin, clone AC-15 | Sigma | A1978 | 1:50,000 (WB) |

IF, immunofluorescence; IHC, immunohistochemistry; WB, western blot; HIER, heat-induced epitope retrieval (in 10 mM sodium citrate, pH 6.0)