Supplementary Data

Novel Dynamics Between Stem Cells and Their Niche in the Hair Follicle.

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Supplemental Figure Legends:

Figure S1. Schematic of an Anagen Hair Follicle, and Proliferation Analyses of Anagen Outer Root Sheath Cells, Related to Figure 1 and 4 (A) Schematic. During anagen, matrix cells (also referred to as transit amplifying or TA cells) are located at the base of the growing follicle. There, they form a bulb that envelops the dermal papilla (DP). Mesenchymal-epithelial interactions maintain matrix cells in a rapidly proliferative and relatively undifferentiated state, but also provide fate-instructive properties. As matrix cells move upward, they terminally differentiate into one of the seven distinct lineages that are spatially organized into concentric rings in an onion-like fashion: the medulla, cortex and cuticle of the hair shaft; the cuticle, Henle and Huxley layers of the inner root sheath (IRS), and the companion layer (Cp), which is sandwiched between the IRS and ORS. Shh, Sonic hedgehog, a marker of the lateral disc, which is a small pocket of cells that gives rise to the IRS layers. K6 is a marker of the anagen-phase companion layer. Sox9, Lhx2, Tcf3 and Lgr5 are all markers that are enriched in the CD34+ bulge cells, but also expressed throughout the ORS cells between the bulge and the matrix (Greco et al., 2009). K5 and K14 are markers of the basal cells of the epidermis, ORS, sebaceous gland and bulge.

(B-C). These data pertain to Figures 1 and 4. (B) shows the average epifluorescence intensity of individual cells in the bulge (Bu) and ORS of *Tet-OFF H2BGFP* HFs, which were chased beginning at P21 at the start of the first telogen phase of the hair cycle and then analyzed at P37, the end of the anagen phase. The data are plotted as box-and-whisker diagram, indicating the median (middle line), 25-75 percentile (box), minimum and maximum (whiskers), n=22 HFs. Note that at this time, the upper ORS cells (positions 1-12) display slow-cycling characteristics similar to the initial bulge, but they have undergone on average 1-2X more divisions, reflected in their reduced epifluorescence. Cells below position 30 divided more times, and GFP levels dipped below detection limits. (C) Examples of *Tet-*

OFF H2BGFP HFs chased since P21 and then analyzed at P29-P33. Just prior to analyses, mice were given two short (6hr) BrdU pulses so that we could track the proliferative behavior of all the HF cells at this time (BrdU+) and contrast this with their slow cycling nature (GFP+). Note that at P29 mid anagen, there are many double positive cells in the bulge and in the ORS^{GFP+} zone. At P31, proliferation in the bulge has ceased while proliferation events are still detectable throughout the ORS. By P33, the upper ORS has mostly withdrawn from the cell cycle. By contrast, the lower ORS and matrix are hyperproliferative throughout this period.

Figure S2. Immunogold Labeling of Label Retaining cells in the ORS and the Size and Cell Numbers of the Old bulge, Related to Figure 2.

(A-D) *Tet-OFF H2BGFP* mice were chased from P21 to P42, when their backskins were processed for immunogold EM. (A) Panoramic view of catagen HF (P42) indicating the old bulge and club hair. Black dotted line indicates the basement membrane. Boxed area in red is magnified in B. (B) H2B-GFP label retaining ORS cell (marked by the green line) contains apoptotic debris (Ap) in its cytoplasm. Boxed area in yellow is shown in C. Boxed area in blue is shown in D, showing apoptotic debris in the LRC cytoplasm is enlarged in D. (C) Nucleus of H2B-GFP label retaining cell (LRC) is labeled with anti-GFP conjugated with gold particles. (D) Apoptotic body present in the cytoplasm of the same cell is not labeled for GFP, suggestive that the healthy LRC engulfed a dying cell. (E) Quantifications in two dimensions of the total average size (in area) of the bulge niche and the numbers of CD34+ bulge cells within HFs at catagen (Cat) and telogen (Telo) stages. Skins were sectioned (10μm) and immunostained with CD34 to identify the bulge SCs. Only perfect cross sections through both the old club hair and the new hair shaft were used for quantifications of the overall surface area and for CD34+ SC numbers. In quantifying the cell numbers, the suprabasal CD34+ bulge cells were omitted since they are shared by both the old and new bulge. Littermates of the same sex and the same area of backskin were chosen to minimize any potential variations. Error bars were determined for n>30 HFs. Note that the overall size of the bulge and the numbers of its stem cells don't change appreciably in catagen and telogen, indicating that they cannot contribute

appreciably to the formation of the new bulge. Rather, as shown in the text, the new bulge is derived from LRCs within the upper ORS of the prior hair cycle. Data are mean ± SD.

Figure S3. Lgr5 Expression in the Adult AnaVI, Supporting Data for Tet-OFF H2BGFP System and the Fate of the ORS^{mid}, Related to Figure3.

(A) At full anagen (Anagen VI), Lgr5-CreERT2-IRES-EGFP mice express GFP in cells of CD34+ bulge and entire ORS below the bulge, as shown here by immunostaining. Shown also are FACS profilings, gating on CD34+ and an epithelial marker α6, essential to exclude CD34+ cells in dermis. Note that most anagen CD34+ cells are also Lgr5-EGFP+. Scale bar, 30 μm.

(B) Example of a 90μm thick section of full-anagen HFs of skin from Lgr5-CreER X Rosa26-fl-stop-fl-LacZ mice after a brief dose of tamoxifen. Under this condition where few cells are marked, there is a paucity of LacZ+ cells in bulge (small) relative to ORS (>15X larger). See also Figure 3A.

(C) In unchased (no-doxy treated) HFs from *Tet-OFF/pTRE-H2BGFP* HFs, old Bu, new Bu and HG all express uniform H2BGFP shown by quantifications of GFP intensity for each cells. No H2BGFP was seen in dermis of unchased *Tet-OFF/pTRE-H2BGFP* skin, nor in these mice fed Doxy starting from embryogenesis, nor in *pTRE-H2BGFP* mice alone (Tumbar et al., 2004; Nguyen et al., 2006; Nowak et al., 2008). These data together confirm the tightness of the *Tet-OFF H2BGFP* system. The box and whisker plot indicates the median (middle line), 25-75 percentile (box), minimum and maximum (whiskers), n=14 HFs. On the right, examples of FACS profiles and GFP histograms for bulge and HG of unchased and chased (from P21-P43) *Tet-OFF H2BGFP* mice. In un-chased animals, >90% of the gated population showed uniform high levels of H2BGFP. After the chase, the GFP intensity of HG declined more so than the bulge, but the majority of both populations still remained GFP+.

(D) ORS^{mid} is the source of the ~10-20% of HG cells. H2BGFP/BrdU double-label, double pulse-chase scheme to monitor the fate of cells below ORS^{GFP+}. BrdU was administered to *Tet-OFF H2BGFP* mice at P34-36 (mid to late anagen) and analyzed at anagen's end (P38) or after chasing to telogen (P43). At P38, BrdU+ LRCs (arrowheads) can be found in ORS^{mid}, usually right beneath the end of the

ORS^{GFP+} trail (weak GFP+ cells are marked by arrows). ORS^{low} and matrix also retain some BrdU+ cells. At P43, BrdU+ cells show up mainly in HG cells showing very low H2BGFP, best visualized by more sensitive immunofluorescence rather than epifluorescence. BrdU+ LRCs can also be found in the inner layer of the new bulge; see main text for details. Scale bar, 30 μm. Data are mean ± SD.

Figure S4. At the Catagen/Telogen Transition, the New Bulge Incorporates a K6+ Layer That Is Morphologically Distinct from the K6+ Anagen-Phase Companion Layer, Related to Figure 4.

(A) Schematic of a HF at the 2nd telogen stage. The old bulge is the bulge structure formed at the 1st telogen stage (P21 in CD1 mice). The new bulge is the one that is formed at the start of the 2nd telogen stage (P43 in CD1 mice). Always located at the base of the new bulge, the HG separates the new bulge from DP. Typically, the bulge contains a single layer of "basal" CD34+ cells that adhere to the surrounding basement membrane. However, because of niche topography, CD34+ cells residing between new and old bulge become "suprabasal", a feature previously discussed (Blanpain et al., 2004). The K6+ suprabasal layer is CD34(-), and distinct from other bulge cells described previously.

(B) A 2nd telogen HF immunolabeled for K6 and CD34 to mark the two different bulge layers.

(C) When BrdU was pulsed at the end of anagen, and chased to telogen, BrdU LRCs wound up exclusively in the K6+ layer of the new bulge, not the CD34+ layer, confirmed by immunostaining and FACS analyses. Scale bar, 30 μm. See also Figure 4A,B.

(D-F) Ultrastructural analyses at different stages of the hair cycle to illustrate morphological differences between K6+ bulge and companion layer (Cp) cells. (D) Anagen-phase HF shows typical flattened, elongated K6+ Cp cell. The nucleus and cytoplasm are both elongated along the inner root sheath (IRS). Black dotted line denotes boundary between Cp and ORS layers. Red boxed area is magnified in D' to show desmosomal connections between Cp and Henle's layer of the IRS, which is fully keratinized in this mid-zone of the HF.

(E) Epithelial strand of a catagen-phase HF. In contrast to Cp layer, K6+ cells of catagen are adjacent to club hair, not IRS, and also display more rounded nuclei. Region boxed in blue is shown at higher magnification in E' and at very high magnification in E''. Note that adhesions between K6+ layer and

club hair are unusual half-desmosome connections (arrowheads) to which thick bundles of keratin filaments (Kf) converge. Their extensive interdigitating structure is visible at lower magnification (E'). K6+ cells connect to each other and to ORS through classical desmosomes, which also link to Kfs. Note that neighboring ORS cells display frequent figures of apoptosis (Ap) (E). Der, dermis.

(F) K6+ layer in the telogen HF is now sandwiched between bulge HF-SCs and club hair.

Figure S5. Additional Evidence That the K6+ Bulge Layer Forming in Telogen Is Derived from the ORS of Anagen HFs, Related to Figure 4.

(A) HF-SC transcription factors Lhx2 and Tcf3 are nuclear in anagen ORS cells. Note that expression is restricted to ORS, and is not seen in K6+ companion layer cells. DAPI counterstain to visualize the nuclei. Scale bar, 30 μm.

(B) Confocal immunofluorescence microscopy from the *Lgr5-CreER/Rosa-LacZ* lineage tracing experiment (see also Figure 3A). Examples of βgal+ CD34+ new bulge cells (left) and βgal+ K6+ cells (right).

(C) Lineage tracing results from *K14-CreER/Rosa-LacZ*. When tamoxifen was given close to the end of anagen and traced to telogen, βgal+ cells are found in the K6+ bulge layer. Continuous white line delineates DP, below HG.

(D) When H2BGFP is induced in *K14TetON-H2BGFP* mice at the start of telogen, H2BGFP is only seen the outer (CD34+) and not inner (K6+) layer of the bulge, confirming the specificity of the *K14* promoter.

(E) TUNEL staining of a HF in catagen VI, near the end of the destructive phase. At this stage, it is clear that the K6+ layer that encases the newly formed club hair has been spared from destruction, even though the K5+ cells surrounding it are dying (left). Ultrastructure analysis is in agreement with this (right). Cells containing apoptotic bodies are circled in red.

(F) In contrast to the anagen Cp layer, the K6+ cells surrounding the club hair in catagen express HF-SC markers. Boxed areas are shown at higher magnification below each immunostained image.

(G) Cat VII HFs of the *Tet-ON H2BGFP* and BrdU double pulsed-chase experiments (See also Figure 4C). ORS was the only double positive populations in this double labeling. When chased to late catagen, GFP+ BrdU+ cells were largely confined to the K6+ layer surrounding the club hair, supporting their lower ORS origin.

Figure S6. Expression of NFATc1 during Different Stages of the Hair Cycle and Verification of Sorting Strategy to Isolate K6+ cells, Related to Figure 5 and 6.

(A) In anagen, *NFATc1* is expressed in the bulge but not in the mid-zone or lower ORS (Horsley et al., 2008). In catagen, NFATc1 can be seen not only in bulge (Bu), but also in the K6+ layer surrounding the newly formed club hair (boxed area, shown at higher magnificence). By telogen, NFATc1 is detected in both K6+ and CD34+ bulge layers. Scale bar, 30 μm.

(B) When BrdU is pulsed at the end of anagen to preferentially mark the lower ORS precursors, and then chased to telogen, K6+ bulge cells become preferentially BrdU+. When followed into the next hair cycle, BrdU label remains high in K6+ bulge cells, which do not venture from the bulge, in contrast to CD34+ HF-SCs. See also Figure 5B.

(C) In *Lhx2-EGFP* BAC transgenic mice, GFP is expressed in both the CD34+ bulge and the K6+ bulge, faithfully representing endogenous Lhx2. E-cadherin serves as an additional marker enriched in K6+ bulge. The purity of FACS-sorted cells from the two bulge layers (see main text) was verified by anti-K6+ cytospin.

(D) Real-time PCR analyses of mRNAs from FACs-sorted K6+ (red) and CD34+ (blue) bulge cells. The α6+ population was used to normalize expression levels at 1. Data are mean ± SD. Note near equivalent levels of HF-SC transcription factors but not CD34 in the two populations.

Figure S7. Additional Evidence Supporting the Functional Analyses of K6+ Bulge, and Accompanying Schematic Depicting Where They and Other Bulge and HG Cells Come From, Related to Figure7 and Discussion.

(A) Immunostaining of K6 and CD34 of a club hair plucked out from the telogen skin surface (left) and the remaining skin (right). Note that although the K6+ bulge layer was removed together with the club hair, most of the CD34+ bulge cells remained in the skin.

(B) Quantifications of the apoptotic (CP3+) cells in the bulge (K6+ and CD34+ layers) at D4 after administering DT to the backs of the various different mouse strains, which were first treated with 1% or 2% RU486 for 3 or 5d (*K15-iDTR)* or high or low doses of tamoxifen (*Sox9-iDTR)*. 1% RU486 treated for 5d to activate K15-iDTR was selected as a comparison standard for Sox9-iDTR^{low} because the total CP3+ cells showed no significant difference between these two conditions. In striking contrast to *Sox9-iDTR* animals, *K15-iDTR* mice showed no hair loss or precious anagen entry under any of these conditions used. *: $P<0.05$; ** $P<0.01$.

(C) BrdU was administered for 1d prior to analyzing *K15-iDTR* mice at D8-D12 after DT treatment, and the % HFs with BrdU+ cells in the bulge was quantified. Note that by D12, most bulges had returned to complete quiescence.

(D) *Sox9-iDTR* mice at early telogen were treated with DT as described in the main text, either with or without FGF18 or BMP6, administered at the levels indicated along with Cy5 coated beads (to track sites of treatment). 3D later, animals were given a 1d pulse of BrdU and then analyzed for CD34 and BrdU. Shown are representative examples. Note that administration of either FGF18 or BMP6 alone were sufficient to suppress the otherwise considerable cycling activation response of the CD34+ bulge upon ablation of K6+ bulge. See also Figure 7G for more data and details.

(E) A HF stained for Mitf, a key transcription factors for melanocytes (K14/K5-negative), which are located above DP in the pre-medullary matrix of full anagen HFs. Melanocytes are derived from melanoblasts in the bulge, and are thought to migrate to the matrix and differentiate at the start of anagen. These cells are among non-epithelial candidates that along with the DP and dermal sheath, might influence HF-SCs after they leave the niche and progress along the ORS to the matrix.

(F) Schematic summarizing our data which delineates how the HG, new CD34+ bulge and K6+ bulge layer arise. During anagen, HF-SC bulge descendents appear in the single layer of ORS. At full-anagen (stages VI-VII), the ORS exists as a concentric layer of ~85 cells that trail from the bulge to

the TA matrix. It can be divided into three segments based on proliferation status and distance from the niche: ORS^{GFP+}, ORS^{mid} and ORS^{low}. ORS^{GFP+} cells are the slow-cycling bulge descendents, having divided only a few times since departing the niche. The upper part of ORS^{GFP+} (green cells with black circles), induce CD34 expression at catagen and contribute substantially to the new bulge. The lower part of ORS^{GFP+} does not express CD34 (green cells with red circles) but rather contributes to the new HG. Most cells in the ORS^{mid} and ORS^{low} zones do not survive catagen. The few surviving ORS^{mid} cells end up as $GFP^{low/neg}$ HG cells, while the surviving ORS^{low} cells home back to the bulge, where they become the K6+ layer. Like their ORS origins, these cells express many HF-SC markers. However, despite the fact that they are descendents from the lower ORS, which at the end of anagen were still proliferative and had not yet even reached the TA-matrix pool along the lineage, the K6+ bulge cells are not bona-fide stem cells. Instead, they act to anchor the hair and keep the HF-SCs in their quiescent state. See main text and discussion for more details.

Supplementary Experimental Procedures

Mice

pTRE-H2BGFP, K5-tTA($K5^{tetOFF}$), K14-rtTA($K14^{tetON}$), K14-CreER, $Rosa26^{Flox\text{-}Stop\text{-}Flox\text{-}lacZ}$ (*Rosa-LacZ*), *K15-CrePGR Sox9-CreERT2, Lgr5-CreERT2-IRES-EGFP, $Rosa26^{Flox\text{-}Stop\text{-}Flox\text{-}iDTR}$* (*Rosa-iDTR), Lhx2-EGFP* (Barker et al., 2007; Buch et al., 2005; Diamond et al., 2000; Mao et al., 1999; Nguyen et al., 2006; Soeda et al., 2010; Tumbar et al., 2004; Vasioukhin et al., 1999) were described before. All the mice are housed and bred in the CBC ALAAC-accredited animal facility at The Rockefeller University.

Immunohistochemistry

The following antibodies and dilutions were used: NFATc1 (mouse (7A6), 1:100, Santa Cruz), TCF3 (guinea pig, 1:100, Fuchs Lab), Sox9 (rabbit, 1:1000, Fuchs Lab), Lhx2 (rabbit, 1:2500, Fuchs Lab), Ki67 (rabbit, 1:500, Novocastra), CD34 (rat, 1:100, eBioscience), BrdU (rat, 1:100, Abcam), K6 (guinea pig, 1:2000, Fuchs Lab), K6 (rabbit, 1:500, Fuchs Lab), βgal (rabbit, 1:5000, Cappel), active-Caspases3 (rabbit, 1:500, R&D), GFP (rabbit, 1:500, invitrogen), E-cadherin (rat, 1:250, Fuchs lab), and P-cadherin (goat, 1:200, R&D or rat, 1:100, Fuchs lab). Nuclei were stained using 4'6'-diamidino-

2-phenylindole (DAPI). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed using the in situ cell death detection kit (Roche) according to manufacturer's directions.

Histology and Immunofluorescence

For immunofluorescence microscopy of sagittal sections, backskins were embedded in OCT, frozen, cryosectioned (10–90 μm) and fixed for 10 min in 4% paraformaldehyde in PBS. Sections were permeabilized for 10 min in PBS + 0.1-0.3%Triton (PBST) and blocked for 1 h in 2.5% fish gelatin, 2.5% normal donkey serum, 2.5% normal goat serum, (or 0.5% normal donkey serum and no goat serum when a goat primary antibody was used) 0.5% BSA, 0.1-0.3% Triton in PBS. Primary antibodies (Abs) were incubated overnight 4°C and secondary Abs were incubated 1-2 hrs r.t. LacZ stainings of thick OCT sections were performed as described in Zhang et al, 2009.

FACS

Subcutaneous fat was removed by a scalpel and the whole skin was placed dermis side down in Trypsin (GIBCO) at 4°C O/N then move to 37°C for 1hr the next day. Single cell suspensions were obtained by scrapping the skin gently. The cells were then filtered with strainers (70μM, followed by 40μM). Cell suspensions were incubated with the appropriate antibodies for 30 miuntes at RT. The following antibodies were used: Ecad-Alexa700 (1:150, Fuchs lab), CD34-Alexa647 (1:100, eBioscience), α6-PE (1:100, BD), β1-PE_Cy7 (1:250, eBioscience). DAPI was used to exclude dead cells. Cell isolations were performed on FACSAria sorters equipped with DIVa software (BD Biosciences). FACS analyses were performed using LSRII FACS Analyzers and then analyzed with FlowJo program.

Real-Time PCR

Total RNAs were purified from FACS-sorted cells by directly sorting into Trizol^{LS} (invitrogen) followed by chloroform extraction or by using the Absolutely RNA Micro kit (Stratagene). Equal amounts of

RNA were reverse-transcribed by Oligo-dT (Superscript III, invitrogen). cDNAs were normalized to equal amounts using primers against Ppib2.

Intradermal Injections of Growth Factors

Recombinant mouse BMP6 (R&D) and human FGF18 (abcam) were injected intradermally together with Cy5 beads using 30g syringe into the back skin of Sox9-iDTR mice together with i.p. injections of Diphtheria Toxin (200ng/injection) for 3 days. The skin was analyzed the 4^{th} day. BrdU was injected 2X in the last 24 hrs before harvesting the skin.

Confocal Microscopy and Image Processing

Images were acquired with a Zeiss LSM510 laser-scanning microscope (Carl Zeiss MicroImaging) through a 40X water objective or a 25X objective. Representative single Z-planes are presented and co-localizations were interpreted only in single Z-stacks. Z-stacks were projected using ImageJ software. RGB images were assembled in Adobe Photoshop CS3 and panels were labeled in Adobe Illustrator CS3.

Statistics

For all quantified data, mean value ± one standard deviation was presented. To determine significance between two groups, indicated in figures by asterisks, comparisons were made using student's t-test. For all statistical tests, the 0.05 level of confidence were accepted for significant differences.

Electron Microscopy

Samples were fixed in 2% glutaraldehyde, 4% PFA, and 2 mM CaCl2 in 0.05 M sodium cacodylate buffer, pH 7.2, at room temperature for >1 h, postfixed in 1% osmium tetroxide, and processed for Epon embedding. Ultrathin sections (60–70 nm) were counterstained with uranyl acetate and lead citrate. For immunogold labeling, catagen backskin samples from Tet-OFF H2BGFP doxy from P21

were fixed in 4% formaldehyde, 0.05% glutaraldehyde in 0.05M sodium cacodylate buffer pH 7.2,Ca^{2+} 2 mM. Samples were embedded in LR White resin (London Resin Company, Ltd). Ultrathin sections were immunolabeled for GFP using an anti-GFP antibody followed by secondary antibody (goat anti-rabbit) coupled to 10nm gold particles (Aurion, The Netherlands). Images were taken with a transmission electron microscope (Tecnai G2-12; FEI) equipped with a digital camera (model XR60; Advanced Microscopy Techniques, Corp.).

Additional note

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Supplemental References

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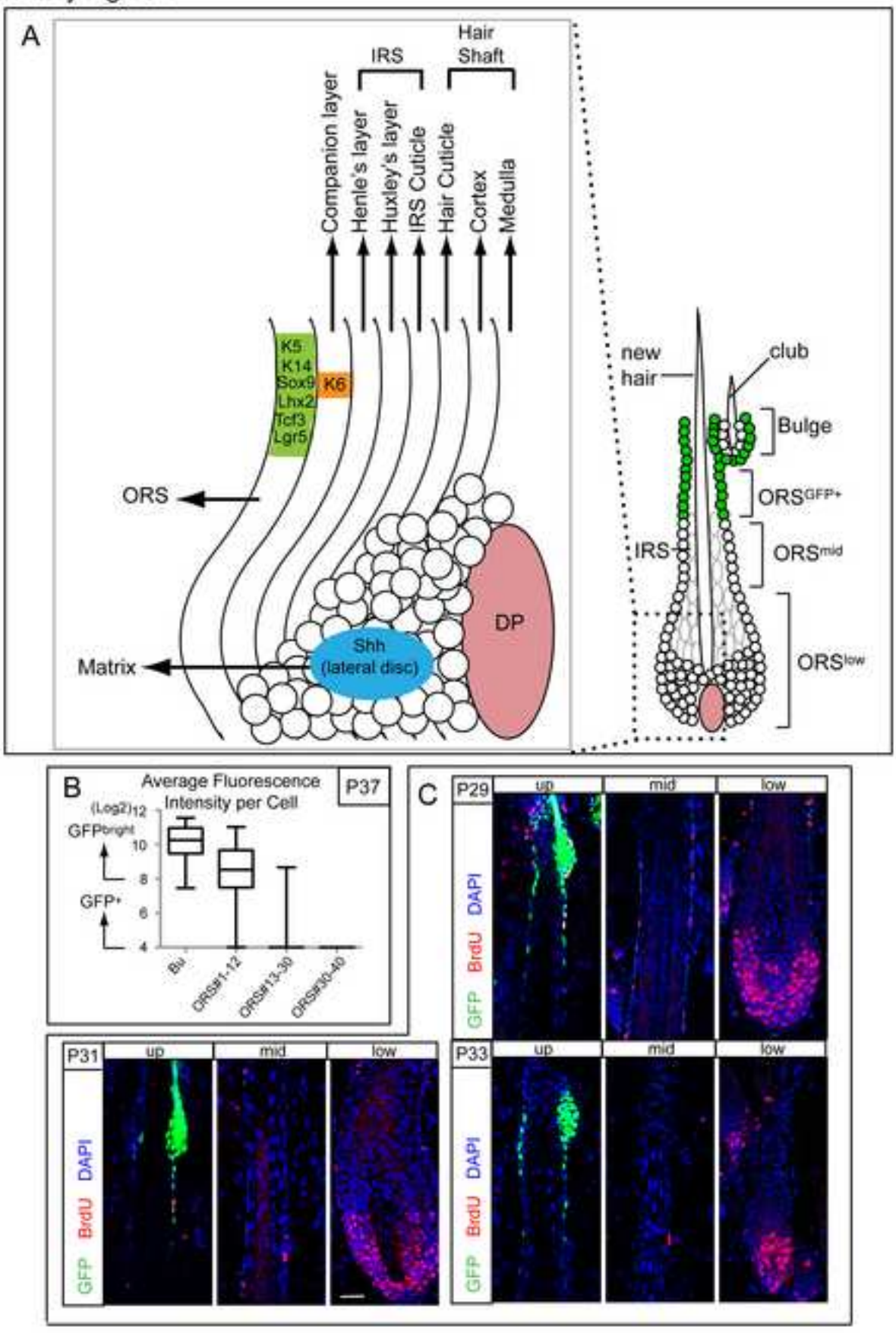
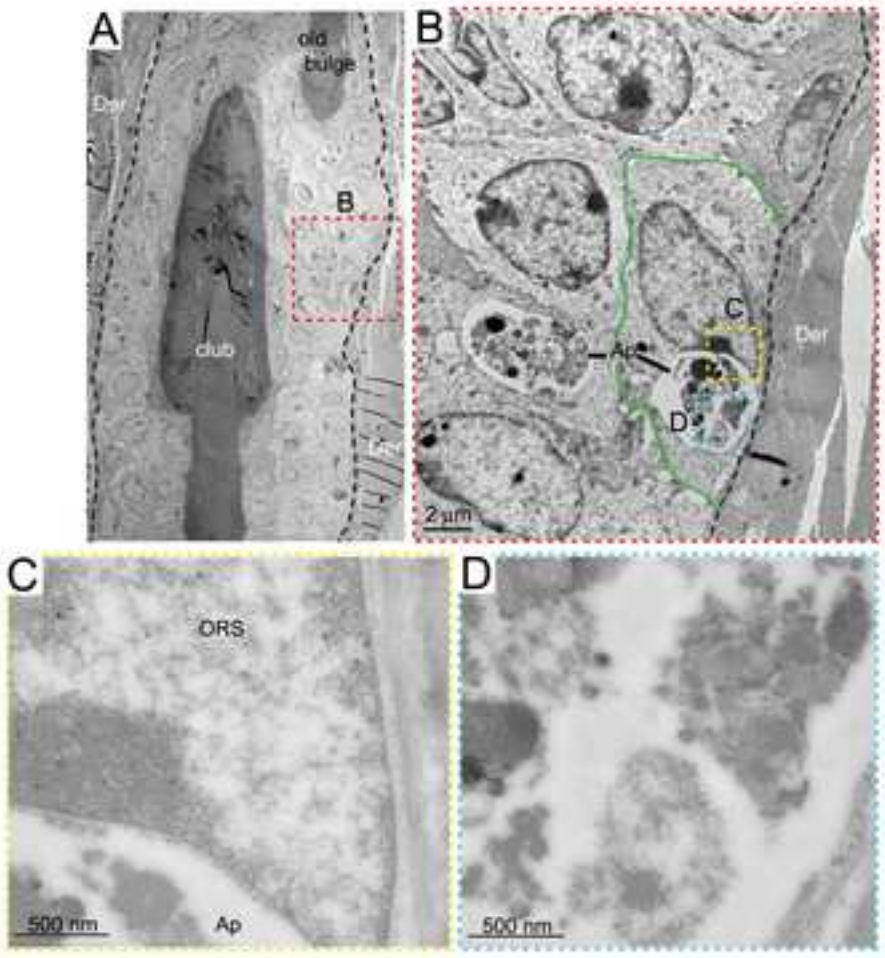
Supplemental Figure1

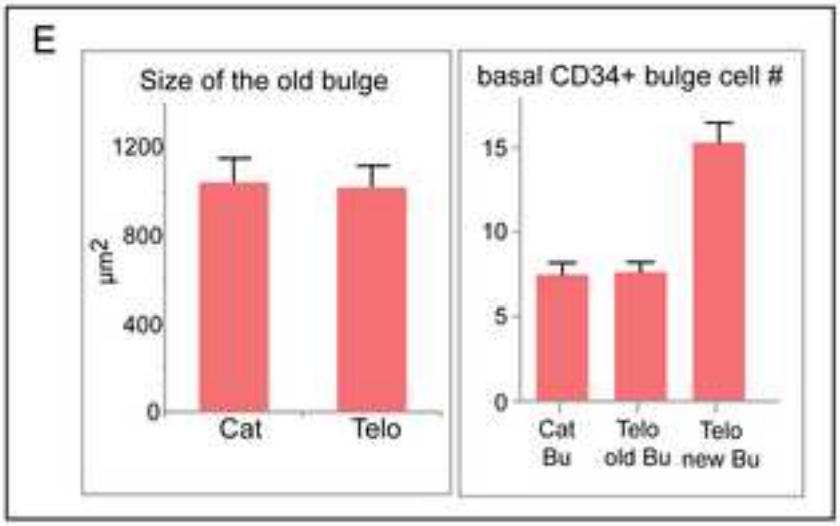
Supplementary Figure 1



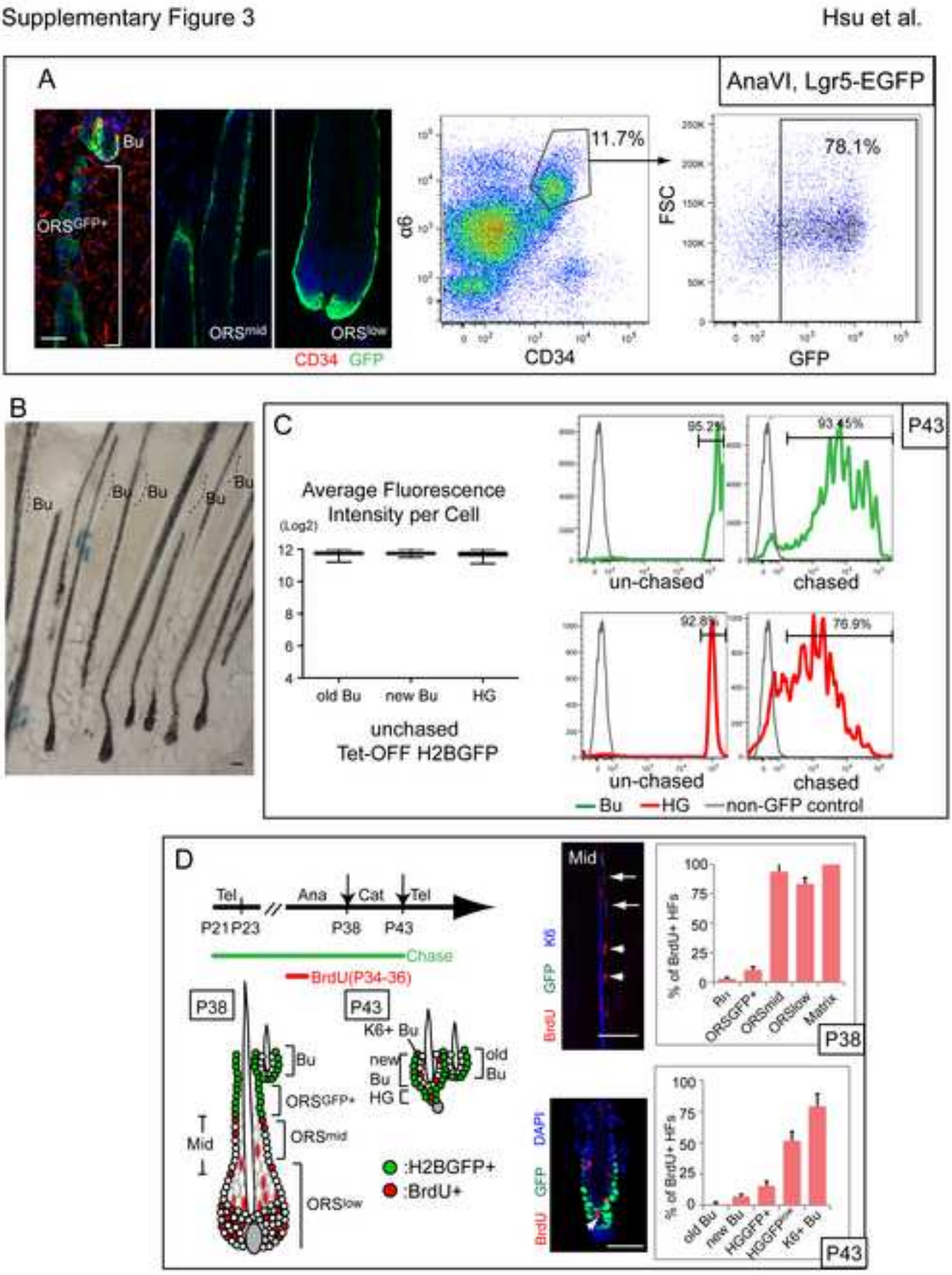
Supplemental Figure2

Supplementary Figure 2 Hsu et al.





Supplemental Figure3



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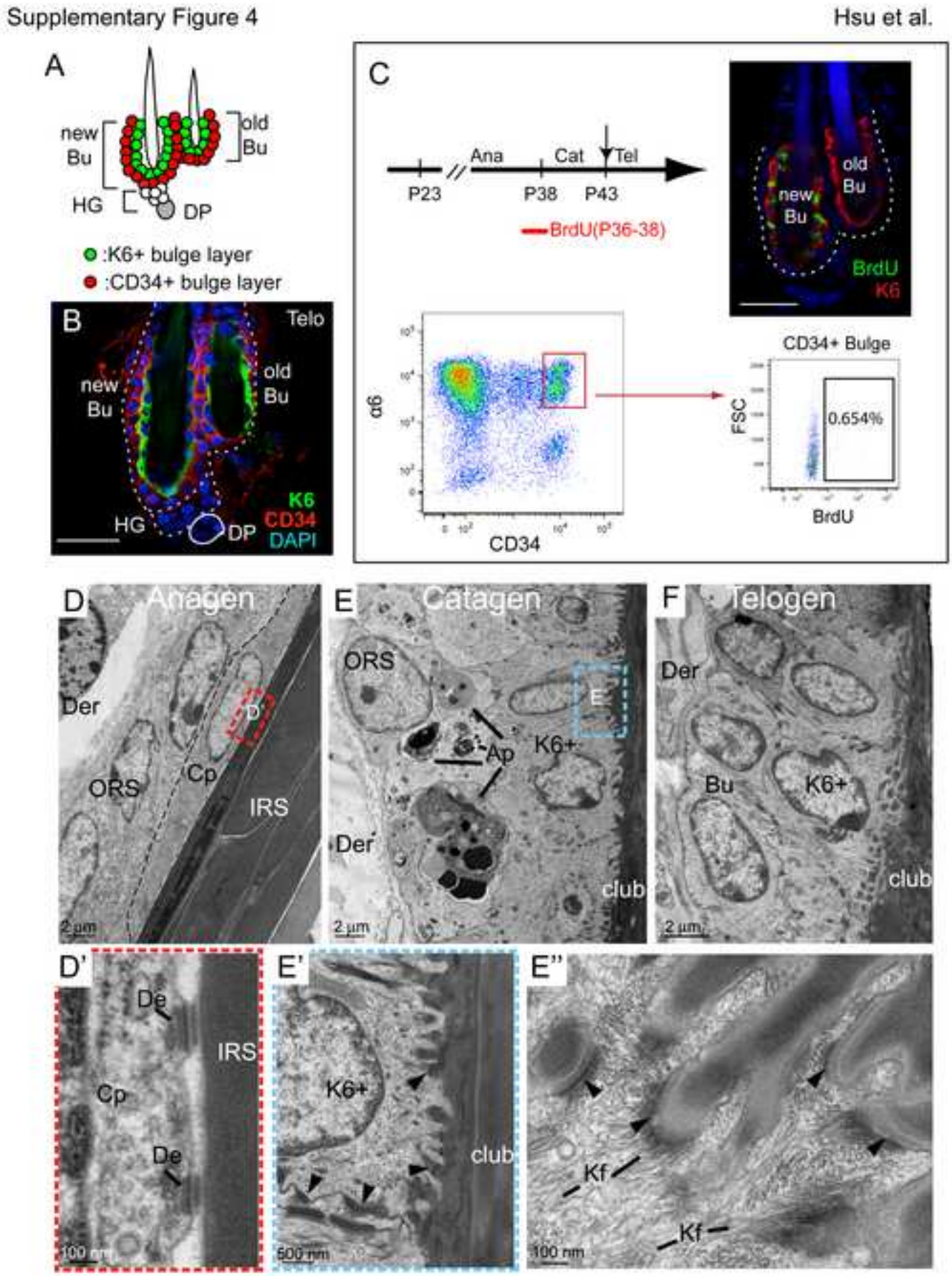

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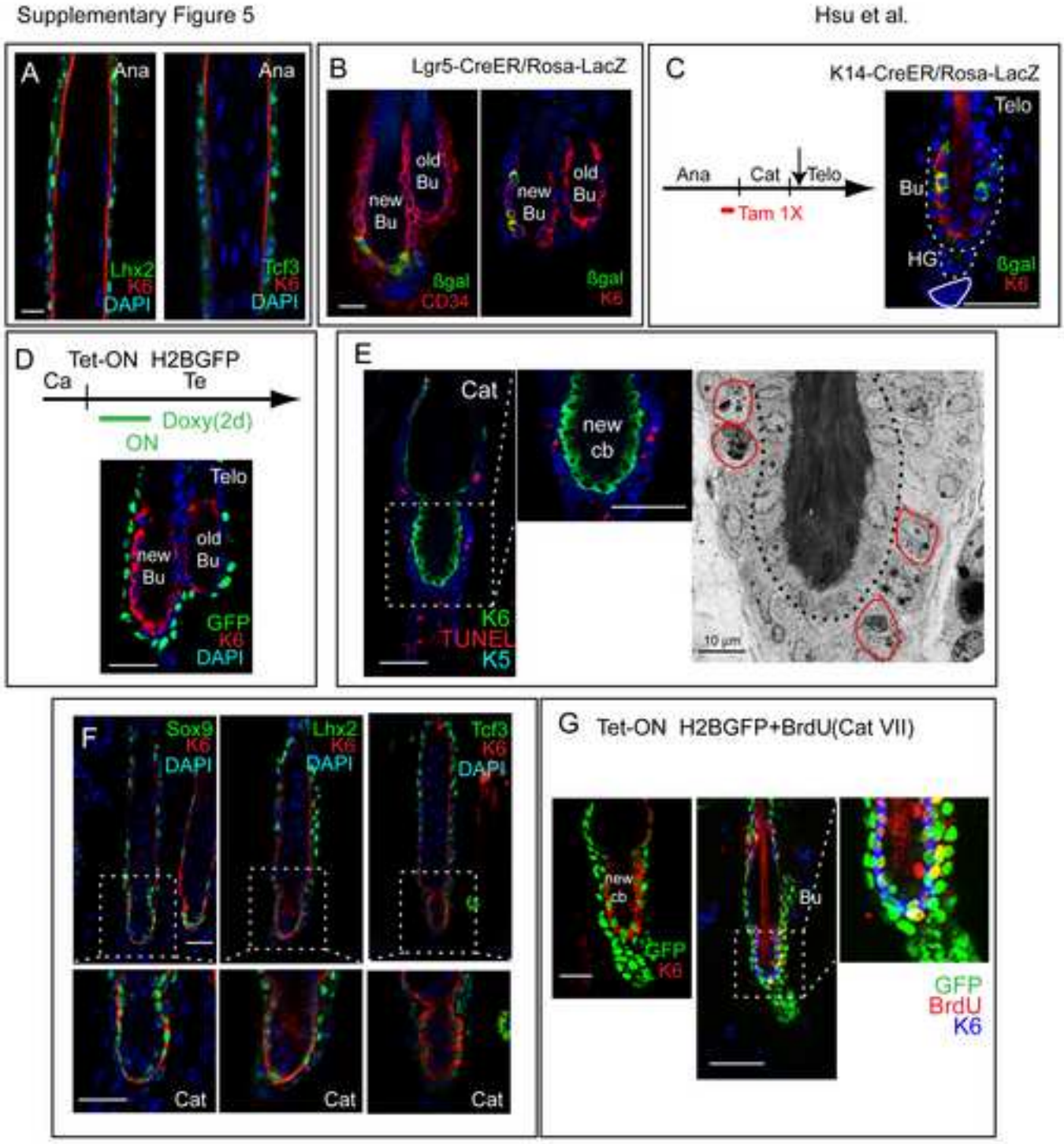
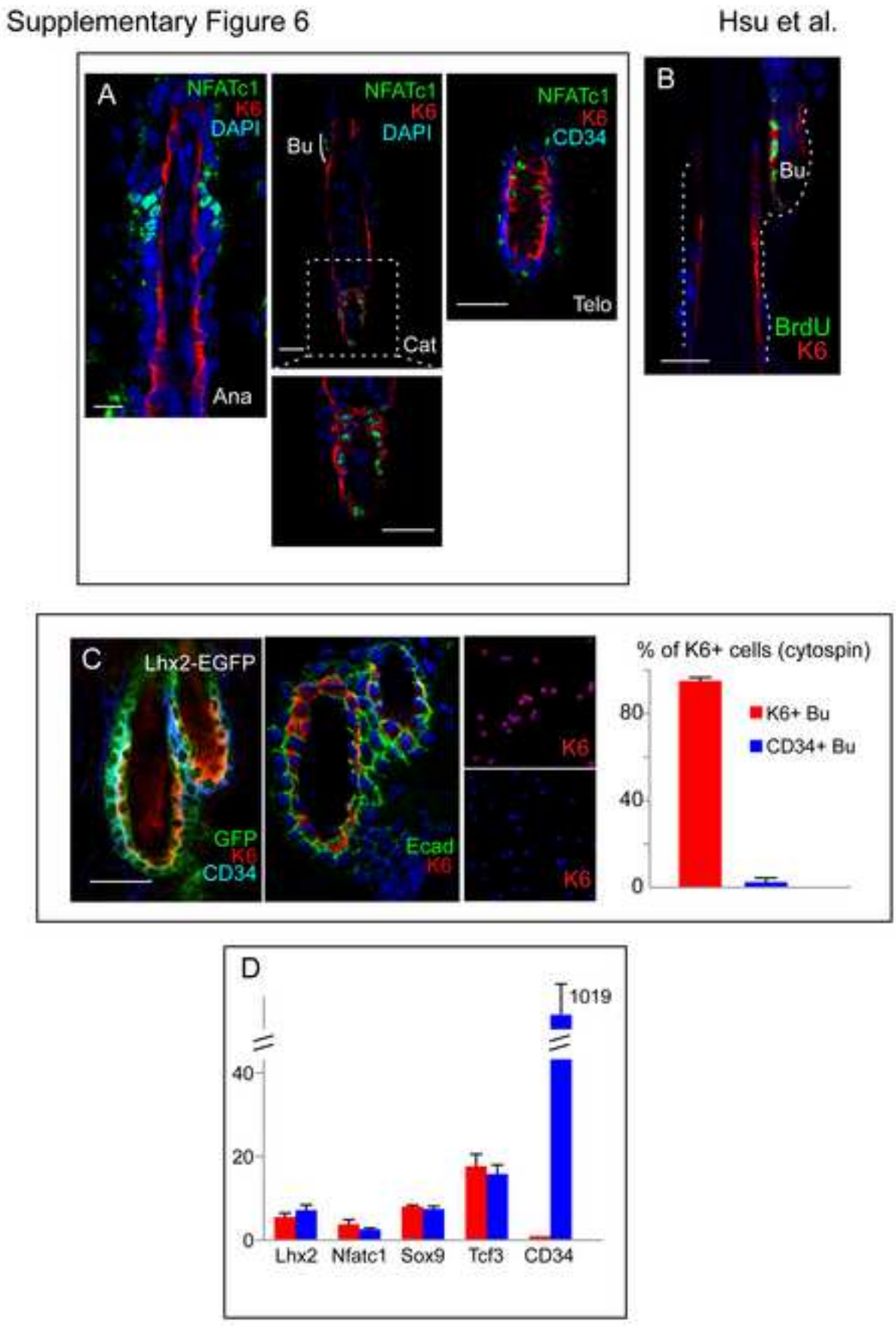


Supplemental Figure6



Supplemental Figure7