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Supplemental Data

NFATc1 Balances Quiescence and Proliferation of Skin Stem Cells

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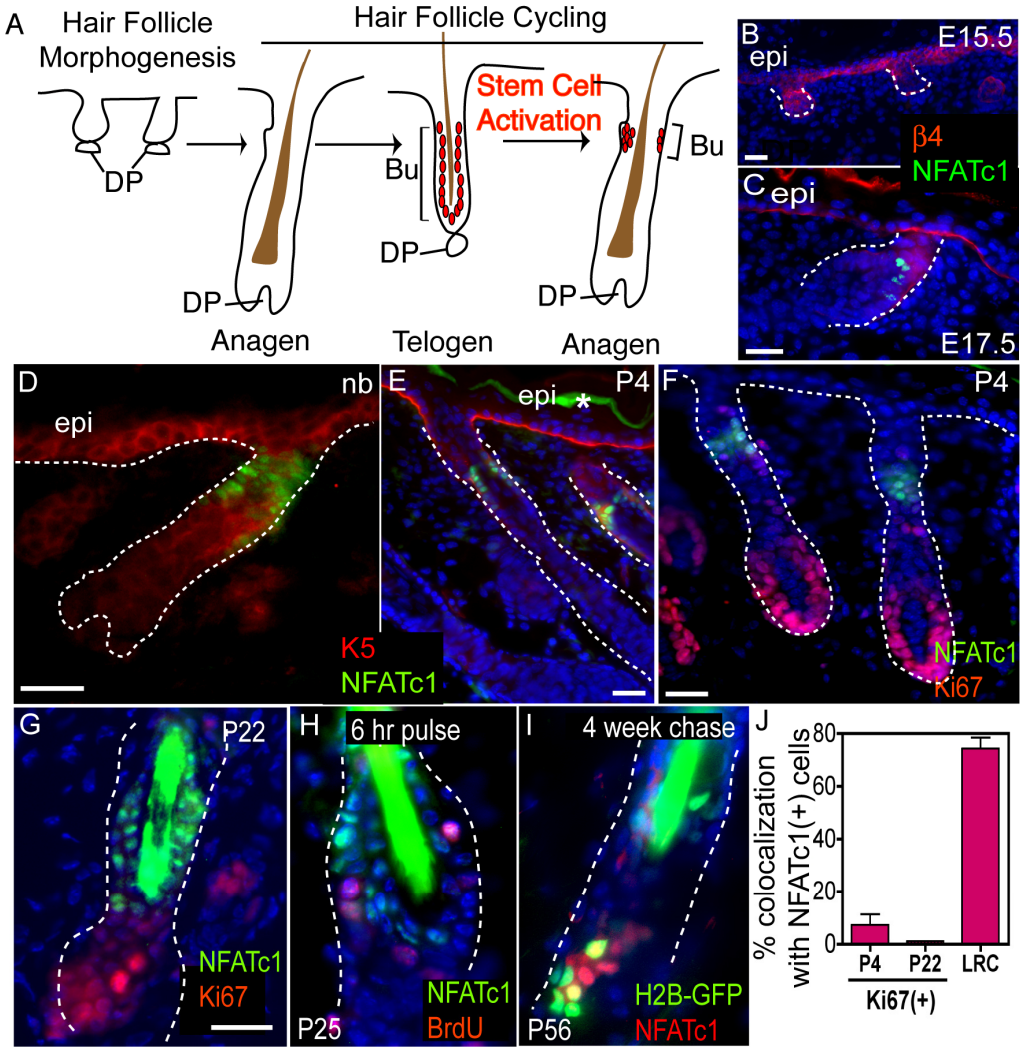
Supplemental Figures



Horsley et al., Supplemental Figure 1

Figure S1. NFATc1 is expressed during morphogenesis and in slow-cycling cells of the hair follicle. (A) Schematic illustrating hair follicle generation during morphogenesis and cycling. Morphogenesis produces hair follicles in the growth phase, anagen, which regresses to the resting phase, telogen. Activation of bulge (Bu) stem during hair follicle cycling results in the production of new anagen hair follicle. DP, dermal papillae. (B-E) NFATc1 expression during hair follicle morphogenesis at E15.5 (B), E17.5 (C), newborn (nb) (D), and P4 (E). Asterisk indicates background staining in the stratum corneum. (F-J) NFATc1 and Ki67 expression during hair follicle morphogenesis at P4 (F) and anagen initiation at P22 (G). (H) NFATc1 and BrdU localization (6 hour pulse) in the 2nd hair cycle. (I) Immunolocalization of NFATc1 in mice expressing a tetracycline controlled H2B-GFP that has been chased for 4 weeks. (J) Quantification of the percentage of NFATc1(+) cells that colocalize with the proliferative marker, Ki67 or the label retaining cells (LRC) after a 4 week chase of a tetracycline controlled H2B-GFP. N=71-180 cells Abbreviations: epi, epidermis, K5, keratin 5, LRC, label retaining cells. Dapi staining (blue) shows nuclear localization. Scale Bars, 30 μm.

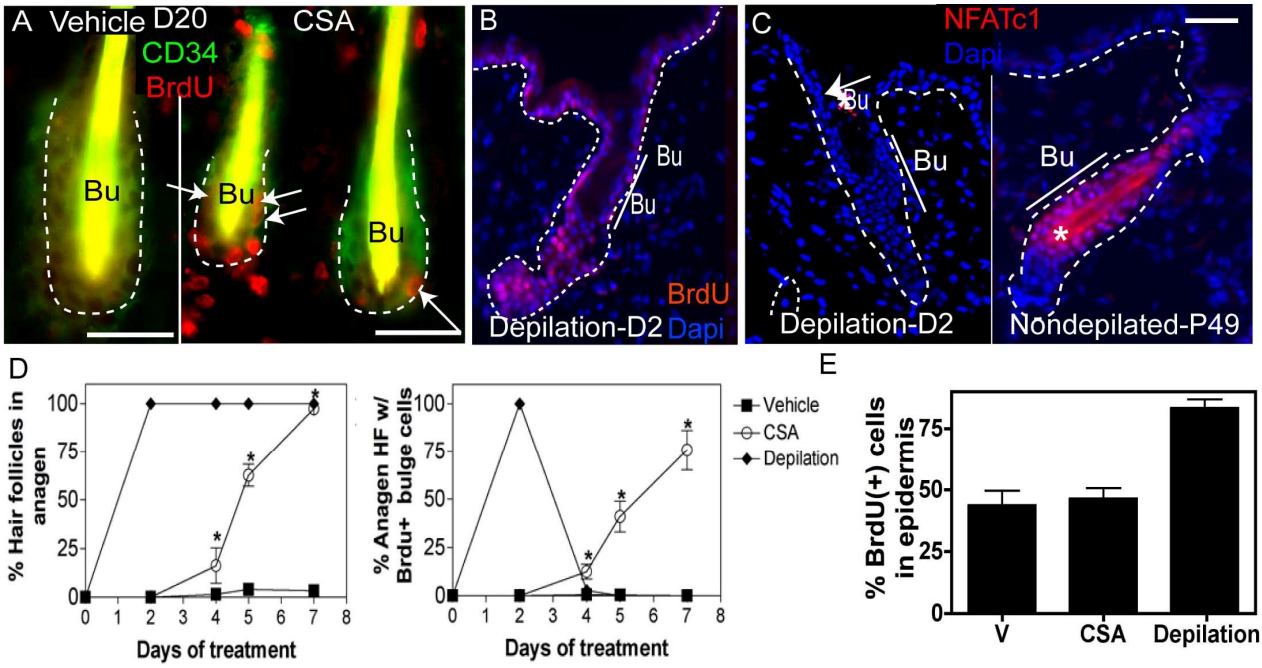


Figure S2. CSA activates hair follicle growth and stem cell proliferation. (A) Treatment of follicles with CSA from D18-D20 during telogen of the first hair cycle results in BrdU incorporation during a 48 hr pulse in the CD34(+) bulge cells. Scale Bar, 30μm. (B-D) BrdU labeling 48 hrs prior to analysis reveals that hair follicle depilation at P49 results in rapid induction of anagen, including proliferation in the bulge at D2 (B) and loss of *NFATc1* expression (C). This activity of the bulge is short-lived and by D4 extending to D7, the bulge becomes quiescent (D). (E) Quantification of BrdU incorporation into epidermal cells during vehicle, CSA treatment or depilation at D2 reveals the effect of depilation on epidermal cells but no effect with CSA treatment. Dapi staining (blue) shows nuclear localization.

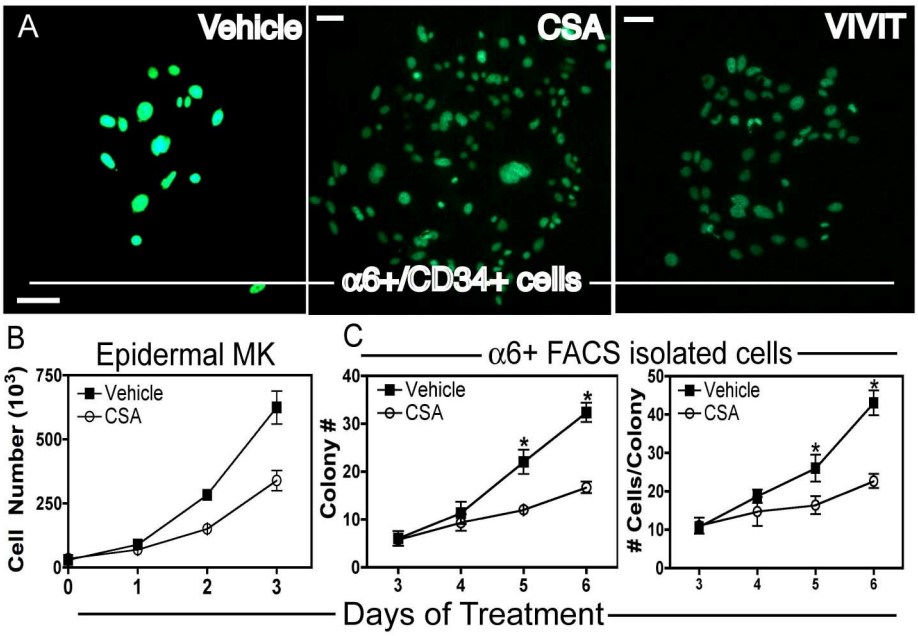
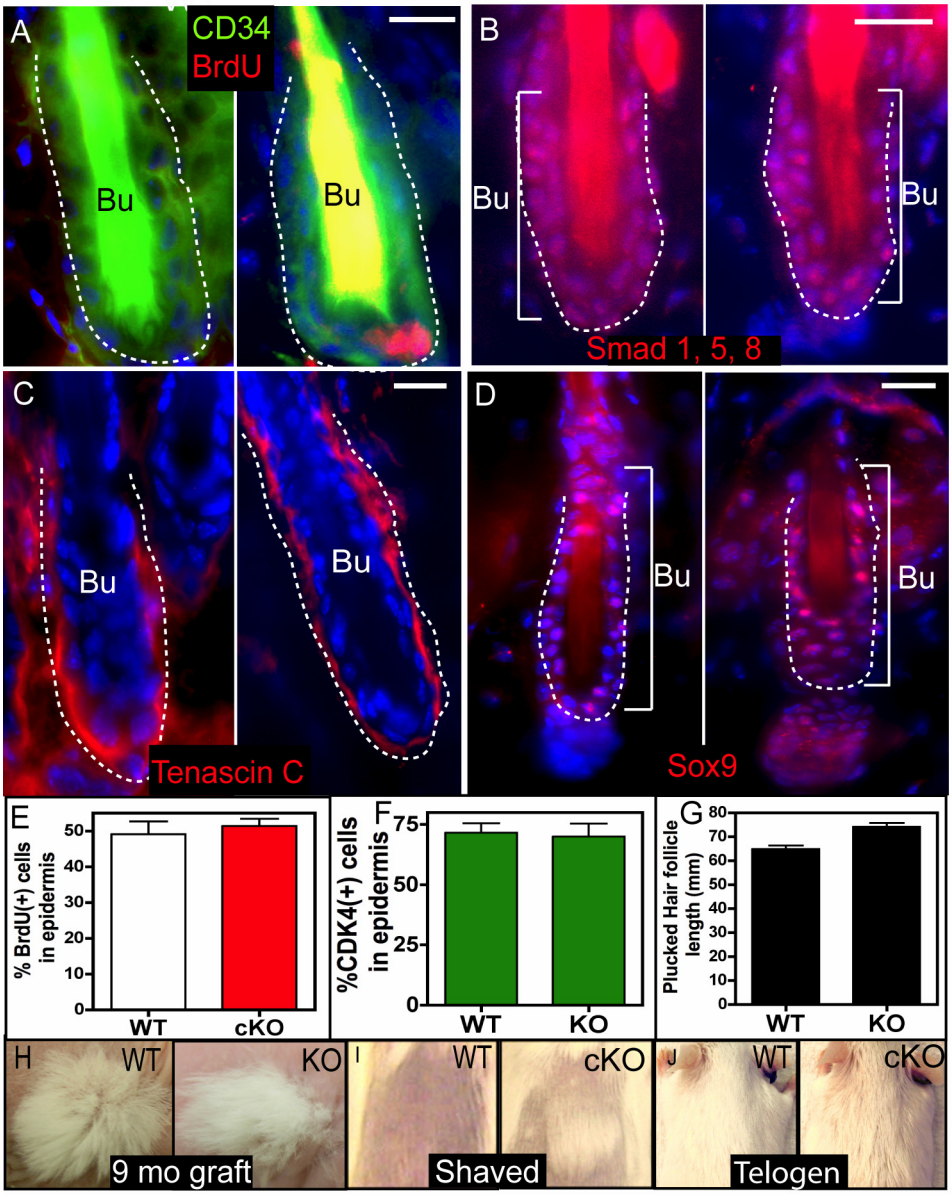


Figure S3. In vitro effects of inhibition of calcineurin on bulge cells and epidermal keratinocytes. (A) Images of a single colony illustrating average colony size of FACS purified bulge cells (α6+/CD34+) from *K14-H2BGFP* mice in culture for 5 days after treatment with vehicle, CSA or 11R-VIVIT. Scale Bar, 100μm. (B) Western analysis of CSA treated skin reveals a reduction in NFATc1 expression following 3 d of CSA treatment. (C) Treatment of epidermal

keratinocytes (MK) with CSA results in suppression of cell proliferation. (D) Isolation of α6+ cells from 1 mo old mice and treatment with CSA results in suppression of colony formation and growth.



Horsley et al., Supplemental Figure 4

Figure S4. Bulge stem cells of *NFATc1* null hair follicles are more active but retain normal niche and markers. (A) *NFATc1* null hair follicles exhibit BrdU (+) CD34(+) cells in the bulge

after a 24 hour BrdU pulse. (B-D) Normal expression of bulge (Bu) markers in the *NFATc1* KO follicles. *NFATc1* KO bulge cells retain the BMP responsive transcription factors, SMAD 1, 5, 8 (B), tenascin C (C) and SOX9 (D) expression. Scale Bar, 20μm. (E) Epidermal proliferation in *NFATc1* cKO skin is comparable to WT. N=200-350 cells from 3 individual mice for each genotype. Abbreviations; SG, sebaceous gland; Bu, Bulge; (F) Expression of CDK4 in the epidermis (epi) of WT and *NFATc1* KO skin is similar. (G) Length of plucked WT and *NFATc1* KO follicles is shown. Data are mean HF length (100-120 follicles) from 3 individual grafts for each genotype. (H) Images of 7-month old grafts from WT and *NFATc1* KO skin, show long-term maintenance of null follicles. (I) Images of 8 week old WT and *NFATc1* cKO skin after shaving during telogen phase of the hair cycle (D54). (J) Images of 7 week old WT and *NFATc1* cKO skin showing the retention of HFs in both genotypes.

Supplemental Experimental Procedures

Skin grafting

Full thickness skins were removed from the torsos of sex-matched wild-type and *NFATc1* null E15.5 embryos, and placed onto the backs of anesthetized female *nu/nu* (Nude) recipient mice, with each recipient receiving a WT and KO graft. Grafts were secured by sterile gauze and cloth bandages, which were removed after healing (14 days). Hairs typically appeared within 1 week after grafting. A total of 20 grafts were placed (10 WT and 10 KO), and each showed a consistent phenotype dependent on the presence or absence of NFATc1 in the donor skin.

Production of Retroviral Supernatants

Retroviral supernatants were produced by transfection of Phoenix cells with Fugene 6. Supernatants containing infectious retroviruses were filtered through 0.45 um filters, snap frozen in liquid nitrogen and stored at –80ºC. Primary MKs were infected by adding retroviral supernatant supplemented with FBS and 4ug/ml polybrene and centrifugation at 1100 X g for 30 min. After

replacing the retroviral supernatant with MK growth media, the cells were replated for appropriate experimental conditions.

Western analysis

For analysis of retrovirally infected cells, proteins were extracted in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1mM PMSF, 1mM EDTA, 5ug/ml aprotinin, 5ug/ml Leupeptin, 1% Triton X-100, 1% Sodium deoxycholate, 1% SDS). For analysis of NFATc1 expression, epidermis and dermis were separated by treatment of P4 skin in dispase at 4ºC overnight. The isolated epidermis and dermis were placed in Ripa buffer and homogenized to lyze the cells. NFATc1 expression in CSA treated skins was performed by isolating proteins from whole skins derived from mice which were injected with CSA for 3 days. Equal amounts of protein were subjected to SDS-PAGE, blotted onto nitrocellulose membranes and developed by chemiluminescence (Vasioukhin et al., 1999). The following antibodies were used: NFATc1 (mouse (7A6), 1:250, Santa Cruz; rabbit (H110), 1:250), CDK4 (rabbit (C22), 1:200, Santa Cruz), CDK6 (rabbit (C21), 1:200, Santa Cruz), pRB (goat, 1:200, Santa Cruz), β-actin (mouse (CL.AC15), 1:1000, Sigma).

Real Time PCR

For Real Time PCR (Rendl et al., 2005), total RNAs were isolated (Absolutely RNA, Stratagene) from keratinocytes and FACS purified hair follicle cell populations as described (Blanpain et al., 2004; Rendl et al., 2005), and equal amounts (1μg) were added to reverse transcriptase reaction mix (Stratagene) with oligo-dT(12) as primer. RT-PCRs of RNA were used as negative controls, and glyceraldehyde phosphate dehydrogenase (GAPDH) was used to control for equal cDNA inputs. Real-time PCR was conducted with a LightCycler system (Roche Diagnostics, Basel Switzerland) using the LightCycle DNA master SYBR Green kit for 45 cycles. LightCycle analysis software was used for quantifications. The number of cycles needed to reach the crossing point

for each sample was used to calculate the amount of each product using the $2^{-\Delta\Delta CP}$ method. Levels of PCR product were expressed as a function of GAPDH.

The following primer sequences were used for real time PCR analysis: NFATc1 (F: 5'-AACGCCCTGCTGACCACCGATAGCACT-3', R: 5'-CCCGGGTGCCTTCCGTCTCATA-3') , TGM3 (F: 5'- TAACCACGCCGAAAGACAAGAGTA-3', R: 5'- GTGATCACCCGAGAGCGAACC-3'), TGM1 (F: 5'- GCGGAGGGCTGTGGAGAAGG-3', R: 5'-GGGTGCGCAAACGGAAGGTG-3'), Filaggrin (F: 5'- GTGGCCAACAGAGAATGAG-3', R: 5'- ATGATGCCCAGAACTATGTGAC-3'), Involucrin (F: 5'-GTCCGGTTCTCCAATTCGTGTTT-3', R: 5'- GCAATTGGAAGAGAAGCAGCATCAG , Cyclin D1 (F: 5'- GCTACCGCACAACGCACTTTCTTT-3', R: 5'- GCACCCCCTGGCTCCCTACTCTC-3'), Cyclin D2 (F: 5'- CCTAGTGCATATGCCCCCTTTAGTAGA-3', R: 5'-GGCCCATAGCGAATTCCCTCCAT-3', p27 (F: 5'-TTCGGCCCGGTCAATCATGAAGAACTAA-3', R: 5'-TTGGCCCTTTTGTTTTGCGAAGAAGAAT-3'), E2F (F: 5'-TGGGCCTGGAGCAAGAAGC-3', R: 5'-GCAGCAACCAAACCCCAGAGC-3'), CDK4 (F: 5'-TGGCTGCCACTCGATATGAAC-3', R: 5'-CCTCAGGTCCTGGTCTATATG-3'), CDK6 (F: 5'- GGCCCGCGACTGAAGAA-3', R: 5'-GGGGTGGCATAGCTGGACTGG-3'), GAPDH (F: 5'- CGTAGACAAAATGGTGAAGGTCGG-3', R: 5'- AAGCAGTTGGTGGTGCAGGATG-3').

Immunohistochemistry

The following Abs and dilutions were used: NFATc1 (mouse (7A6), 1:100, Santa Cruz), β4 (rat, 1:100, Pharmingen), TCF3 (guinea pig, 1:300, Fuchs Lab), Sox9 (rabbit, 1:100, Santa Cruz), Lhx2 (rabbit, 1:1000, Jessell Lab), Ki67 (rabbit, 1:300, Novocastra), CD34 (mouse, 1:100, Pharmingen), BrdU (rat, 1:100, Abcam), Tenascin C (mouse, 1:500, IBL), SMAD 1,5,8 (rabbit, 1:100, Cell Signaling), CDK4 (rabbit (C22), 1:100, Santa Cruz), and CDK6 (rabbit (C21), 1:100, Santa Cruz), K14 (rabbit, 1:100, Fuchs Lab). Nuclei were stained using 4'6'-diamidino-2-phenylindole (DAPI). Imaging was performed using Zeiss Axioskop and Axiophot microscopes equipped with Spot RT (Diagnostic Instruments) and Axiocam (Zeiss) digital cameras, respectively.