NFATc1 balances quiescence and proliferation of skin stem cells

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Abstract

Quiescent adult stem cells reside in specialized niches where they become activated to proliferate and differentiate during tissue homeostasis and injury. How stem cell quiescence is governed is poorly understood. We report here that NFATc1 is preferentially expressed by hair follicle stem cells in their niche, where it's expression is activated by BMP signaling upstream and it acts downstream to transcriptionally repress *CDK4* and maintain stem cell quiescence. As stem cells become activated during hair growth, NFATc1 is downregulated, relieving *CDK4* repression and activating proliferation. When calcineurin/NFATc1 signaling is suppressed, pharmacologically or via complete or conditional *NFATc1* gene ablation, stem cells are activated prematurely, resulting in precocious follicular growth. Our findings may explain why patients receiving cyclosporine A for immunosuppressive therapy display excessive hair growth, and unveil a functional role for calcium-NFATc1-CDK4 circuitry in governing stem cell quiescence.

Keywords

NFATc1; bulge; hair follicle; stem cells; proliferation; skin

Introduction

The hair follicle (HF) is an excellent model for studying stem cell activity because it continuously proceeds through rounds of tissue regeneration. This cyclic nature of HF formation requires a subset of stem cells within a specialized niche called the bulge, located within the HF outer root sheath (ORS) (Claudinot et al., 2005; Cotsarelis et al., 1990; Oshima et al., 2001; Taylor et al., 2000). Following embryonic HF morphogenesis and postnatal completion of the first round of hair growth (anagen), the cycling portion of the follicle dies (catagen) regressing up to the bulge region, where the HF then remains dormant during the resting phase (telogen) (Figure S1A).

The mechanisms by which the bulge niche environment changes to induce new follicular growth are still unfolding. BMP inhibitory signals likely emanating from the dermal papillae (DP) at the base of the HF have been implicated, as have Wnt signals. Both signaling pathways contribute to the stabilization of β-catenin (Kobielak et al., 2003; Andl et al., 2004; Zhang et al., 2006; Kobielak et al., 2007), a transcription cofactor for the TCF/Lef1 family of DNA binding proteins (Clevers, 2006). Activation of β-catenin/TCF/Lef1 target genes is required for bulge stem cell activation and maintenance (Andl et al., 2004; Andl et al., 2002; Huelsken et al., 2001; Kobielak et al., 2003; Kobielak et al., 2007; Lo Celso et al., 2004; Lowry et al., 2005; Van Mater et al., 2003; Zhang et al., 2006). That said, mice conditionally targeted for loss of *BMPr1a* (Kobielak et al., 2007) lose the ability of the bulge to regulate quiescence. These data suggest that the mechanisms controlling stem cell behavior in the niche are complex.

Microarray profiling has identified genes that are preferentially expressed in the bulge relative to the proliferative basal cells of the epidermis (Blanpain et al., 2004; Morris et al., 2004; Tumbar et al., 2004). One of the upregulated genes in these profiles and also in an array distinguishing embryonic hair buds from epidermis (Rhee et al., 2006) is the transcription factor nuclear factor of activated T cells c1 (NFATc1). NFATc1 (also referred to as NFAT2) belongs to the NFAT family of transcription factors which consists of four calcium sensitive members, NFATc1−4, that are broadly expressed in many different tissues and organs (Crabtree and Olson, 2002). The subcellular regulation of NFAT transcription factors can influence their activity, and in many cell types, NFAT proteins are phosphorylated and confined to the cytoplasm under basal conditions. In response to increases in intracellular calcium, the serine/ threonine phosphatase calcineurin becomes activated, dephosphorylating NFAT proteins and allowing their nuclear translocation. Once in the nucleus, NFAT proteins in association with other transcription factors bind to consensus DNA sequences to regulate gene transcription.

NFATc1's prominence as a HF stem cell signature gene is intriguing, given that the immunosuppressant drug cyclosporine A (CSA), which inhibits calcineurin upstream of NFAT, can induce hair growth in humans during organ transplantation (Yamamoto and Kato, 1994) and in mice (Paus et al., 1994; Sawada et al., 1987). In addition, hair growth is precociously activated in *calcineurin B1* null skin (Mammucari et al., 2005). A role for NFAT proteins has been postulated for the catagen (destructive) phase of the hair cycle (Gafter-Gvili et al., 2003), but whether NFAT proteins function in HF stem cells and if so how, remains unexplored.

To date, mouse genetics have not revealed a role for NFATs in skin. Epidermal mouse keratinocytes (MKs) in vivo and in vitro can respond to CSA treatment by blocking nuclear localization of NFATc2 (Al-Daraji et al., 2002; Canning et al., 2006; Gafter-Gvili et al., 2003; Yarosh et al., 2005), and NFATs have been positioned downstream of Notch signaling in cultured epidermal cells (Mammucari et al., 2005). However, in contrast to *NFATc2* (*NFAT1*) null mice, which are viable and fertile (Hodge et al., 1996; Xanthoudakis et al., 1996), *NFATc1* null mice die early in embryogenesis, and display defects in cardiac valve, bone and pancreatic development (de la Pompa et al., 1998; Graef et al., 2001; Heit et al., 2006; Ranger et al., 1998; Winslow et al., 2006).

Here, we focused on addressing the function of NFATc1 in the skin. We show that NFATc1 is expressed exclusively in the bulge region of the HF and using gain- and loss-of function studies, we identify an inhibitory role for NFATc1 in stem cell activation in the HF. Moreover, we find that NFATc1-mediated quiescence involves transcriptional repression of the *cyclin dependent kinase 4* gene encoding CDK4, which is required for progression through the G1/ S phase of the cell cycle (Sherr and Roberts, 2004). Finally, we find that NFATc1 gene expression is linked upstream to BMP signaling. Taken together, these findings provide significant new insights into how stem cell activation is orchestrated in the HF.

Results

NFATc1 is specific to the quiescent stem cell niche of the hair follicle

To determine whether NFATc1 protein is expressed in the bulge region of the HF as suggested from our microarray analyses (Blanpain et al., 2004; Tumbar et al., 2004), we analyzed its expression by immunofluorescence microscopy (Figure 1 and Figure S1). Nuclear NFATc1 was first detected during the late stages of HF morphogenesis (Figures S1B-C). As HFs matured, their mid-segments became marked by nuclear NFATc1(+) (Figure 1A). NFATc1-expressing cells persisted not only through the growing phase (anagen) of postnatal HFs, but also the resting (telogen) phase (Figures 1A-D and Figure S1E).

To more precisely define the cells marked by nuclear NFATc1, we compared the localization of NFATc1 with other proteins expressed in the bulge. NFATc1 colocalized with CD34, which is highly upregulated in quiescent bulge cells and has been used as a marker for purifying these cells (Blanpain et al., 2004)(Figures 1E and 1I). NFATc1 also overlapped substantially with Lhx2, required for HF stem cell maintenance (Rhee et al., 2006)(Figures 1F and 1I). In addition, NFATc1(+) cells partially colocalized with TCF3 and Sox9, transcription factors expressed by bulge cells (Nguyen et al., 2006; Vidal et al., 2005)(Figures 1G-1I). NFATc1 was not detected in the TCF3/Sox9-positive cells of the lower ORS, thought to be less quiescent stem cells which have exited the bulge (Oshima et al., 2001). Real-time PCR of fluorescence activated cell sorted (FACS) skin populations confirmed the enrichment of NFATc1 mRNAs in neonatal ORS (Rendl et al., 2005) (Figure 1J) and more specifically in the adult (α6 integrin +/CD34+) bulge cells at both the 1st and 2nd telogen stages (Blanpain et al., 2004)(Figure 1K).

Interestingly, in both embryonic and adult skin, NFATc1 protein and mRNA appeared to be specific for bulge cells (Figure 1). NFATc1 antibodies did not immunolabel cells in epidermis, sebaceous glands or dermis (Figures 1A-1H and 1L). Western analysis confirmed the absence of NFATc1 protein in epidermis (Figure 1M), and real-time PCR of mRNAs isolated from FACS-purified skin cell populations revealed high levels of *NFATc1* mRNA in P4 ORS and adult a6(+)/CD34(+) bulge cells, in comparison to background levels in P4 epidermal, DP, melanocyte (MC), dermal fibroblast (DF) and adult α6(+)/CD34(-) cells (Figures 1J and 1K). *NFATc1* mRNA expression in FACS-purified α6(+)/CD34(+) bulge MKs was sequentially lost with proliferation and passage in culture (Figure 1N). Appreciable *NFATc1* mRNA was also not observed in cultured epidermal MKs (Figure 1N). These data provide compelling evidence that NFATc1 expression in skin is specific for the HF bulge. The correlation between nuclear NFATc1 and *NFATc1* mRNA expression in the bulge also correlated with NFATc1's known ability to regulate its own transcription (Pan et al., 2007).

Consistent with the slow-cycling nature of bulge cells, nuclear NFATc1(+) cells did not co-label the proliferative cells marked by Ki67 during HF morphogenesis nor during anagen initiation following the first hair cycle (Figure S1G and S1J). In addition, short pulses of BrdU did not label NFATc1(+) cells either during the first growth phase (not shown) nor during the transition to the second hair cycle (Figure S1H). However, when slow cycling cells were labeled by expression of tetracycline-regulatable H2B-GFP and then chased for 4 weeks, NFATc1 was observed in the majority of label retaining cells (LRCs) (Figures S1I and S1J). Based upon these data, cells marked by nuclear NFATc1 exhibit the characteristics of bulge LRCs.

Cyclosporine A enhances stem cell activity in the bulge niche

CSA is known to stimulate precocious entry of telogen HFs into anagen (Paus et al., 1994; Sawada et al., 1987), and is also known to inhibit NFAT activity (Clipstone and Crabtree, 1992). These data led us to wonder whether CSA might act on bulge stem cells to influence the telogen to anagen transition and if so, how.

To evaluate whether CSA affects the proliferation of bulge cells during anagen induction, we co-administered CSA and a short BrdU pulse during the 2nd telogen (Figure 2A). As expected, within 5d, the majority of CSA-treated HFs had precociously entered anagen (Figure 2B). Irrespective of whether the experiments were performed on HFs in their 1st or 2nd hair cycles, most HFs of CSA-treated skin contained BrdU(+) cells in both the bulge and the hair germ (Figures 2B, 2C and Figure S2A). By contrast, the majority of proliferation at the onset of a spontaneous anagen was in the hair germ, while only 10% of HFs showed proliferative activity in the bulge (Figures 2B, 2C and Figure S2A). This was consistent with prior studies showing that in normal HFs, most slow cycling bulge cells (marked by pulse-chase with H2BGFP) do not lose their quiescent status even in the anagen phase of a hair cycle (Tumbar et al., 2004).

Since most CSA-treated bulge cells incorporated BrdU over multiple days, the effects could not be attributed to an acceleration in the timing at which an otherwise normal bulge undergoes a telogen to anagen transition (Figure 2B). The effects of CSA also contrasted with hair depilation, another treatment which induces anagen. While depilation resulted in a short-lived burst of proliferation within the bulge at D2, proliferation in the upper ORS and surrounding epidermal cells also occurred (Figures S2B-S2E). We did not observe enhanced epidermal proliferation with CSA treatment (Figure S2E). Consistent with CSA's ability to inhibit calcineurin, required for nuclear NFATc1 localization, CSA resulted in the disappearance of NFATc1 in the bulge (Figure 2D and S2I). Nuclear NFATc1 was also lost when bulge cell proliferation and anagen were precociously induced by depilation (Figure S2C).

To further explore the relation between calcineurin, NFAT activity and bulge cell proliferation, we purified α6(+)/CD34(+) bulge cells from 1 month old *K14-H2BGFP* mice and cultured them in the presence of CSA or cell-permeable VIVIT (11R-VIVIT), a specific NFAT inhibitor (Aramburu et al., 1998; Noguchi et al., 2004). CSA or 11R-VIVIT-treated cultures formed larger colonies and with greater efficiency than vehicle or scrambled VIVIT (11R-VEET) treated cells (Figures 2F and S3A). The proliferative effects of CSA on primary bulge cells contrasted with CSA's growth suppressive effects observed with cultures of either epidermal MKs (see also Karashima et al., 1996) or α6(+)CD34(-) cells, containing primarily epidermal and non-bulge ORS cells (Figures S3B and S3C). The ability of CSA and VIVIT to regulate calcium-sensitive calcineurin/NFAT activity in keratinocytes was confirmed by *NFAT* reporter gene assays in conjunction with the calcium ionophore ionomycin (I) and phorbol myristate acetate (PMA), required together for full activation of NFAT transcriptional activity, and a constitutively active form of NFATc1 (Figure 2G). Taken together, these results suggest that calcium signaling and calcineurin activity may also influence NFATc1's localization in the bulge and its ability to activate target genes.

NFATc1 regulates bulge stem cell quiescence

To determine whether *NFATc1* gene expression is essential for maintaining HF stem cell quiescence, we performed loss of function studies. Since most *NFATc1* null embryos die between E14.5 and E17.5 (Ranger et al., 1998), skins from *NFATc1* null and control embryos were grafted onto the backs of *Nude* mice to monitor their effects on HF morphogenesis and cycling. HFs formed and underwent cycling normally, including reentry into telogen in both WT and KO skin grafts (Figure 3C). This should not have been observed had NFATc1's effects been exerted on catagen, the hair cycle stage that Gafter-Givili et al. (2003) postulated CSA inhibited through NFATc2.

To address whether loss of NFATc1 affects stem cell activation, we shaved or dyed hairs at D60 after skin engraftments during what is normally an extended telogen phase. By D75 when WT HFs were in telogen, *NFATc1* null HFs had entered anagen, as visualized by growth of new (white) hairs (Figure 3A and B) and shedding of old (blue dyed) hairs (Figure 3B). Histological analyses confirmed that D60 HFs of both genotypes were in telogen as indicated

by the close proximity of DP to bulge (Figure 3C). However, by D75 only KO HFs were in full anagen as indicated by the distance of the DP from the bulge due to new hair growth (Figure 3D). Taken together, these data indicated that the loss of NFATc1 affected hair cycling by shortening telogen and prompting precocious entry into anagen.

To evaluate whether these aberrations in hair cycling reflect a specific alteration in bulge stem cells, we conducted BrdU pulse and pulse-chase experiments. Even at P60, after WT and KO HFs had regressed and were morphologically in telogen, nearly half of the *NFATc1* null bulge cells abnormally incorporated BrdU during short labeling periods, and most bulge cells failed to retain label in BrdU-pulse chase experiments (Figure 3E and Figure S4A). Despite these marked changes in proliferative status within the bulge, CD34, Sox9, tenascin C and nuclear phospho-Smad 1, 5, 8 (reflective of active BMP signaling) as well as activated caspase 3, indicative of apoptotic cells (data not shown), appeared normal in the absence of NFATc1 (Figures 3F and Figures S4B-D). In addition, hair shaft length was slightly longer in KO follicles (Figure S4G) but long-term grafts maintained hair growth (Figure S4H). Thus, the quiescent status and initiation of hair growth were selectively altered upon loss of NFATc1, while stem cell self-renewal seemed to be maintained.

Since hair cycling requires mesenchymal-epithelial interactions, it was important to ablate *NFATc1* specifically in the skin epithelial cells. To this end, we bred *NFATc1^{fl/fl}* mice (to be described further elsewhere) to transgenic mice expressing the Cre recombinase under the control of the keratinocyte-specific *K14* promoter, active by E15.5 (Vasioukhin et al., 1999). *NFATc1* cKO mice developed normal HFs (Figure S4F) which lacked detectable NFATc1 immunolabeling (Figure 3G). In contrast to the *calcineurin B1* cKO (Mammucari et al., 2005), telogen follicles were maintained in NFATc1 cKO mice (Figure S4J). However, consistent with the phenotype of the complete *NFATc1* null, while hair germs of WT HFs were still in telogen, a majority of *NFATc1* cKO HFs entered anagen at D56, as revealed by BrdU labeling in hair germs (Figures 3H and 3I) and the growth of hairs after shaving (Figure S4I). Quantification documented that ~75% of *NFATc1* cKO bulges were BrdU-labeled within a 48 hr pulse (Figures 3H and 3I). FACS analysis further substantiated the significant increase of S-phase bulge cells in *NFATc1* cKO HFs (Figure 3I). This activation of bulge cell proliferation was specific as no effect on epidermal proliferation was noted with loss of NFATc1 (Figure S4E). Together, these data suggest that in the absence of NFATc1, the slow cycling properties are selectively lost without perturbing other features of HF stem cells.

To determine whether NFATc1 can confer slow cycling characteristics upon MKs, we used retroviral delivery to express a constitutively nuclear form of NFATc1 (caNFATc1; (Neal and Clipstone, 2003)) in cultured epidermal MKs, which as shown in Figure 1, lack endogenous NFATc1. caNFATc1 markedly reduced the proliferation rate of MKs grown in low-calcium medium, which is favorable to proliferation of WT cells (Figure 4A). The reduction in proliferation rate was confirmed by BrdU incorporation and FACS analyses of cell cycle profiles: caNFATc1-expressing cultures showed 50−80% fewer BrdU-labeled (Figure 4B) and S phase MKs (Figure 4C).

The effects of caNFATc1 appeared to be specific for the reduction in cell cycling rather than an effect on terminal differentiation or apoptosis. caNFATc1 did not appreciably induce terminal differentiation markers transglutaminase (TGM) 1 and 3, filaggrin and loricrin, which are induced by elevated calcium (Figure 4D), nor did it increase the percentages of dead or apoptotic cells in MK cultures (Figure 4E).

NFATc1 represses the expression of CDK4 in hair follicle stem cells

The effects of NFATc1 on cell cycle kinetics both *in vitro* and *in vivo* suggested that NFATc1 may control key genes involved in promoting cell cycle progression. Of the cell cycle genes

which are downregulated in the telogen bulge relative to proliferating progeny (Blanpain et al., 2004; Morris et al., 2004; Tumbar et al., 2004), *cyclin-dependent kinase 4 (CDK4)* stood out. NFATc2 represses *CDK4* promoter activity in T-lymphocytes (Baksh et al., 2002), and injection of a TAT-p16INK4a protein, which inhibits CDK4/6, impairs hair growth in mice (Yu et al., 2003).

By immunohistochemistry, CDK4 appeared to be upregulated in the telogen to anagen transition of WT HFs, where it appeared in a few cells at the base of the bulge, in the developing hair germ and later in the matrix cells of the anagen hair bulb (Figure 5A). The elevated CDK4 protein during HF stem cell activation was reflected at the mRNA level, as judged by real time PCR on FACS-purified cell populations (Figure 5B). By contrast, *NFATc1*-null HFs displayed anti-CDK4 staining within the bulge region even prior to visible signs of anagen progression *in vivo* (Figure 5C), and constitutively nuclear caNFATc1 *in vitro* elicited a reduction in both *CDK4* mRNA (Figure 5D) and protein (Figure 5E). The regulation of *CDK4* expression was at the level of transcription, since NFATc1 repressed the activity of a luciferase reporter driven by the wild-type *CDK4* promoter but not the promoter harboring mutations in the NFAT binding sites (Baksh et al., 2002)(Figure 5F).

The elevation in CDK4 appeared to be specific for the bulge, as no change in CDK4 was noted in *NFATc1* null epidermis (Figure S4F). The effects also appeared to be specific for CDK4, as other cell cycle regulators, including the closely related homologue CDK6, did not exhibit this behavior (Figures 5B, 5D and 5E). Importantly, the major downstream target of CDK4/6, namely retinoblastoma protein (RB), was not phosphorylated in caNFATc1-infected cells (Figure 5E). Moreover, when a *CDK4* transgene was co-expressed with caNFATc1, MK proliferation and S phase progression were restored to WT levels (Figure 5G). Taken together, these findings indicated that the suppression of *CDK4* by NFATc1 was sufficient to inhibit G1/S progression in quiescent stem cells..

Finally, we addressed whether NFATc1 acts downstream of either of two endogenous signals, BMP and Wnt, known to promote the telogen to anagen transition. As shown in Figure 6 (frames A and B), NFATc1 was still present and nuclear in bulge cells from mice expressing a constitutively stabilized β-catenin (ΔNβcatenin). The HFs of these mice precociously activate anagen, but the bulge stem cells to return to quiescence (Lowry et al., 2005; see also Lo Celso et al., 2004;van Mater et al., 2003). In addition, treatment of ΔNβcatenin mice with CSA resulted in loss of NFATc1 expression, suggesting that its regulation was not affected (data not shown). Interestingly, NFATc1 localization was lost in HFs conditionally targeted for loss of the BMP receptor 1a (Kobielak et al., 2003;Andl et al., 2004), required for BMP signaling. This was true both for the presumptive bulge of newborn HFs by NFATc1 (Figure 6C) and for 22d HFs of *BMPr1a* cKO skin (Figure 6D). Importantly and in contrast to ΔNβcatenin, *BMPr1a* ablation causes bulge cells to lose quiescence (Kobielak et al., 2007).

To address how BMP signaling might affect NFATc1, we first tested whether exogenous BMP could activate *NFATc1* gene expression *in vitro*. As shown in Figure 6E, BMP4 increased *NFATc1* mRNA levels by 7−9 fold, and this effect was blocked by the BMP inhibitor, noggin. We next addressed whether BMP signaling acted directly upon the activity of the *NFATc1* promoter (Zhou et al., 2002). *NFATc1* promoter activity was enhanced >100 fold by BMP4, and again these effects were abrogated by noggin (Figure 6F). Interestingly, despite these profound BMP-specific effects on *NFATc1* gene expression, NFAT reporter activity was only slightly upregulated by BMP4 treatment and required calcium/CN activity (Figure 6G).

Discussion

Our data support a model whereby transcriptional repression of *CDK4* by NFATc1 in WT HFs maintains bulge stem cells in a quiescent state in all stages of the hair cycle (Figure 7). Our studies *in vitro* and *in vivo* suggest that BMP signaling and calcineurin activity are both required for NFATc1 expression. While BMP signaling affects *NFATc1* gene expression, calcium signaling activates NFAT's action as a transcription factor, suggesting that both of these upstream activators, BMP and calcium, impinge on NFATc1's role in the bulge. As cells are activated to proliferate and form the new HF, nuclear NFATc1 and *NFATc1* gene expression are downregulated and *CDK4* expression is upregulated. Finally, when NFATc1 activity are inhibited, either by CSA treatment or conditional ablation of *NFATc1*, CDK4 levels are elevated in the bulge cells and their slow cycling character is compromised.

CSA has pleiotropic effects on hair growth, although most studies have concentrated on CSA's in enhancement of hair growth by preventing the apoptosis that occurs in the catagen phase of the hair cycle (Gafter-Gvili et al., 2004; Maurer et al., 1997; Paus et al., 1994). Our studies confirm the ability of CSA to precociously induce the telogen to anagen transition in resting HFs (Maurer et al., 1997), and now demonstrate that this transition is mediated at least in part by promoting proliferation within the bulge stem cell compartment. Furthermore, our data reveal that NFATc1, previously unstudied in the skin, is likely responsible for the effects of CSA on bulge stem cell activation. The stimulation of bulge proliferation in response to CSA or loss of NFATc1 contrasts with the normal behavior of bulge stem cells, which typically are slow cycling even during the growing phase of the hair cycle.

Calcineurin may have additional substrates in the skin that control catagen or HF retention, since *NFATc1* null mice do not have defects in either process. *Calcineurin B1* cKO mice display alopecia due to the inability to retain telogen HFs, but the downstream targets of calcineurin that control this phenotype are unknown (Mammucari et al., 2005). In addition, other groups have postulated that inhibition of calcineurin by CSA may influence HF regression during the catagen phase of the hair cycle by acting on NFATc2 nuclear localization (Gafter-Gvili et al., 2004; Gafter-Gvili et al., 2003). Although *NFATc2* null mice are viable and display no overt abnormalities on the hair coat (Hodge et al., 1996; Xanthoudakis et al., 1996), further studies will be required to determine the mechanisms by which calcineurin and CSA might affect other substrates in the skin, including NFAT proteins.

It has also been shown that *in vitro*, calcineurin/NFATc2 regulates terminal differentiation of epidermal MKs by acting downstream of Notch signaling to activate the *p21* promoter in terminally differentiation (Santini et al., 2001). NFATc1 did not appear to be expressed in normal epidermis *in vivo*, nor did its loss result in any discernable defects in the epidermis. Moreover, in keratinocytes *in vitro,* activated NFATc1 did not appear to affect terminal differentiation. Thus, both *in vivo* and *in vitro*, NFATc1's function in bulge cells was distinct from that previously attributed to other NFAT family members in epidermal cell cultures.

Our data suggest that calcium signaling may contribute to the regulation of NFATc1 activity in the HF stem cell niche. Calcium is a well-known regulator of homeostasis and differentiation in the epidermis *in vitro* (Hennings et al., 1980; Pillai et al., 1990) and *in vivo* (Menon et al., 1985). Intriguingly, the bulge signature contains a number of genes encoding calcium binding proteins and ion channels (Blanpain et al., 2004; Morris et al., 2004; Tumbar et al., 2004). Future studies will be necessary to dissect the mechanisms that regulate calcium signaling in HF stem cells to regulate their activity and fate.

Despite the identification of bulge stem cell populations based on their relative quiescence (Claudinot et al., 2005; Cotsarelis et al., 1990; Trempus et al., 2003; Tumbar et al., 2004), insights into how quiescence is controlled have only recently begun to emerge. BMP signaling

appears to participate in maintaining quiescence within the bulge, as conditional ablation of *BMPr1a* results in sustained activation of HF stem cells without apparent loss of many stem cell features, including Sox9 (Kobielak et al., 2007). Our data suggest that part of BMPs role in the stem cell niche may be to control *NFATc1* expression. This is supported by the loss of NFATc1 in *BMPR1a* cKO skin as well as the marked elevation in *NFATc1* promoter activity and mRNA expression in BMP4-treated MKs. In this regard, it is noteworthy that 10 putative SMAD sites reside within the *NFATc1* promoter. While NFATc1 is not likely the only downstream target of BMP signaling, our findings suggest that NFATc1 acts downstream of BMPR1A signaling in the bulge, where it participates in stem cell quiescence.

In contrast to the effects of BMP signaling and NFATc1 on the slow-cycling properties of bulge stem cells, inhibition of Wnt signaling seems to act primarily by repressing the differentiation lineages afforded to them (Merrill et al., 2001; Nguyen et al., 2006). Conversely, while both inhibition of BMP signaling and elevated Wnt signaling seem to promote the telogen to anagen transition, gene targeting and transgenic studies are suggestive that they activate proliferation and lineage commitment by different means (Gat et al., 1998; Lowry et al., 2005; Kobielak et al., 2007). Our findings provide new insights as to how BMP signaling may function in controlling the quiescent state of HF stem cells and in their transition to an activated state at the beginning of the new hair cycle.

It is intriguing that the epidermis seems to be largely unaffected irrespective of whether *NFATc1*, *BMPR1a* or *β-catenin* is targeted for ablation. The cell lineage-specific differences in stem cell regulation between the epidermis and HF may explain why in the epidermis, quiescence and homeostasis appear to be regulated by different mechanisms involving EGF receptor signaling (Jensen and Watt, 2006) and NFkB-mediated regulation of *CDK4* (Zhang et al., 2005). Given that CSA affects proliferation, albeit differentially, in both epidermal and HF stem cells, it will be interesting in the future to evaluate whether calcineurin signaling and other NFAT proteins play general roles in regulating stem cell proliferation in other tissues and organs.

In closing, our data suggest that excessive hair growth in human patients during immunosuppressive therapy during CSA treatment might result from inhibition of NFATc1, which in turn leads to CDK4 expression and HF stem cell activation. If the mechanism we've uncovered in mouse skin occurs in human HFs, NFATc1 activation could have clinical significance by providing a new possible target for controlling hair growth. Additionally, by specifically inhibiting or activating NFATc1 in skin, it may be possible to pharmacologically uncouple the immunosuppressive and hair growth effects caused by general calcineurin inhibitors like CSA.

Experimental Procedures

Mice, Cyclosporine A treatments, grafts and BrdU incorporation

NFATc1-null, K14-H2BGFP, ΔN-β-catenin transgenic mice, and *BMPR1a* conditional null mice have been described previously (Gat et al., 1998; Kobielak et al., 2003; Kobielak et al., 2007; Lowry et al., 2005; Tumbar et al., 2004). A conditional allele of *NFATc1* (*NFATc1* fl) was generated by flanking exon 3 with loxP sites (to be described further elsewhere). Exon 3 encodes the NFAT regulatory domain and deletion results in a null allele ((de la Pompa et al., 1998) and data not shown). *NFATc1*fl/fl, *K14-Cre* negative littermates were used as WT controls for all experiments.Grafting of E15.5 skins was performed as described (Rhee et al., 2006). For 5-Bromo-2'-deoxyuridine (BrdU) (Sigma-Aldrich) pulse-chase experiments (Blanpain et al., 2004), mice were injected intraperitoneally with 50 μg/g BrdU (Sigma-Aldrich) and animals were then sacrificed thereafter either directly, or following a chase period. For *in vivo* experiments using CSA, mice were injected with 100mg/kg CSA solution (Bedford

Laboratories) intraperitoneally daily at P49 for 6 days. Hair dye was used according to manufacturer's directions (L'Oreal).

Retroviral Infection, Cell culture and FACS

MSCV-GFP (Vector control), constitutively nuclear caNFATc1, a NFAT reporter construct and CDK4 retroviral vectors were previously described (Abbott et gal., 1998; Baksh et al., 2002; Matsushime et al., 1992; Neal and Clipstone, 2003). Retrovirus production is described in the Supplemental Methods. Growth assays using infected cells were performed by trypsizing and counting cells with a Z1 Particle Coulter Counter at indicated intervals. Quantification of BrdU and cell cycle was performed using FACS as described previously (Blanpain et al., 2004). Differentiation of MKs was induced by adding MK growth media with 1.5mM calcium. FACS analysis of apoptosis was performed via Annexin V (Invitrogen) staining according to manufacturer's instructions. As a positive control for the apoptosis experiments, cells were treated with TNFα (100 ng/ml) and cycloheximide (5ug/ml) for 24 hours prior to staining.

For experiments using cultured bulge stem cells, α6+ cells alone and α6+/CD34+ bulge cells were FACS purified as described (Blanpain et al., 2004) from K14-H2BGFP mice. Following FACS isolation, a minimum of 1×10^5 cells were seeded onto mitomycin treated fibroblast feeder layers and cultured in MK growth media with 0.3mM calcium. After the first passage, the MKs were treated with vehicle, cyclosporine A (1μM; Sigma), cell-permeable VIVIT (11R-VIVIT; 1μM; Calbiochem), or cell-permeable scrambled VIVIT, (11R-VEET; generous gift from Masayuki Matsushita and Rockefeller Proteomics Resource Center)(Noguchi et al., 2004) and analyzed for colony size (>5 cells) or cell number for the indicated days. No toxicity was noted with the use of VIVIT on MKs.

Reporter assays

For *CDK4* and *NFATc1* promoter reporter assays, MKs were grown to 30−40% confluency before Fugene 6 (Roche) reagent-assisted transfections of cytomegalo virus–*Renilla* luciferase DNA (control) and the CDK4 or NFATc1 promoter firefly luciferase constructs. The WT CDK4 promoter construct (F(B)) consisted of −404 to +43 (Baksh et al., 2002). This construct was used to generate a mutated version of F(B) lacking a NFAT binding site using the Quickchange Kit (Stratagene) using the complementary oligonucleotide, F'-CCCGCCTCCCAGA<u>GAGT</u>GCGCGCCTCTTTGGC-3' (mutational changes are underlined). 48 h after transfection, luciferase assays with the *CDK4* promoter were performed as described previously (Kaufman et al., 2002).

The *NFATc1* promoter construct consisted of 3.7kb upstream of the NFATc1 transcriptional start site (Zhou et al., 2002). 48h post-transfection, cells were treated with BMP4 (100ng/ml; R&D Systems) and/or noggin (1000ng/ml; R&D Systems) for 48 hours. 6h prior to performing the luciferase assay, cells were treated with PMA (10nM) and ionomycin (1μm).

For NFAT reporter assays, MKs were infected with retroviruses encoding the NFAT responsive plasmid in control or caNFATc1 infected cells (Abbott et al., 1998). After 48 h, PMA

(10nM), ionomycin (1μM), VIVIT (1μM) and CSA (1μM) were added to the cultures as indicated for 6 h. PMA acts to induce AP-1 transcription factors and ionomycin increases intracellular calcium, activates calcineurin, and leads to nuclear translocation of NFAT family members. Luciferase assays were performed as described above.

Histology and Immunofluorescence

Skins were embedded in OCT, frozen, sectioned and fixed in 4% formaldehyde. For paraffin sections, skins were incubated in 4% formaldehyde at 4°C overnight, dehydrated with a series of increasing concentrations of ethanol and xylene, and embedded in paraffin. Paraffin sections were rehydrated in decreasing concentrations of ethanol and subjected to antigen unmasking in 10 mM Citrate, pH 6.0. For histological analysis, sections were stained with hematoxylin and eosin. Sections were subjected to immunofluorescence microscopy. For immunohistochemistry, HRP conjugate secondary antibodies (Abs) were used followed by the HRP substrate, diaminobenzidine. When applicable, the MOM Basic Kit (Vector Laboratories) was used to prevent nonspecific binding of mouse monoclonal Abs. Antibodies and dilutions are included in the Supplemental Data.

For each type of analysis performed in this study, we assayed for hair cycle by both histology and proliferation status as indicated by BrdU incorporation during short pulses.

Statistics

To determine significance between groups, comparisons were made using Students t tests. Analyses of multiple groups were performed using One-Way ANOVA with Bonferroni's posttest with GraphPad Prism version for Macintosh (GraphPad Software). For all statistical tests, the 0.05 level of confidence was accepted for statistical significance.

Acknowledgments

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Supplementary Material

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Figure 1. NFATc1: a marker of hair follicle stem cells
(A-D) NFATc1 is expressed the upper HF during anagen at P4 and P27 and at the base of the follicle during telogen (P21 and P49). β4 integrin marks the dermo-epidermal interface. (C-H) Immunohistochemistry showing NFATc1 colocalization with bulge cell markers, CD34, Lhx2, TCF3 and Sox9. Arrows denote examples of co-expression. (I) Quantification of the % of cells with bulge cell markers that colocalize with NFATc1. Data are mean ± SEM. N=50−176 cells. (J-K) Real-time PCR analysis of NFATc1 and Sox9 mRNA in FACS isolated populations at P4 (J) and NFATc1 mRNA in the α6/CD34-positive bulge compartment during the 1st (P19) and 2nd (P49) telogen. (K). Data are mean ± SEM. N=2 (J) and N=3 (K) FACS isolated populations. Data are mean ± SEM. Asterisks indicate significance, p<0.05. (L) Expression of

NFATc1 in the keratin 5 (K5)(+) epidermis at E14.5 or newborn (nb). (M) Isolated P4 epidermis and the dermis (containing HFs) were subjected to western analysis. (N) Real Time PCR analysis of NFATc1 mRNA from FACS isolated α6(+)/CD34(+) bulge cells, after passage (P1, P4) and in epidermal keratinocytes (MK). Abbreviations: DP, dermal papillae; Bu, bulge; ORS, outer root sheath, epi, epidermis, derm, dermis HG, hair germ; FB, fibroblasts; MX, matrix, MC, melanocytes. Dapi staining (blue) shows nuclear localization. Scale Bars, 30 μm.

Figure 2. Impairing calcineurin/NFAT signaling results in hair growth and stem cell proliferation
(A) Schematic illustrating the experimental design of CSA experiments. (B-C) Quantification of anagen induction and BrdU incorporation in follicle stem cells of the bulge (Bu) with cyclosporine A (CSA) treatment. The anagen control used was the first hair cycle (P18-P25). Data are the mean ± SEM for 50−100 follicles for 3 individual mice for each timepoint. Asterisks in (C) indicate hair shaft autofluorescence. (D-E) Immunohistochemistry and western analysis of NFATc1 expression following 3d treatment with vehicle or CSA. (F) Colony formation and cell number of bulge cells following treatment with vehicle, CSA, 11R-VIVIT, or 11R-VEET. N= 3 experiments with independent sorted populations. (G) NFAT reporter activity in response to calcium ionophore (I), phorbol myristate acetate (PMA), caNFATc1, CSA or 11R-VIVIT. Data are mean ± SEM. N=3−5 individual experiments for each treatment. Asterisks indicate significance, p<0.05. Scale bars, 30μm. Dapi staining (blue) shows nuclear localization. Abbreviations: HF, hair follicle; Bu, bulge; HG, hair germ; DP, dermal papillae.

Figure 3. Full and conditional *NFATc1* deletion activates precocious follicular growth and enhanced stem cell activity

(A-B) When shaved or dyed blue during telogen (D60), the KO follicles regrow white hair (A and B) and shed their dyed hairs (B) by D75. (C-D) Histological analysis of the WT and KO HFs at D60 (C) and D75 (D). (E) Immunostaining of WT and KO follicles after BrdU label retaining experiments (BrdU pulse at D24−27 post-graft followed by 28 days of chase). Quantification of bulge cell proliferation (24h BrdU pulse) and label retention at D60 in WT and *NFATc1*-null follicles. Data are mean ± SEM. N=3 mice for each genotype. (F) Immunostaining for NFATc1 and CD34 in WT and *NFATc1* null follicles. (G) Immunolocalization of NFATc1 in WT and *NFATc1* cKO mice at P56. (H) BrdU

immunolabeling after a 48 hour pulse in WT and *NFATc1* cKO follicles at P56. Data are mean ± SEM. N=6 mice for WT and N=5 mice for NFATc1 cKO. (I) Quantification of BrdU incorporation and cell cycle analysis of a6(+)/CD34(+) bulge cells from WT and NFATc1 cKO mice. Data are mean ± SEM. Asterisks indicate significance, $p < 0.05$. Scale Bars, 30μm. Dapi staining (blue) shows nuclear localization. Abbreviations: Bu, bulge; SG, sebaceous gland; DP, dermal papillae; HG, hair germ.
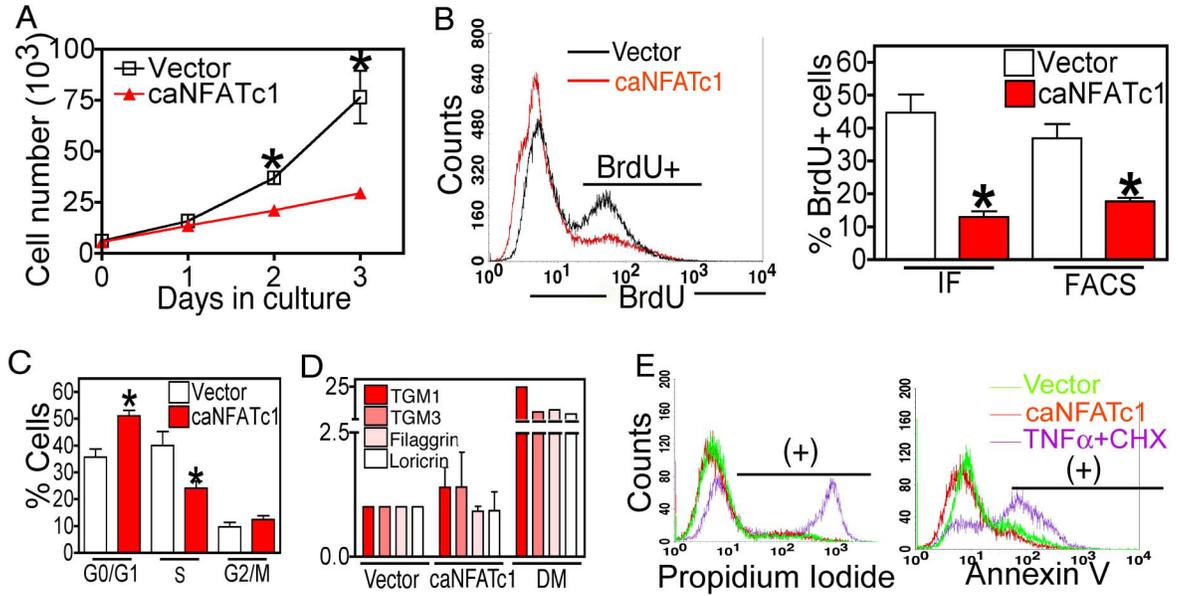
Figure 4. NFATc1 inhibits cellular proliferation by blocking G1/S phase progression
(A) Retroviral expression of constitutively active NFATc1 (caNFATc1) represses cell proliferation in epidermal MKs. N= 3 individual experiments for each timepoint. (B-C) Quantification of BrdU incorporation as analyzed by FACS or immunostaining (IF) (B) or combined with DNA content (C) in control or caNFATc1 infected MKs. Representative FACS histogram plot of BrdU immunostaining is shown (left). Data are mean ± SEM. N= 3−4 individual experiments. (D) Real time PCR analysis of the epidermal terminal differentiation markers transglutaminase (TGM) 1 and 3, filaggrin, or loricrin in control, caNFATc1 infected MKs, or cells in high calcium media (Diff. Media). Data are mean ± SEM. N=3 individual experiments. (E) FACS analysis of propidium iodide (dead cells) and Annexin V (apoptotic cells) in control, caNFATc1 expressing cells, or cells treated with TNFα (100 ng/ml) and cycloheximide (CHX) (5ug/ml). Data are mean ± SEM. Asterisks indicate significance, p<0.05.
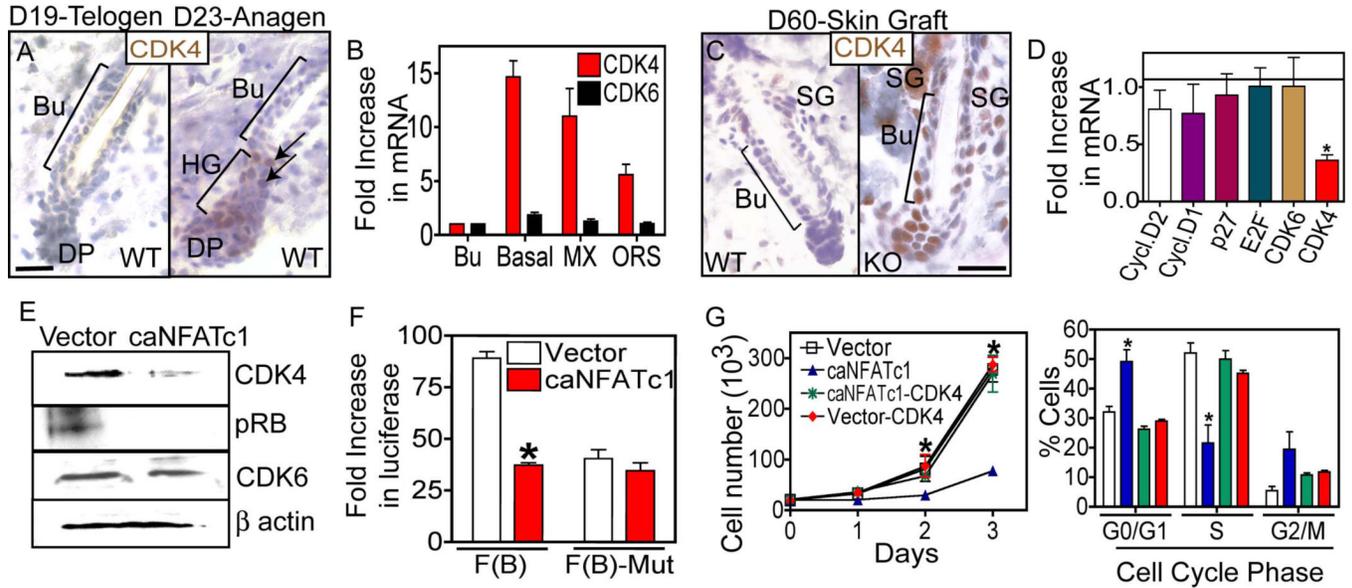
Figure 5. NFATc1 acts by repressing CDK4 expression to regulate G1/S progression in hair follicle stem cells
(A) Anti-CDK4 immunohistochemistry during the telogen (D19) to anagen (D23) transition. Hematoxylin counterstain. (B) Real Time PCR analysis of *CDK4* and *CDK6* mRNA expression in bulge (Bu), all basal (α6+), ORS and matrix (MX) cell populations. Data are mean ± SEM. N=3 for each sample. (C) Immunostaining of CDK4 in WT and *NFATc1*-null follicles. Bu, bulge. (D) Expression of cell cycle regulatory genes in control or caNFATc1 expressing cells. Data are mean ± SEM. N=3 individual experiments. (E) Western analysis for CDK4, phosphorylated retinoblastoma protein (Rb), and CDK6 in control and caNFATc1 expressing keratinocytes (MKs). (F) Reporter assays using the *CDK4* promoter with NFAT sites (F(B)) or mutated NFAT sites (F(B)-mut) in conjuction with caNFATc1 in MKs. Data are mean ± SEM. N=4 individual experiments. (G-H) Expression of *CDK4* rescues MK growth and S phase progression of cells expressing caNFATc1. N=3 individual experiments. Data are mean ± SEM. Asterisks indicate significance, p<0.05. Scale Bar, 30 μm.
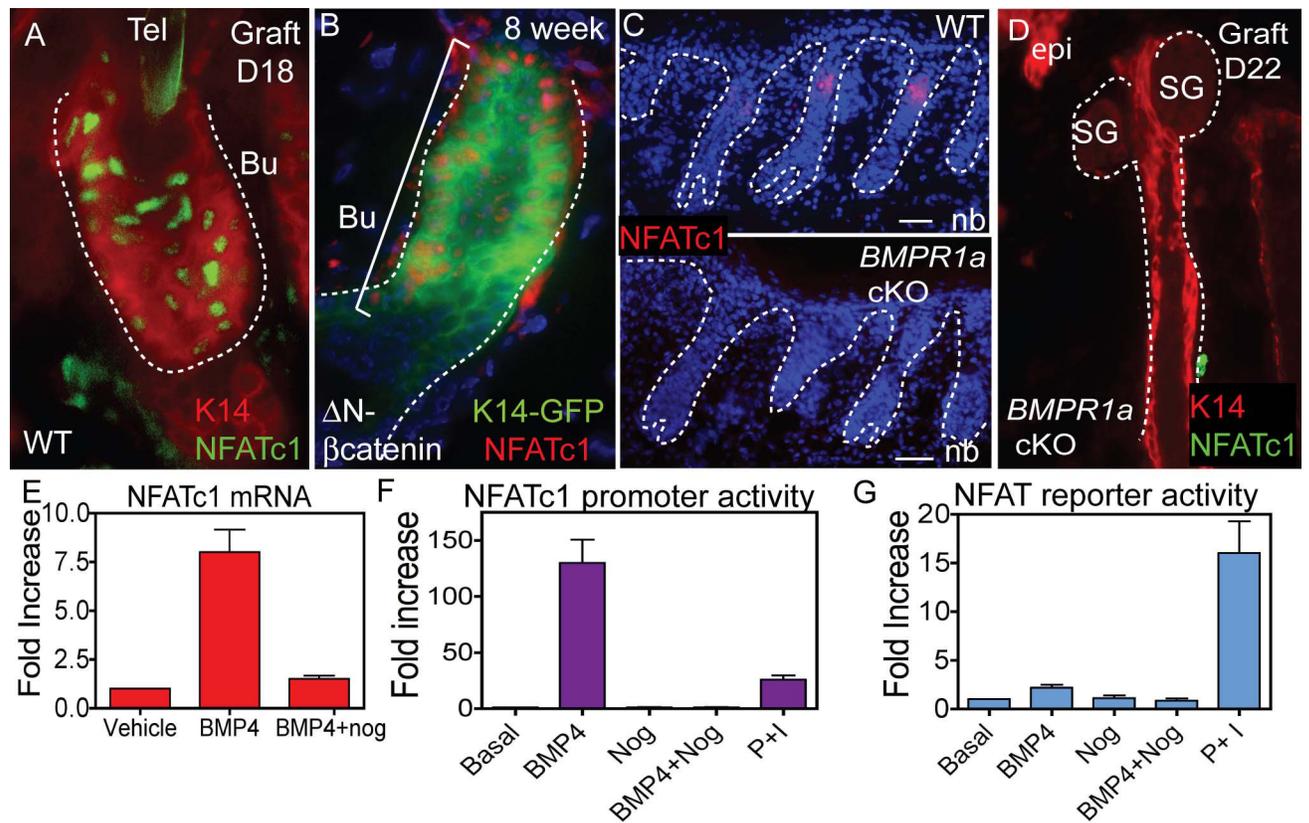
Figure 6. Expression of NFATc1 in mutant mouse models with altered hair follicle stem cell biology
(A-D) K14 expression (immunolocalization and K14-GFP) and NFATc1 immunolocalization
are shown for grafted skin from WT (A), heterozygous stabilized β-catenin (ΔN) (B) and
BMPR1a conditional knockout (cKO) mice (C and D). (E) Real Time PCR of *NFATc1* mRNA
levels upon MK treatment with BMP4 and/or the BMP inhibitor, noggin. Data are mean ±
SEM. N=3 individual experiments. (F) The activity of a 3.6-kb *NFATc1* promoter construct
following treatment of MKs with BMP4 and/or noggin or PMA (P) and ionomycin (I). Data
are mean ± SEM. N=3 individual experiments. (G) NFAT reporter activity upon BMP4, noggin
or P/I treatment of MKs. Data are mean ± SEM. N=3 individual experiments. Dapi staining
(blue) shows nuclear localization. Bu, bulge, CN, calcineurin, DP, dermal papillae, HG, hair
germ, epi, epidermis.
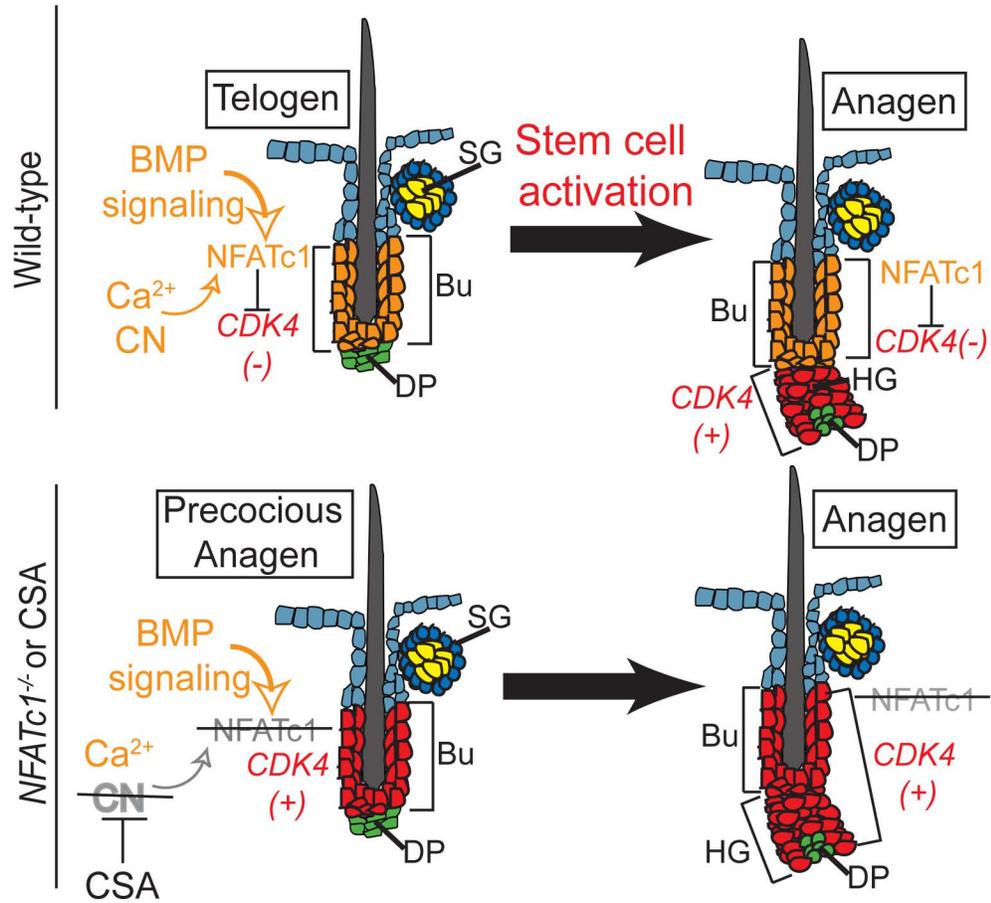
Figure 7. Model describing the role of NFATc1 signaling in bulge stem cell quiescence
Calcium/calcineurin (CN) activity and BMP signaling are required to maintain *NFATc1*
expression and activity, which transcriptionally represses *CDK4* gene expression. Upon
activation of hair growth, BMP signaling is inhibited, leading to a loss in NFATc1 expression,
and relief of *CDK4* repression. When CN signaling is blocked by CSA or loss of *NFATc1*
expression, *CDK4* is expressed and precocious activation of the stem cells occurs within the
bulge.