

Calcineurin/Nfatc1 signaling links skin stem cell quiescence to hormonal signaling during pregnancy and lactation

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In most tissues, the prevailing view is that stem cell (SC) niches are generated by signals from within the nearby tissue environment. Here, we define genetic changes altered in hair follicle (HF) SCs in mice treated with a potent SC activator, cyclosporine A (CSA), which inhibits the phosphatase calcineurin (CN) and the activity of the transcription factor nuclear factor of activated T cells c1 (Nfatc1). We show that CN/Nfatc1 regulates expression of *prolactin receptor (Prlr)* and that canonical activation of *Prlr* and its downstream signaling via Jak/Stat5 drives quiescence of HF SCs during pregnancy and lactation, when serum prolactin (Prl) levels are highly elevated. Using Prl injections and genetic/pharmacological loss-of-function experiments in mice, we show that Prl signaling stalls follicular SC activation through its activity in the skin epithelium. Our findings define a unique CN–Nfatc1–Prlr–Stat5 molecular circuitry that promotes persistent SC quiescence in the skin.

[*Keywords:* calcineurin; Nfatc1; cyclosporine; hair follicle; stem cell; prolactin; Stat5]

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Adult stem cells (SCs) govern tissue homeostasis and repair; thus, their function is essential for the prevention of tissue dysfunction and disease. Within tissues, SCs localize to specific niches (or microenvironments) that regulate SC activity through physical and molecular cues (Spradling et al. 2008). In most tissues, the prevailing view is that the SC niche is composed of signals provided by cells within the nearby tissue environment. However, an emerging question in SC biology is how systemic hormonal signaling controls SC function (Gancz and Gilboa 2013).

Systemic endocrine signals have been shown to regulate germline and intestinal SC activity in *Drosophila* (Hsu and Drummond-Barbosa 2009; Ables and Drummond-Barbosa 2010; Gancz et al. 2011; Zeng and Hou 2012). In mammals, endocrine signals can alter the tissue environment in the intestinal and hematopoietic SC niche (Calvi et al. 2003) and can promote hematopoietic SC proliferation (Jung et al. 2006; Adams et al. 2007; Pirihi et al. 2010) and self-renewal (Nakada et al. 2014). Additionally, endocrine hormones can regulate mammary gland development in part by directly altering mammary SC proliferation during puberty, pregnancy, and lactation (Asselin-Labat et al. 2010; Joshi et al. 2010) and epithelial

cell fate during development (Wysolmerski et al. 1998; Foley et al. 2001). Despite this accumulating evidence, hormonal control of SC activity in many tissues is not well understood.

The hair follicle (HF) contains a SC niche in the bulge region that is an excellent model to understand the mechanisms that control SC function. HF SCs are responsible for the cyclic growth (anagen) and regeneration of the follicle following its death (catagen) and rest (telogen) during the hair cycle (Cotsarelis et al. 1990; Blanpain et al. 2004; Tumber et al. 2004; Blanpain and Fuchs 2006). During native HF activation, bulge SCs migrate to the adjacent hair germ (HG) (Zhang et al. 2009), a compartment containing bulge SC progeny where cell proliferation initiates (Greco et al. 2009; Hsu et al. 2011). As cells in the HG proliferate to promote hair growth, bulge SCs divide to replenish the follicular SC pool (Zhang et al. 2009). To regulate their activity, follicle cells receive signals from surrounding dermal cells, including those in the dermal papilla (DP), a mesenchymal compartment associated with the HF (Greco et al. 2009; Rompolas et al. 2012; Chi et al. 2013); neurons (Brownell et al. 2011); smooth muscle cells (Fujiwara et al. 2011);

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and dermal adipocytes (Plikus et al. 2008; Festa et al. 2011). Interestingly, hormonal changes associated with pregnancy and lactation can alter skin tissue biology, notably through the stalling of HF growth in mice (Plikus et al. 2008). However, whether hormonal changes associated with pregnancy and lactation signal directly to follicular SCs or other components of the SC environment to regulate hair growth remains unknown.

Several hormones are altered during pregnancy and lactation (Augustine et al. 2008), yet the precise molecular mechanisms that modulate HF SC activity during these physiological states remain unclear. Prolactin (Prl) is highly elevated during pregnancy and lactation, and several lines of evidence have implicated a function for Prl signaling in the control of hair growth outside of pregnancy. In humans, patients with elevated serum Prl levels (or hyperprolactinemia) can experience hair loss (Orfanos and Hertel 1988; Foitzik et al. 2009; Lutz 2012). Additionally, Prl has been implicated in the regulation of epidermal appendage growth in several avian and mammalian species (Duncan and Goldman 1984; Pearson et al. 1996; Dawson and Sharp 1998; Nixon et al. 2002; Dawson et al. 2009). Prl receptors (Prlrs) are expressed within the HF in both mice and humans, and Prl can induce follicle death in cultured skin tissue from both species (Foitzik et al. 2003, 2006). Exogenous Prl delays hair growth in murine skin associated with inhibition of Prl secretion during the native hair cycle (Craven et al. 2006), and *Prlr* knockout mice exhibit precocious hair growth during native hair cycling and in skin grafts (Craven et al. 2001, 2006), indicating a functional role for Prl signaling in the skin. Despite these findings, whether Prl signaling acts directly on follicular SCs to regulate hair growth under native conditions or during pregnancy is not clear.

One key regulator of SC activity in the HF is the transcription factor nuclear factor of activated T cells c1 (*Nfatc1*), which is expressed in bulge SCs (Tumbar et al. 2004; Rhee et al. 2006; Horsley et al. 2008). *Nfatc1* activity is directly regulated by the phosphatase calcineurin (CN), which dephosphorylates *Nfatc1* to induce its nuclear translocation and transcriptional activity (Rao et al. 1997; Horsley and Pavlath 2002). Inactivation of *Nfatc1* through skin-specific genetic knockout or treatment with cyclosporine A (CSA), an inhibitor of CN (Mattila et al. 1990), results in precocious activation of HF growth in mice (Paus et al. 1989; Gafter-Gvili et al. 2003; Horsley et al. 2008). *Nfatc1* inhibition induces hair growth through precocious bulge cell proliferation (Horsley et al. 2008), and it is not clear whether *Nfatc1* inhibition accelerates bulge cell migration to induce HF growth, as in the native hair cycle (Zhang et al. 2009). Although CN and *Nfatc1* have been implicated in epidermal SC function via distinct molecular events (Horsley et al. 2008; Wu et al. 2010; Keyes et al. 2013), the global role of this signaling pathway in regulating skin SC quiescence has not been fully elucidated.

In this study, we identified genetic changes associated with SC activation following administration of CSA, a robust activator of follicular SCs. Gene expression pro-

filings revealed a novel molecular interplay between *Nfatc1* and *Prlr*, implicating Prl signaling as a regulator of SC activity in the hair. Using pharmacological and genetic experiments in mice, we revealed that Prl signaling and *Nfatc1* expression are required for the abrogation of hair growth during pregnancy. Furthermore, we demonstrated that direct administration of Prl promotes HF quiescence. Finally, we showed that Prl signaling activates Jak/Stat5 signaling in follicular SCs to maintain their dormancy. Our findings unveil a novel CN-*Nfatc1*-*Prlr*-Stat5 pathway that governs skin SC quiescence downstream from a systemic hormone.

Results

Inhibition of Nfatc1 with CSA induces unique gene expression changes in bulge cells

To identify novel mechanisms that control SC activation in the HF prior to anagen induction, we developed a strategy to characterize mRNA expression in bulge SCs shortly after their activation. To this end, we took advantage of the robust ability of CSA to stimulate hair growth in mice (Paus et al. 1989; Gafter-Gvili et al. 2003) and alter SC quiescence (Horsley et al. 2008) even when follicular SCs are refractory to tissue-level activation signals (Plikus et al. 2008). Mice treated with a 3-d course of CSA during the second telogen, a time when HFs are in an extended period of quiescence, lose nuclear *Nfatc1* expression within the bulge SC compartment (Fig. 1A). Furthermore, a 3-d treatment with CSA does not induce proliferation in either bulge or HG cells, as evidenced by the lack of 5-bromo-2'-deoxyuridine (BrdU) incorporation following a 48-h pulse (Fig. 1B). Continued treatment with CSA induces bulge cell proliferation and anagen induction following 5 d of drug treatment (Horsley et al. 2008). Therefore, a 3-d treatment with CSA induces molecular changes in bulge SCs prior to the onset of cell proliferation and follicular growth, allowing for the analysis of the transcriptional changes associated with earliest events of SC activation.

To identify genes that are regulated by *Nfatc1* and play a role in HF activation, we performed transcriptional profiling of bulge SCs following 3 d of CSA or vehicle (VEH) treatment. We focused our analysis on HF SCs because *Nfatc1* expression is restricted to the bulge compartment (Horsley et al. 2008). Characterization of gene expression in FACS-purified bulge cells based on their dual expression of integrin $\alpha 6$ and CD34 (Supplemental Fig. S1A; Blanpain et al. 2004) confirmed the up-regulation of several molecular markers in sorted bulge populations relative to basal cells of the interfollicular epidermis (IFE; integrin $\alpha 6^+$ /CD34 $^-$) (Supplemental Fig. S1B; Blanpain et al. 2004; Tumbar et al. 2004). Furthermore, the bulge cell populations did not display mRNAs associated with HG cells (Supplemental Fig. S1C; Greco et al. 2009). Microarray analysis of mRNAs expressed by bulge cells from VEH- and CSA-treated mice with a *P*-value cutoff of 0.05 revealed ~200 genes that were significantly altered >1.5-fold between CSA- and VEH-treated bulge cells (Fig. 1C; Supplemental Table S2).

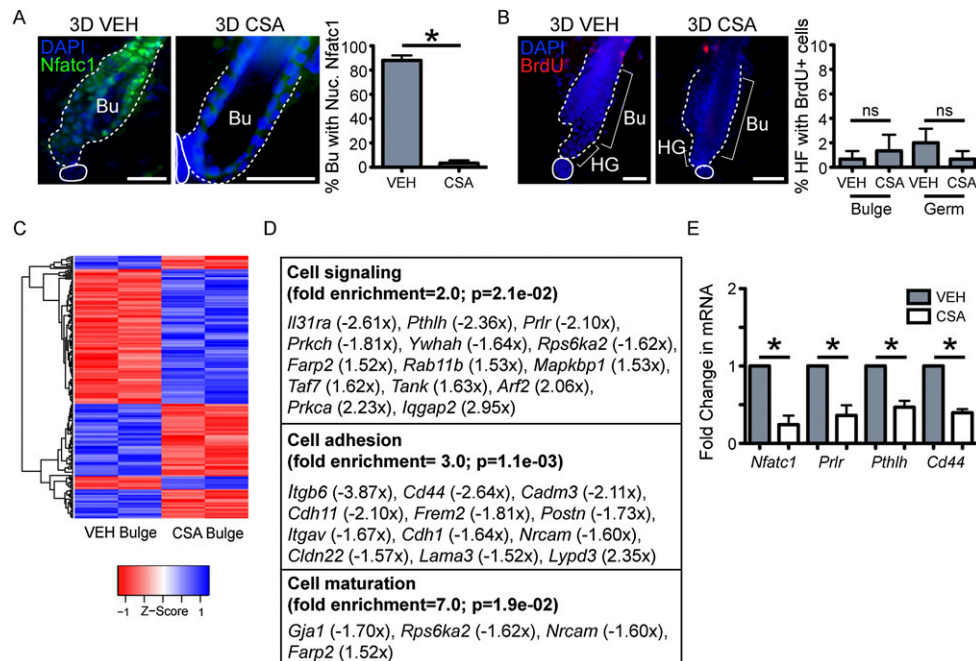


Figure 1. Pharmacological inhibition of *Nfatc1* alters bulge cell gene expression. (A,B) Immunostaining and quantification of *Nfatc1* (A) and BrdU (B) following 3 d of CSA or VEH treatment. Data are mean \pm SD ($n = 50$ – 100 HF; $N = 3$ mice). (Blue) DAPI. Bar, $30 \mu\text{m}$. The white dotted line denotes the epithelial–dermal barrier. The white solid line delineates the DP. (Bu) Bulge. (C) Heat map and hierarchical clustering analysis of microarray data from sorted CSA- and VEH-treated bulge cells. (D) Gene ontology analysis of biological processes enriched in CSA-treated bulge cells. (E) Real-time PCR validation of differentially regulated genes in CSA-treated bulge cells. Data are mean \pm SEM ($n = 3$). (*) $P < 0.05$; (ns) not significant.

To probe the functional characteristics of genes that change with CSA treatment in bulge SCs, we performed gene ontology analyses using the online Database for Annotation, Visualization, and Integrated Discovery (DAVID) tool (Huang et al. 2009a,b). In relation to the whole genome, we observed a significant enrichment in genes involved in cell signaling, cell adhesion, and cell maturation (Fig. 1D). We validated a subset of these differentially regulated genes by real-time PCR and confirmed their changes in expression level with CSA treatment (Fig. 1E). Additionally, we confirmed that *Nfatc1* expression was significantly reduced in CSA-treated bulge cells (Fig. 1E; Horsley et al. 2008), which is consistent with the finding that *Nfatc1* promotes its own gene expression (Pan et al. 2007). Moreover, these findings illustrate that pharmacological inhibition of CN/*Nfatc1* signaling in bulge SCs induces gene expression changes prior to proliferation of HG cells.

Nfatc1 promotes *Prlr* expression in bulge SCs

From our microarray analysis, we observed that *Prlr* mRNA expression decreases in bulge SCs after pharmacological inhibition of *Nfatc1* (Fig. 1D,E). We further validated that CSA treatment decreases *Prlr* protein levels in the skin (Fig. 2A). This finding was particularly interesting given that Prl signaling has previously been implicated in the regulation of hair growth (Craven et al. 2001, 2006; Foitzik et al. 2003, 2006). However, a role for

Prl signaling in the regulation of bulge SC activity has not been reported. Indeed, both *Nfatc1* and *Prlr* expression levels are elevated in bulge SCs compared with HG cells at early telogen (GSE15185) (Fig. 2B; Greco et al. 2009) as well as during mid- and late telogen (data not shown). Together, these data reveal that *Nfatc1* and *Prlr* levels are up-regulated in bulge SCs relative to HG cells during the native hair cycle, and pharmacological inhibition of *Nfatc1* can reduce *Prlr* expression in the bulge.

To determine whether genetic loss of *Nfatc1* leads to decreased *Prlr* expression in HF SCs, we analyzed *Prlr* expression in a *Keratin14-Cre;Nfatc1^{fl/fl}* (*Nfatc1* conditional knockout [cKO]) mouse model, which deletes *Nfatc1* conditionally in epithelial cells of the skin (Horsley et al. 2008). Real-time PCR analysis in FACS-purified bulge cells revealed a significant down-regulation of *Prlr* mRNA expression in *Nfatc1* cKO mice relative to littermate controls (Fig. 2C). To determine whether *Nfatc1* can promote *Prlr* expression in the skin, we expressed a constitutively active form of NFATc1 (caNFATc1) in primary mouse keratinocytes using retroviruses encoding a recombinant human NFATc1 containing mutations in the autoinhibitory domain that cause persistent nuclear localization (Neal and Clipstone 2003; Horsley et al. 2008). Real-time PCR revealed that *Prlr* mRNA levels are significantly elevated following expression of caNFATc1 in cultured keratinocytes (Fig. 2D).

We next performed in situ hybridization analysis to validate *Prlr* expression in the HF bulge. In wild-type

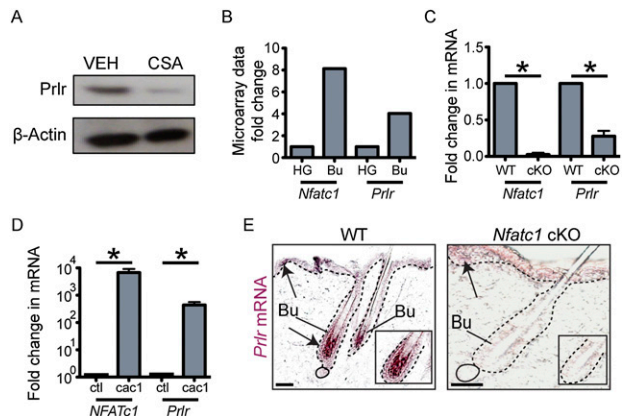


Figure 2. *Nfatc1* promotes *Prlr* expression in bulge cells. (A) Western analysis of *Prlr* expression from CSA- and VEH-treated mice. (B) Fold change in mRNA for *Nfatc1* (probe 1417621_at) and *Prlr* (probe 1425853_s_at) between bulge and HG cells at early telogen (postnatal day 42 [P42]) from published microarray data (GSE15185) (Greco et al. 2009). (C) Real-time PCR for *Nfatc1* and *Prlr* from *Nfatc1* cKO bulge cells and littermate controls. Data are mean \pm SEM ($N = 3$ mice). (D) Real-time PCR for *NFATc1* and *Prlr* from mouse keratinocytes infected with caNFATc1 (*cac1*) or empty vector (*ctl*). Data are mean \pm SEM ($n = 9$). (*) $P < 0.05$. (E) In situ hybridization of *Prlr* (maroon) in wild-type (WT) and *Nfatc1* cKO skin. The black dotted line denotes the epithelial-dermal barrier. The solid black line delineates the DP. Arrows mark the *Prlr* signal. *Insets* show the bulge signal. (Bu) Bulge. Bar, 30 μ m.

mice, hybridization with antisense probes revealed *Prlr* expression in the HF bulge and IFE (Fig. 2E). *Prlr* expression was also noted in the HG and DP, although to a lesser extent than within the bulge (Fig. 2E), which is consistent with previous reports (Rendl et al. 2005; Greco et al. 2009). In situ hybridization analysis in *Nfatc1* cKO mice revealed diminished *Prlr* expression within the bulge but persistent expression within the IFE (Fig. 2E). We confirmed the maintenance of *Prlr* mRNA expression in FACS-sorted IFE keratinocytes of *Nfatc1* cKO mice compared with littermate controls (1.06 ± 0.59 fold change) (Fig. 2E), which is consistent with the restriction of *Nfatc1* expression to bulge cells in the epidermis (Horsley et al. 2008). Thus, distinct mechanisms seem to regulate *Prlr* expression in different keratinocyte populations. While *Nfatc1*-independent mechanisms control *Prlr* expression in the IFE, *Nfatc1* promotes *Prlr* expression in HF SCs, which is likely due to a direct mechanism, since *Nfatc1* physically associates with the *Prlr* promoter in bulge cells (Keyes et al. 2013).

Prl signaling is activated in follicular SCs during pregnancy

To determine whether *Prl* signaling is activated in follicular SCs, we analyzed the activation of *Stat5*, which is the canonical downstream transducer of *Prl* in several tissues (Liu et al. 1995; Brown et al. 2010; Rouet et al. 2010). Surprisingly, we did not detect p-*Stat5* in bulge SCs during telogen or in other native hair cycle

stages (Fig. 3A; data not shown) despite the expression of *Prlr* in the bulge (Fig. 2E) and the hair cycling phenotype of *Prlr*-null mice during the native hair cycle (Craven et al. 2001, 2006). Outside the epithelium, p-*Stat5* was detected in the DP throughout the native hair cycle (Fig. 3A).

Since serum *Prl* levels are elevated during pregnancy and lactation (Fig. 3D; Augustine et al. 2008), we next analyzed p-*Stat5* activity in the skin of pregnant and lactating mice. We found high p-*Stat5* activity in bulge SCs during pregnancy and lactation and little to no detectable p-*Stat5* in the HG (Fig. 3B,C). These data indicate that *Stat5* is not detected in bulge SCs under basal conditions but rather becomes activated during pregnancy and lactation when systemic *Prl* levels are elevated. In contrast, *Stat3* was not detected during pregnancy or lactation within the HF, indicating that *Stat* proteins are not activated generally during pregnancy/lactation (Supplemental Fig. S2A).

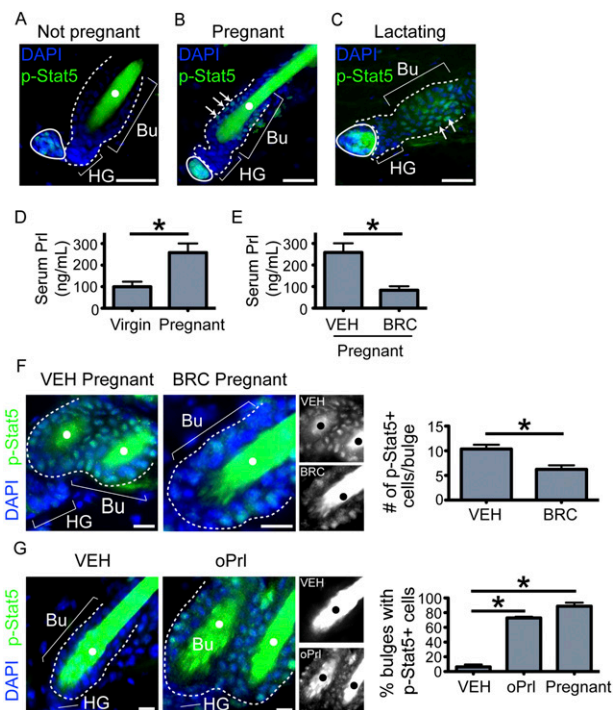


Figure 3. Elevated *Prl* signaling activates *Stat5* in bulge SCs. (A–C) Immunostaining of p-*Stat5* in the HF. (A) Nonpregnant mice. (B) Pregnant mice. (C) Lactating mice. In A–C, the dotted line denotes the epithelial-dermal barrier, and the solid line delineates the DP. Bar, 30 μ m. (D,E) Serum *Prl* levels in virgin and pregnant wild-type (WT) mice (D) and pregnant mice (E) following 3-d BRC or VEH treatment. Data are mean \pm SEM ($N = 9$ mice). (F,G) Immunostaining and quantification of p-*Stat5* in pregnant mice following a 3-d treatment with BRC or VEH (F) and nonpregnant mice injected with o*Prl* or VEH for 3 d (G). Data are mean \pm SEM ($n = 50$ – 100 HF; $N = 4$ mice). Bar, 10 μ m. Close-ups of p-*Stat5* staining in the bulge are shown in black and white. In F and G, the dotted line denotes the bulge. (Blue) DAPI. White and black dots indicate hair shaft autofluorescence. (Bu) Bulge. (*) $P < 0.05$.

Since multiple hormones change during pregnancy and lactation, Stat5 activation in bulge SCs could be due to Prl or another molecular factor. To test whether p-Stat5 activity is downstream from Prl signaling, we reduced systemic Prl levels with bromocriptine (BRC), a dopamine agonist that depletes secretion of pituitary Prl (Craven et al. 2006; Larsen and Grattan 2010). We confirmed that treatment of mice with a low dose of BRC during pregnancy diminished serum Prl levels to nonpregnant levels while still permitting successful lactation (Fig. 3D,E). Analysis of p-Stat5 activity in pregnant BRC-treated mice revealed a significant decrease, but not a complete abrogation, of p-Stat5 activation in bulge SCs relative to VEH-treated controls (Fig. 3F). Furthermore, local injection of ovine Prl (oPrI) in the skin of both male and female mice during the quiescent stage of the hair cycle induced p-Stat5 activation in the HF bulge, which is similar to the increased Stat5 activation seen during pregnancy (Fig. 3G). Consistent with the lack of Stat3 activation during pregnancy and lactation, Stat3 was not induced by oPrI injections (Supplemental Fig. S2B). Taken together, these data indicate that Prl signaling directly activates p-Stat5 in HF SCs.

Based on these data, we hypothesized that the abrogation of hair growth during pregnancy (Plikus et al. 2008) results from elevated Prl signaling within follicular SCs. To test this hypothesis, hair regrowth was analyzed in mice treated with BRC during lactation. We administered a low dose of BRC by subcutaneous injection to reduce systemic Prl levels at a time when it is continually elevated without terminating lactation (Augustine et al. 2008). Females in the extended telogen phase of the hair cycle were shaved at the initiation of pregnancy, and hair regrowth was monitored externally throughout pregnancy and lactation and after weaning (Fig. 4A). While VEH-treated lactating mice remained in telogen until after weaning, BRC-treated mice displayed precocious hair regrowth during lactation (Fig. 4B; Supplemental Fig. S3A). Histological analysis of skin sections from lactating mice confirmed the persistence of telogen in VEH-treated mice and the induction of anagen in mice treated with BRC (Fig. 4C). Furthermore, we confirmed the persistence of telogen throughout the dorsum in pregnant and VEH-treated lactating mice (Supplemental Fig. S3B,C) and the consistent induction into anagen throughout the dorsum of BRC-treated lactating mice (Supplemental Fig. S3D).

To rule out any secondary effects from our BRC experiments and determine whether Prl is sufficient to inhibit hair regrowth during pregnancy and lactation, we performed local injections of purified oPrI into the back skin of telogen stage nonpregnant mice. At postnatal day 42 (P42), wild-type mice were shaved and injected daily in the mid-back skin with oPrI or VEH. External analysis of hair regrowth revealed that the majority of mice injected with oPrI did not regrow hair in the injection site despite regrowth in the uninjected surrounding skin and in VEH-injected mice. Furthermore, these bald areas in mice injected with oPrI started to regrow 1 wk after the injections were stopped (Fig. 4D). These data are consistent with previous findings that

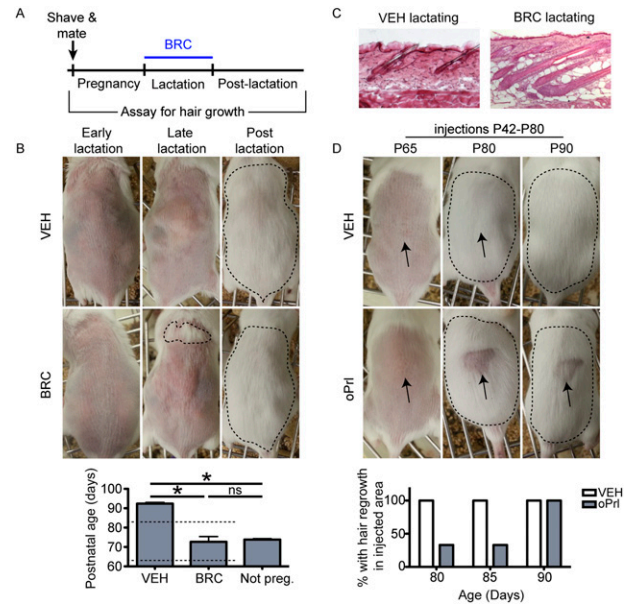


Figure 4. Prl signaling delays hair regrowth during pregnancy. (A) Schematic for hair regrowth assay during pregnancy/lactation. (B) External hair regrowth analysis of VEH- or BRC-treated animals during lactation. Quantification of hair regrowth in VEH/BRC-treated lactating mice and age-matched nonpregnant controls. The *bottom* dotted line denotes the end of pregnancy. The *top* dotted line denotes the end of lactation. Data are mean \pm SEM ($N = 3$ mice). (C) H&E-stained skin sections from VEH- or BRC-treated mice during lactation. (D) External hair regrowth analysis of mice treated with VEH or oPrI in the middle of the back skin during native telogen (P65–P80). Quantification of hair emergence in the injected area at the designated postnatal ages ($N = 3$ mice). Black dotted lines mark sections of hair regrowth. Arrows indicate injected regions. (*) $P < 0.05$.

inhibition of Prl in adolescent mice advances hair regrowth (Craven et al. 2006) and extend these findings to implicate Prl as a major hormone responsible for stalling HF growth in adult mice during pregnancy and lactation.

CN/Nfatc1 signaling is required for SC quiescence during pregnancy

Since *Nfatc1* regulates *PrIr* expression, we evaluated whether pharmacological and genetic inhibition of *Nfatc1* results in decreased Prl signaling during pregnancy. *Nfatc1* expression is maintained in HF bulge cells during pregnancy (Fig. 5A). However, activation of Stat5 was significantly reduced within the bulge compartment of CSA-treated mice during pregnancy (Fig. 5B), which is consistent with the down-regulation of *PrIr* with CSA treatment. We further substantiated these findings in our *Nfatc1* cKO mice during pregnancy, which displayed reduced Stat5 activation (Fig. 5C). Additionally, dermal injections of oPrI induced a slight increase in detectable p-Stat5 activity in *Nfatc1* cKO bulge cells, but this effect was more than fourfold less than the activation of Stat5 in wild-type mice injected with oPrI (Fig. 5D). Collectively, these data indicate that impaired CN/*Nfatc1* signaling in

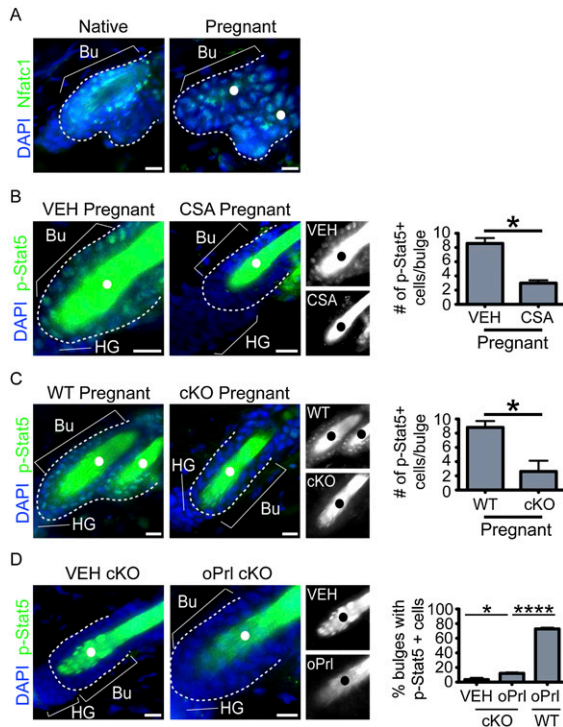


Figure 5. Inhibition of *Nfatc1* decreases Prl signaling in bulge SCs. (A) Immunostaining of *Nfatc1* in native and pregnant HF. (B) Immunostaining and quantification of p-Stat5 in pregnant mice following topical treatment with VEH or CSA. (C) Immunostaining and quantification of p-Stat5 in bulge SCs of pregnant wild-type (WT) and *Nfatc1* cKO mice (E3). (D) Immunostaining and quantification of p-Stat5 in *Nfatc1* cKO mice injected for 3 d with VEH or oPrI. HF are in telogen. (B–D) Close-ups of p-Stat5 staining in the bulge shown in black and white. Data are mean \pm SEM ($n = 50$ –100 HF; $N = 3$ mice). (Blue) DAPI. Bar, 10 μ m. The white dotted line denotes the bulge. White and black dots indicate hair shaft autofluorescence. (Bu) Bulge. (*) $P < 0.05$; (****) $P < 0.0001$.

the epithelium results in diminished Prl signaling in bulge SCs.

To determine whether mice lacking CN activity or epithelial *Nfatc1* expression display precocious hair growth during pregnancy, we performed hair regrowth assays in shaved mice after mating. While the HF of VEH-treated mice remained in the resting stage, CSA-treated mice regrew hair during pregnancy, as indicated by the presence of anagen follicles (Fig. 6A) and enhanced bulge and HG cell proliferation (Fig. 6B). Similarly, pregnant *Nfatc1* cKO mice precociously regrew their hair coat during lactation in contrast to wild-type mice, which regrew hair after weaning (Fig. 6C). Furthermore, *Nfatc1* cKO mice injected with oPrI outside of pregnancy failed to exhibit delayed hair regrowth relative to VEH-treated cKO mice (Fig. 6D), emphasizing the requirement of *Nfatc1* and *Prlr* expression in follicular SCs to stall hair growth. These data indicate that CN/*Nfatc1* activity in epithelial cells is required for Prl signaling to inhibit hair growth during pregnancy and lactation.

Activated *Jak/Stat5* signaling regulates hair growth during pregnancy

Since Prl and CN/*Nfatc1* signaling promotes Stat5 activation in bulge SCs during pregnancy, we assessed the function of *Jak/Stat5* activation on hair growth during pregnancy. We inhibited *Jak* signaling in the epidermis using topical application of the pan-*Jak* inhibitor pyr6 during pregnancy. Immunofluorescence staining and quantification of p-Stat5⁺ cells confirmed the reduction of p-Stat5 in the HF bulge of pyr6-treated mice relative to VEH-treated controls, while p-Stat5 localization in the DP was maintained (Fig. 7A). To determine whether the inhibition of *Jak/Stat* signaling in the bulge controlled SC activation, we assayed proliferation in mice treated with BrdU and either VEH or pyr6. Consistent with the absence of activated Stat proteins in the bulge during the native hair cycle of young, adult mice (Fig. 3A; Supplemental Fig. S2; Doles et al. 2012), pyr6 treatment did not induce proliferation in the HF in the native state (Fig. 7B). However, treatment of pregnant mice with pyr6 induced precocious proliferation of follicular cells, indicating that SC activation was induced (Fig. 7C). Taken together, these data indicate that *Jak/Stat* signaling in HF SCs promotes follicular quiescence during pregnancy (Fig. 7D).

Discussion

Endocrine control of skin SC function

This study provides several lines of evidence that extend the role of the endocrine hormone Prl as a major regulator of SC activity in the skin during pregnancy and lactation in mice. We provided *in vivo* evidence that endocrine secretion of Prl enforces HF SC quiescence to prevent hair growth. We elucidated an upstream regulatory pathway via CN/*Nfatc1* signaling that promotes *Prlr* expression/signaling and regulates follicular SC quiescence during pregnancy/lactation. Finally, we demonstrated that Prl activates Stat5 in follicular SCs during pregnancy/lactation, and *Jak/Stat5* signaling promotes HF SC quiescence during pregnancy. Moreover, these findings reveal an important role for endocrine signals in controlling SC activity in the skin.

While previous work has implicated Prl signaling in the control of hair growth during the native hair cycle (Craven et al. 2001, 2006; Foitzik et al. 2003), several questions remain regarding the global mechanisms of Prl signaling in the skin. Although *Prlr* is up-regulated in bulge cells relative to HG cells (Fig. 2B), it is not clear whether the down-regulation of *Prlr* in the HG is required for controlling hair cycling in either native or pregnant conditions. Additionally, neither the downstream signaling pathways nor the cellular targets of Prl in the native hair cycle have been elucidated. Prl has been reported to signal through Akt (Acosta et al. 2003; Chakravarti et al. 2005; Chen et al. 2012) and Map kinase (Das and Vonderhaar 1995), both of which are activated in bulge cells during the native hair cycle (Affara et al. 2006; Festa et al. 2011) and may act downstream from Prl. Adding

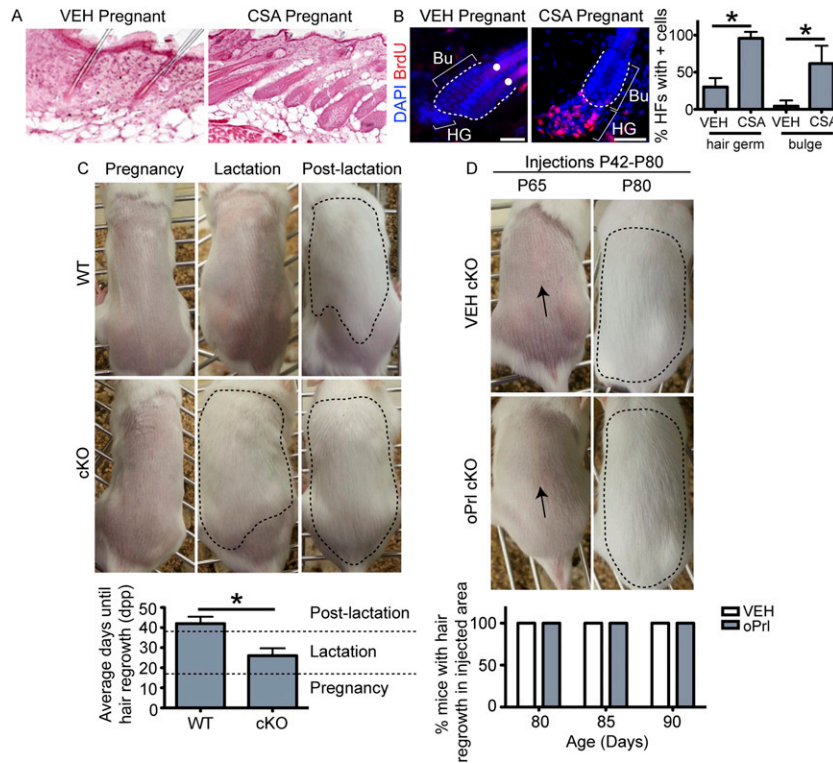


Figure 6. *Nfatc1* signaling delays hair regrowth during pregnancy. (A) H&E staining of skin sections from VEH- and CSA-treated mice during pregnancy. (B) BrdU immunostaining and quantification in VEH- and CSA-treated mice during pregnancy. Data are mean \pm SEM ($n = 50\text{--}100$ HF; $N = 3$ mice). (C) External hair growth analysis in wild-type (WT) and *Nfatc1* cKO mice throughout pregnancy and lactation and after lactation. Quantification of hair regrowth in wild-type and *Nfatc1* cKO mice relative to days post-plug (dpp). Data are mean \pm SEM ($N = 5$ mice). (D) External hair regrowth analysis in VEH- and oPrl-injected *Nfatc1* cKO mice. Arrows represent injected area. Quantification of hair regrowth relative to postnatal age ($N = 3$ mice). White dots indicate hair shaft autofluorescence. (Blue) DAPI. Bar, 30 μ m. White dotted line denotes the bulge. Black dotted lines represent regions of hair regrowth. Arrows indicate injected areas. (Bu) Bulge. (*) $P < 0.05$.

further complexity to the matter are reports of local Prl expression in human skin (Foitzik et al. 2006) that suggest that Prl can also signal in an autocrine or paracrine manner. Ultimately, elucidating the global molecular mechanisms of Prl signaling in the skin in both native and pregnant conditions may have relevance for other epithelial tissues that respond to this pleiotrophic hormone.

Prl: a conserved and global regulator of tissue regeneration

Our study highlights the ability of systemic hormones to control mammalian SC function during pregnancy. Similar paradigms have been shown during pregnancy in the hematopoietic system, in which estrogen promotes hematopoietic SC self-renewal (Nakada et al. 2014), and the forebrain, in which Prl induces neural SC proliferation (Shingo et al. 2003). Other known effects of Prl are thought to derive from extrapituitary Prl production within the local tissue environment. In the prostate, local Prl induces the proliferation of basal prostate cancer SCs (Rouet et al. 2010). Autocrine Prl signaling can also control mammary gland differentiation into lactogenic epithelial cells (Chen et al. 2012). In the skin, Prl addition to culture human HF can induce catagen, and *Prlr*-null mice display altered hair cycling in the absence of pregnancy (Craven et al. 2001, 2006; Foitzik et al. 2006). Given the broad expression of *Prlr* in different cell types (Bole-Feysot et al. 1998) and the numerous tissue changes that occur during pregnancy and lactation, tissue-specific disruption of Prl signaling will help to elucidate the likely broad role of systemic Prl in SC regulation.

Epidermal tissue regeneration is controlled by Prl in numerous species. Prl can control skin molting in newts (Dent et al. 1973) and feather molting in avian species (Dawson and Sharp 1998; Dawson et al. 2009; Crossin et al. 2012). Seasonal Prl in combination with other hormones is thought to modulate annual changes in hair growth of several species, including sheep, deer, and voles (Duncan and Goldman 1984; Curlewis et al. 1988; Smale et al. 1990; Dicks et al. 1996; Nixon et al. 2002; Randall 2007). Our data are consistent with these roles in epidermal renewal and suggest that Prl may have evolved as a general regulator of regeneration in the epidermis and its appendages.

In addition to Prl, estrogen has also been deemed to be a potent hormonal regulator of hair growth in multiple species (Ohnemus et al. 2006). Pharmacological experiments in mice revealed that topical estrogen treatment arrests HF in telogen, while treatment with an estrogen receptor (ER) antagonist induces anagen (Oh and Smart 1996). Both ER α and ER β are highly expressed in the DP during telogen (Oh and Smart 1996; Ohnemus et al. 2006), suggesting that estrogen may inhibit anagen via the follicular mesenchyme. Furthermore, ER β is highly expressed throughout the HF, including the bulge region (Ohnemus et al. 2006). Like Prl, estrogen levels increase during pregnancy (Augustine et al. 2008), which may contribute to the alterations in HF growth during pregnancy. Estrogen has been suggested to induce *Prl* and *Prlr* expression in cultured human skin (Langan et al. 2010), but further work is needed to elucidate whether Prl and estrogen act synergistically to modulate murine hair growth dynamics during the native or pregnant hair cycle.

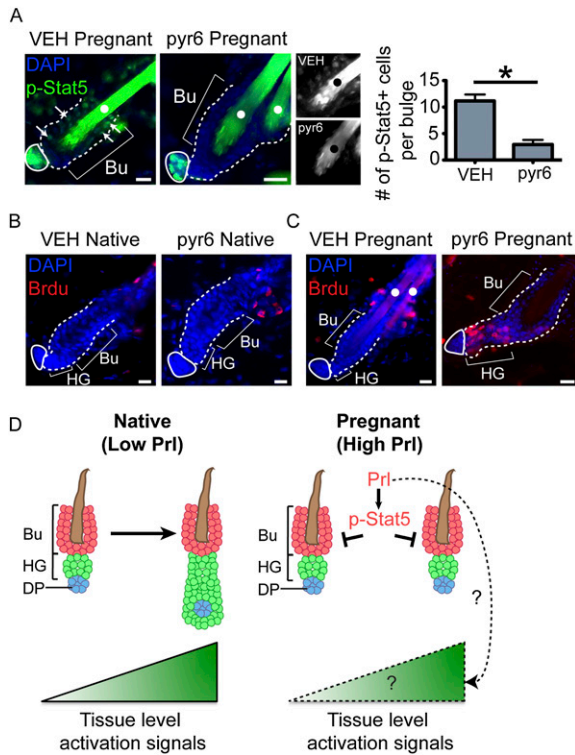


Figure 7. Stat5 signaling delays hair regrowth during pregnancy. (A) Immunostaining and quantification of p-Stat5 in pregnant mice following 7-d treatment with pyr6 or VEH. Data are mean \pm SEM ($n = 50\text{--}100$ HF; $N = 3$ mice). (B,C) BrdU immunostaining in mice following a 2-d BrdU pulse and 7-d treatment with pyr6 or VEH in the native (B) or pregnant (C) state. (D) Model depicting the molecular pathway regulating hair growth during pregnancy. Dashed lines and question marks denote potential effects. White and black dots indicate hair shaft autofluorescence. (Blue) DAPI. Bar, 10 μm . The white dotted line denotes the epithelial–dermal barrier. The white solid line delineates the DP. (Bu) Bulge. (*) $P < 0.05$.

Endocrine hormones trump local signals

Our data suggest an interesting possibility that Prl production during pregnancy may have the ability to override local signals within a tissue to control SC behavior and reset the hair cycle (Fig. 7D). The cyclic regeneration of the HF in mice occurs in a reproducible wave-like pattern in the native state, and this effect is stalled during pregnancy (Plikus et al. 2008). We found that Prl stalls the progression of hair growth in mouse back skin, and suppression of systemic Prl signaling with BRC treatment induces anagen entry throughout the back skin during lactation (Supplemental Fig. S3D). Interestingly, we first observed external hair growth at the nape of the neck (Fig. 4B; Supplemental Fig. S3A), suggesting that when Prl signaling is reduced, the regular wave-like pattern of hair regrowth is reinstalled.

Regulation of the HF growth wave is achieved by local signals within the skin's dermis, providing periods of competence and refraction for HF SC activation (Plikus et al. 2008). While dermal production of Fgfs, Bmps, and

Pdgfs have been implicated in activating the HF cycle (Blanpain and Fuchs 2006; Plikus et al. 2008; Greco et al. 2009; Festa et al. 2011), the identity of these signals and the temporal mechanisms that control their expression within the local skin environment have not been fully identified. Our data suggest that Prl-induced Stat5 signaling induces bulge SCs to be refractory to these local signals, resulting in a resetting of the hair cycle. This dominance occurs in part via the epithelium, since deletion of *Nfatc1* in the epithelium can promote SC activation during pregnancy. Furthermore, CSA and pyr6 treatment maintained activation of Stat5 in the DP, suggesting that inhibition of epithelial Stat5 is sufficient to induce follicular activation during pregnancy. It remains unclear whether tissue-level activation signals change during pregnancy relative to the native state (Fig. 7D). Furthermore, it is not clear whether Prl regulates tissue-level activation signals during pregnancy (Fig. 7D). While our data indicate that Prl activates bulge cell intrinsic signals to control HF activation, future studies should investigate how systemic factors regulate hair growth during pregnancy via the local microenvironment.

Jak–Stat signaling: a global regulator of SC function

Our data illustrate the functional role of Jak–Stat5 signaling downstream from systemic Prl in controlling SC quiescence in the skin. HF SC activation requires Stat3 during the native hair cycle (Sano et al. 1999), and Stat3 represses follicular SC activation in aged skin (Doles et al. 2012). In the hematopoietic system, Stat5 functions to maintain hematopoietic SC quiescence (Wang et al. 2009) and is required for the maintenance and expansion of SCs in both normal and leukemic hematopoiesis (Li et al. 2007; Liu et al. 2008). Loss of Stat5 also diminishes luminal progenitor cells in the mammary gland (Yamaji et al. 2009). In germline SCs of the *Drosophila* testis, local niche cells activate Jak–Stat signaling to maintain SC self-renewal (Kiger et al. 2001; Tulina and Matunis 2001). *Drosophila* intestinal SCs also require Jak–Stat signaling to activate proliferation (Jiang et al. 2009; Beebe et al. 2010). Our finding that Stat5 signaling contributes to SC quiescence in the HF during pregnancy (Fig. 6C) uncovers a novel role for Stat5 in coupling hormonal signaling to tissue regeneration and implicates Stat proteins as central regulators of SC function during tissue regeneration.

Prl and hair cycling in humans

Our results may have implications for the mechanisms by which hair growth is altered in humans during pregnancy. In rodents, serum Prl levels oscillate during the first half of pregnancy and remain low during the second half of pregnancy until spiking prior to birth. This effect is distinct in humans, in which Prl levels steadily increase throughout pregnancy. Despite these differences, Prl secretion is maintained at continually elevated levels during lactation in both rodents and humans (Ben-Jonathan et al. 2008). Interestingly, the patterns of hair growth also differ between rodents and humans during pregnancy and lactation. While hair growth is stalled in

mice during pregnancy and lactation (Figs. 4B, 6C; Plikus et al. 2008), the percentage of anagen follicles increases during human pregnancy and significantly drops following delivery (Lynfield 1960). Despite the differences in Prl secretion and hair growth between rodents and humans during pregnancy and lactation, maximal serum Prl levels correlate with HF quiescence in both species.

Increased hair shedding (or telogen effluvium) is thought to occur due to post-partum hormonal changes (Lynfield 1960; Schiff and Kern 1963; Gizlenti and Ekmekci 2013) or stress (Kligman 1961). Our findings that Prl promotes HF quiescence by directly regulating bulge SC activity support a previously hypothesized role for Prl during post-partum hair shedding (Randall 2007). Telogen effluvium may result from the transition of a large population of HFs into telogen and/or the premature release of the club hair during exogen (Milner et al. 2002). The latter scenario involves inner bulge cells, which anchor the club hair to the HF, and ablation of this cell layer results in precocious hair shedding (Hsu et al. 2011). Interestingly, we observed *Nfatc1* (Fig. 1A) and *Prlr* (Fig. 2E) expression and Stat5 activation (Fig. 3B,C,F) in both the inner and outer bulge compartments during pregnancy/lactation. Whether deletion of *Nfatc1* or inhibition of Prl signaling influences hair shedding is an important question for future studies.

Outside of pregnancy, patients with hyperprolactinemia exhibit increased serum Prl levels and also report increased hair loss (Orfanos and Hertel 1988; Foitzik et al. 2009; Lutz 2012). While Prl may promote catagen to increase hair shedding during both pregnancy and hyperprolactinemia (Foitzik et al. 2003, 2006), our finding that Prl stalls HF SC activation may serve as an additional explanation for Prl-induced hair loss in humans. Furthermore, by identifying CN/*Nfatc1* and Stat5 signaling as essential components of the pathway that regulates and responds to *Prlr* in follicular SCs, respectively, our data increase our understanding of the complex hormonal mechanisms that regulate mammalian hair growth.

Materials and methods

Animal use

All animals were housed and handled according to the institutional guidelines of Yale University. CD-1 mice were obtained from Taconic Farms. *K14-Cre/+; Nfatc1^{fl/fl}* mice have been described previously (Horsley et al. 2008). *K14-Cre*-negative; *Nfatc1^{fl/fl}* age-matched littermate animals were used as wild-type controls. For BrdU experiments, mice were injected intraperitoneally with 50 mg/kg BrdU (Sigma-Aldrich) daily for 2 d prior to being sacrificed.

CSA treatments

For microarray experiments, 8-wk-old telogen stage CD-1 male mice were injected intraperitoneally with 100 mg/kg CSA solution (Bedford Laboratories) or a VEH control for 3 d prior to being sacrificed. For CSA treatments during pregnancy, 6-wk CD-1 female mice were shaved on the dorsum and mated to a male. Mice were treated topically with 100 μ L of CSA in 100%

ethanol or a VEH control daily from day 1 to 7 of pregnancy, and skin was harvested at day 8.

BRC injections

For BRC experiments, 50 μ g of 2-bromo- α -ergocryptine methanesulfonate salt (Sigma-Aldrich) was dissolved in a minimum amount of ethanol and brought up to 100 μ L with sesame oil for each injection (Larsen and Grattan 2010). Starting the day after birth, females were injected subcutaneously with BRC or a VEH control. Injections were performed daily until pups were weaned from the mother after 21 d.

oPrl injections

For oPrl experiments, CD-1 female mice were shaved on the dorsum at P42. Intradermal injections of 50 μ g of oPrl (Sigma-Aldrich) in PBS or a VEH control were performed daily. Injections were performed in the middle of the back and continued until hair regrowth was observed.

pyr6 treatments

For pyr6 (JAK inhibitor I, EMD Millipore) experiments, CD-1 female mice were shaved on the dorsum at P42. A 1 mM solution of pyr6 in 20% DMSO/80% acetone was applied topically to shaved dorsal skin daily. Skin was harvested 24 h after final treatment.

Hair regrowth analysis

For experiments during pregnancy, female mice were shaved on their dorsum at P42 and mated to a male. The presence of a vaginal plug indicated an embryonic day 0.5 (E0.5) time point. Hair growth was monitored daily by visual examination and recorded in relation to the date of plug. For experiments outside of pregnancy, mice were shaved on their dorsum and injected daily with PBS or oPrl starting at P42. Hair regrowth was monitored daily by visual examination and recorded in relation to the postnatal age. For all experiments, an area of hair regrowth $\geq 5\%$ of the shaved area was considered positive. ImageJ was used for calculations of shaved and regrown areas.

Microarray analysis

Total RNAs from FACS-purified bulge and basal cells were quantified (Nanodrop), and quality control was assessed using a Bioanalyzer (Agilent). One round of amplification/labeling was performed on 20 ng of RNA to obtain biotinylated single-stranded cDNA (sscDNA) (Ovation Exon Module, Ovation Pico WTA system, and Encore Biotin Module, Nugen). Biotinylated sscDNA (2.5 μ g) was hybridized for 17 h at 45°C to Mouse Gene 1.0 ST array (Affymetrix). Processed chips were read using a Gene Chip Scanner 3000 7G (Affymetrix). FACS purifications and microarray hybridizations were performed in duplicate for 3-d CSA and 3-d VEH bulge and basal cells.

Raw data were normalized using Robust Multichip Average (RMA) (Affymetrix), and GeneSpring GX 11.0 (Agilent) was used to identify differentially regulated genes between CSA- and VEH-treated bulge cells containing probe sets that are ≥ 1.5 -fold increased or ≤ 1.5 -fold decreased with a *P*-value of ≤ 0.05 (Supplemental Table S2). While duplicate arrays were performed for each RNA sample, we validated individual differential abundance data obtained from independent, duplicate samples by quantitative PCR and immunoblotting in lieu of applying corrections for multiple testing, since the microarray experi-

ment was intended to be investigative rather than a definitive analysis of CN/Nfatc1 signaling in the skin.

A heat map was generated using R. For functional gene ontology analyses, gene lists were uploaded to the DAVID tool (Huang et al. 2009a,b). Microarray data have been deposited in the Gene Expression Omnibus database under accession number GSE51194.

Statistical analysis

To determine significance between groups of two, comparisons were made using Student's *t*-tests with GraphPad Prism version for Macintosh (GraphPad Software). For all statistical tests, the 0.05 level of confidence was accepted for statistical significance.

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