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Transcription Factor CTIP2 maintains hair follicle stem cell pool and contributes to altered expression of LHX2 and NFATC1

Shreya Bhattacharya[1,2], **Heather Wheeler**[3], **Mark Leid**[1,2,5], **Gitali Ganguli-Indra**[1,2,*], and **Arup K. Indra**[1,2,4,6,*]

[1]Department of Pharmaceutical Sciences, College of Pharmacy, Oregon State University, Corvallis, Oregon, 97331

[2]Molecular Cell Biology Program, Oregon State University, Corvallis, Oregon, 97331

[3]Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon, 97331

[4]Environmental Health Science Center, Oregon State University, Corvallis, Oregon, 97331

[5]Department of Integrative Biosciences, Oregon Health & Science University, Portland, Oregon, 97239, USA

[6]Department of Dermatology, Oregon Health & Science University, Portland, Oregon, 97239, USA

Abstract

Transcription factor CTIP2 (COUP-TF-interacting protein 2), also known as BCL11B, is expressed in hair follicles of embryonic and adult skin. *Ctip2-null* mice exhibit reduced hair follicle density during embryonic development. In contrast, conditional inactivation of *Ctip2* in epidermis (*Ctip2*$^{ep-/-}$ mice) leads to a shorter telogen and premature entry into anagen during the second phase of hair cycling without a detectable change in the number of hair follicles. Keratinocytes of the bulge stem cells niche of *Ctip2*$^{ep-/-}$ mice proliferate more and undergo reduced apoptosis than the corresponding cells of wild-type mice. However, premature activation of follicular stem cells in mice lacking CTIP2 leads to the exhaustion of this stem cell compartment in comparison to *Ctip2*$^{L2/L2}$ mice, which retained quiescent follicle stem cells. CTIP2 modulates expression of genes encoding EGFR and NOTCH1 during formation of hair follicles, and those encoding NFATC1 and LHX2 during normal hair cycling in adult skin. The expression of most of these genes is disrupted in mice lacking CTIP2 and these alterations may underlie the phenotype of *Ctip2-null* and *Ctip2*$^{ep-/-}$ mice. CTIP2 appears to serve as a transcriptional organizer that integrates input from multiple signaling cues during hair follicle morphogenesis and hair cycling.

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*Correspondence: Arup K. Indra, Telephone: 541 737 5775; Telefax: 541 737 3999; arup.indra@oregonstate.edu; Gitali Ganguli-Indra, Telephone: 541 740 2332; Telefax: 541 737 3999; indrag@onid.orst.edu.

CONFLICT OF INTEREST
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Introduction

The hair follicle (HF) is a complex appendage of epidermis and its formation is regulated by epithelial-mesenchymal interaction (Botchkarev and Paus, 2003; Millar, 2002). Cross-talk between epithelium and mesenchyme initiates thickening of the epidermis to form the hair placode (Botchkarev and Paus, 2003; Millar, 2002), which gives rise to hair germ and then the bulbous hair peg(Paus *et al*., 1999). A mature HF consisting of the hair shaft, root sheaths and dermal papilla arise from the bulbous hair peg (Botchkarev and Paus, 2003; Millar, 2002; Paus *et al*., 1999). The mature HF undergoes cyclic changes of catagen (regression phase), telogen (quiescent phase), and anagen (active growth phase) (Muller Rover *et al*., 2001). Hair cycling homeostasis is maintained by a subset of multipotent, HF stem cells (HFSCs) residing in the bulge region of the HF (Tiede *et al*., 2007; Waters *et al*., 2007). HFSCs also have an important role in epidermal regeneration after injury (Ito *et al*., 2005).

Cell signaling pathways regulate every aspect of HF development and function. The WNT/β catenin, sonic hedgehog (SHH), bone morphogenetic protein (BMP), NOTCH1, and epidermal growth factor receptor (EGFR) pathways regulate HF formation and cycling (Andl *et al*., 2002; Botchkarev *et al*., 1999; Chiang *et al*., 1999; Crowe *et al*., 1998; Crowe and Niswander, 1998; Doma *et al*., 2013; Hansen *et al*., 1997; Huelsken *et al*., 2001; Lee and Tumbar, 2012; Lin *et al*., 2011; Lyons *et al*., 1990; Murillas *et al*., 1995; Nakamura *et al*., 2013; St Jacques *et al*., 1998; Uyttendaele *et al*., 2004; Vauclair *et al*., 2005).

Bulge SC activation and homeostasis is regulated by the β catenin/TCF/LEF1 pathway and downstream proteins (DasGupta and Fuchs, 1999; Merrill *et al*., 2001; Nguyen *et al*., 2006). LIM Homeobox 2 (LHX2) is an important factor in follicular organogenesis and cycling (Rhee *et al*., 2006; Tornqvist *et al*., 2010), whereas NFATC1 and SOX9 regulate HFSCs during hair cycling in the adult (Gafter-Gvili *et al*., 2003; Horsley *et al*., 2008; Vidal *et al*., 2005).

The transcriptional regulator CTIP2 is a C_2H_2 zinc finger protein (Avram *et al*., 2000; Avram *et al*., 2002) that is highly expressed in neonatal and adult skin and plays a significant role in murine skin morphogenesis and homeostasis (Golonzhka *et al*., 2007). Germline deletion of *Ctip2* in mice leads to impaired epidermal proliferation, differentiation, and delayed EPB formation (Golonzhka *et al*., 2009). Epidermal-specific ablation of *Ctip2* triggers atopic dermatitis-like skin inflammation with concurrent infiltration of T lymphocytes, mast cells and eosinophils in adult mice (Wang *et al*., 2012).

CTIP2 is expressed in both developing and adult HFs (Golonzhka *et al*., 2007). It is co-expressed with SC markers, such as K15 and CD34, in HF bulge region (Golonzhka *et al*., 2007). Ablation of *Ctip2* in the epidermis delays wound healing and induces abnormal expression of HFSC markers (Liang *et al*., 2012). However, CTIP2 expression during postnatal follicle establishment and cycling has not been studied extensively and it is also unknown whether CTIP2 controls HFSCs during embryonic follicle development and/or hair cycling. We observed that CTIP2 has a distinct pattern of expression during follicular maturation and cycling. Moreover, CTIP2 regulates HF formation and control NOTCH1 and

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EGFR signaling pathways during embryonic HF development. In this study, we have also demonstrated that CTIP2 plays a critical role in maintaining the bulge SC reservoir and directly regulates NFATC1 and LHX2 expression during postnatal hair cycling. Our results establish CTIP2 as a top-level regulator of hair follicle morphogenesis and cycling.

Results

Impaired HF morphogenesis and dysregulated expression of key players of HF morphogenesis in *Ctip2-null* mice

HF progenitors proceed through eight stages of development that are distinguished by basal to apical length (Paus *et al*., 1999). CTIP2 is expressed in all stages of HF morphogenesis (Golonzhka *et al*., 2007). In order to elucidate the role of CTIP2 in HF morphogenesis, we quantified HFs in wild-type and *Ctip2-null* skin from E14.5 to P0. The number of HFs in the mutant skin was indistinguishable from that of wild-type mice at E14.5 (Figure S1a and S1b). In contrast, HFs was less abundant in *Ctip2-null* skin from E16.5 through P0 (Figure 1a and 1b). Dorsal skin of E16.5 $Ctip2^{-/-}$ embryos also produced fewer hair follicles in stages 1 – 3 (Figure 1c). The reduction in HF density in the mutants was more striking at E18.5 and P0 (Figures 1d and e), when $Ctip2^{-/-}$ skin displayed fewer HFs at Stages 2-4 (Figure 1d and e). $Ctip2^{-/-}$ mice exhibited a reduced number of HFs in Stage 5 and Stage 6 at P0 (Figure 1e). Unlike the abnormality in hair follicle numbers, hair follicles structure was unaltered in $Ctip2^{-/-}$ skin. These results suggest that CTIP2 plays an important role in morphogenesis of the HF during skin development.

The reduced hair follicle density in the $Ctip2^{-/-}$ *mice* during hair formation prompted us to investigate whether loss of CTIP2 alters expression of signaling cues that are important in HF formation. We observed down regulation of NOTCH1 and EGFR in $Ctip2^{-/-}$ HFs, particularly at E18.5 (Figure S1c and S1d), when the difference in HF density between $Ctip2^{-/-}$ and $Ctip2^{+/+}$ was most striking (Figure 1d). In contrast, expression of *Tcf3* and *Lhx2* were up-regulated at E18.5 in $Ctip2^{-/-}$ skin (right panel of Figure S1e). Expression of *Sox9*, which plays an important role in HF cycling but not development (Vidal *et al*., 2005), was upregulated in $Ctip2^{-/-}$ skin at E18.5 (Figure right panel of S1e), whereas there was no change in expression of NFATC1, a transcription factor regulating only HF cycling (S1f-h). Expression of other key regulators, such as *Bmp, Shh* and *Wnt* were unaltered (Figure S1f). These results suggest that CTIP2 controls HF development by modulating expression of specific factors and signaling molecules involved in HF development.

Deregulated hair cycling in *adult* $Ctip2^{ep-/-}$ mice

We analyzed the expression of CTIP2 during postnatal HF establishment and also at different phases of natural hair cycling (Figure S2a and S2b). The expression of CTIP2 was uniform throughout the HF at all stages (Figure S2b). CTIP2 expressions were also detected during depilation-induced hair cycling, with highest expression in anagen and comparably lower levels of expression in catagen and telogen (Liang *et al*., 2012). CTIP2 co-localizes with HFSC markers CD34 and K15 within the HF bulge region (Golonzhka *et al*., 2007), suggesting that CTIP2 may play a role in hair cycling of adult skin. Because *Ctip2-null* mice die shortly after birth, we used $Ctip2^{ep-/-}$ mice, selectively lacking *Ctip2* in the epidermis

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(Golonzhka *et al*., 2009; Liang *et al*., 2012; Wang *et al*., 2012) for all subsequent studies. HF formation and the first postnatal hair cycling were similar in *Ctip2*$^{L2/L2}$ and *Ctip2*$^{ep-/-}$ skin at P0, P7 and P21 (Figure S3a-c). During the second hair cycling, the pink coat color of *Ctip2*$^{L2/L2}$ and *Ctip2*$^{ep-/-}$ skin at P50 confirmed that all the HFs were in telogen (Figure 2a). However, at P75 when *Ctip2*$^{L2/L2}$ follicles were still in telogen, *Ctip2*$^{ep-/-}$ follicles had already entered anagen, as indicated by the black color of the *Ctip2*$^{ep-/-}$ skin (Figure 2b). Mutant mice grew hair much faster than *Ctip2*$^{L2/L2}$ mice two weeks after shaving (Figure 2c), but the anagen-catagen-telogen transition progressed normally in *Ctip2*$^{ep-/-}$ mice at P28, P42 and P50 (Figure 2d, 2e and 2f). However, *Ctip2*$^{ep-/-}$ mice exhibited long anagen HFs compared to the short, resting HFs in the *Ctip2*$^{L2/L2}$ mice at P75 (Figure 2g). To explore the role of CTIP2 in hair cycling further, we analyzed induced hair-cycling post depilation in 8 weeks old *Ctip2*$^{ep-/-}$ and *Ctip2*$^{L2/L2}$ mice. The anagen-catagen-telogen transition proceeded normally in both groups of mice post-depilation (Figure S4a, S4c-e). However, after entering into telogen, *Ctip2*$^{ep-/-}$ HFs spontaneously entered anagen at day 28 post-depilation, whereas *Ctip2*$^{L2/L2}$ HFs remained in telogen (Figure S4b and S4f, compare left and right panels). Based on the above results, we conclude that CTIP2 plays a key role in maintenance of natural and depilation induced hair cycling in adult skin.

Disruption of *Ctip2* in the adult HFs enhances follicular stem cell activity

Normal hair cycling homeostasis is maintained by HFSCs and early induction of anagen is linked to altered activity of HFSCs (Cotsarelis *et al*., 1990; Morris and Potten, 1999; Sun *et al*., 1991). Premature induction of anagen in *Ctip2*$^{ep-/-}$ mice suggests a role of CTIP2 in controlling HFSC activity. To detect slow cycling hair follicle stem cells, BrdU pulse chase experiment was performed as described herein(Braun *et al*., 2003). Although there was no difference in hair morphology at P28, *Ctip2*$^{ep-/-}$ HFs incorporated more BrdU than *Ctip2*$^{L2/L2}$ HFs after a pulse of 24 hrs (Figure 3a and b). However, this finding was reversed after a longer chase period of 47 days. At P75, the percentage of BrdU+ cells which also co-labeled with bulge specific SC marker K15 was significantly reduced in *Ctip2*$^{ep-/-}$ HFs (Figure 3c and d). These results indicate that loss of *Ctip2* increases follicular SC proliferation which may lead to the exhaustion of the bulge stem cells at later stages of hair cycle. Moreover this enhancement of progenitor/stem cell proliferation may lead to their increased differentiation. Therefore we looked at the expression of K6HF,a marker of differentiation that is expressed in companion layer and also in upper matrix cells of hair bulb during anagen (Huelsken *et al*., 2001; Langbein *et al*., 1999; Winter *et al*., 1998). K6hf expression was increased in *Ctip2*$^{ep-/-}$ HFs at P28 and at P30 indicating accelerated differentiation of HF epithelial cells (Figure 3e and f).

Since we observed a loss of BrdU+ label retaining cells in *Ctip2*$^{ep-/-}$ HFs at later time point we performed immunohistochemical analysis for expression of HF bulge specific stem cell markers CD34 and K15. Expression of both CD34 and K15, was significantly reduced at P75 in *Ctip2*$^{ep-/-}$ HFs compared to the *Ctip2*$^{L2/L2}$ HFs (Figure S6a and Figure 4a, b and c). Similar downregulation of CD34 and K15 expression was observed in *Ctip2*$^{ep-/-}$ HFs at P120 (Figure 4a, b and c) when *Ctip2*$^{ep-/-}$ mice displayed a significant loss of dorsal hair (Figure S7a). Although, the total number of HFs between *Ctip2*$^{ep-/-}$ and *Ctip2*$^{L2/L2}$ mice were unaltered at that stage (Figure S7b).

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In addition we examined HF apoptosis in the mutant mice because apoptosis of HF cells plays an important role in anagen-catagen-telogen transition during hair cycling (Botchkareva *et al*., 2006; Lindner *et al*., 1997; Paus *et al*., 1994). TUNEL assay revealed no difference in the number of apoptotic cells between *Ctip2*$^{L2/L2}$ and *Ctip2*$^{ep-/-}$ HFs at P42 (catagen phase) (Figure S5a and b). However, a significant decrease in apoptosis was observed in *Ctip2*$^{ep-/-}$ skin at P75 (Figure S5c and d). Altogether, our data for altered HFSC proliferation, differentiation and reduced expression of bulge specific stem cell markers indicate that CTIP2 is essential for maintaining bulge SC quiescence during hair cycling in adult murine skin.

CTIP2 regulates expression of NFATC1 and LHX2 in bulge SCs during hair cycling in adult skin

The transcription factors NFATC1 and LHX2 maintain the bulge SC pool in murine HFs (Horsley *et al*., 2008; Rhee *et al*., 2006). Grafting of *Nfatc1*- and *Lhx2*-null skin onto nude mice results in premature entry into anagen (Horsley *et al*., 2008; Rhee *et al*., 2006), which closely resembles the phenotype of *Ctip2*$^{ep-/-}$ mice (Figure 2b and g). We therefore investigated expression of both LHX2 and NFATC1 in adult *Ctip2*$^{ep-/-}$ mice throughout the different stages of hair cycling.

Expression of both *Nfatc1* (Fig. 5a and b) and *Lhx2* (Fig. 5d and e) was down-regulated in the HF bulge at P28 and P42 (see also Figure S6b). *Nfatc1* expression continued to be down-regulated at P75 in mutant mice (Figure 5c and Figure S6b), but the levels of *Lhx2* (Fig. 5f) transcripts were indistinguishable and low in *Ctip2*$^{ep-/-}$ and control skin at this stage (Figure S6b). Expression of NOTCH1 and EGFR was unaltered in HFs of adult *Ctip2*$^{ep-/-}$ mice at P28 and P75 (Figure S7c, d and e).

ChIP assays were performed in keratinocytes isolated from the epidermis of newborn (P0) and adult (P28 and P75) mice to determine if CTIP2 interacts with the regulatory regions of the *Lhx2* and *Nfatc1* loci (see Table S3 and Figure S8a and S8b for details). CTIP2 interacted with the proximal promoter region of both *Lhx2* (Figure 6a) and *Nfatc1* (Figure 6b) in primary keratinocytes during HF morphogenesis (P0), and adult hair cycling (P28 and P75; Figure 6a and b). Moreover, the corresponding promoter fragments from both genes were sufficient to confer positive transcriptional regulatory activity by CTIP2 using luciferase-based reporter constructs in *Ctip2-null* keratinocytes (Figure 6c and d). These results suggest that CTIP2 is a direct and positive regulator of *Lhx2* and *Nfatc1* expression, which may underlie the adult HF phenotype of *Ctip2*$^{ep-/-}$ mice.

Discussion

The HF is considered a model system in stem cell (SC) biology because of its occupancy by multipotent SCs that underlie its self-renewal properties (Tiede *et al*., 2007; Waters *et al*., 2007). Here, we have investigated the role of transcription factor CTIP2 in HF morphogenesis and cycling. We show that CTIP2 regulates HF development during embryogenesis likely by regulating the NOTCH1 and EGFR signaling pathways. Expression of CTIP2 in the epithelium is important for maintaining postnatal hair cycle homeostasis. Epidermal-specific ablation of *Ctip2* results in inappropriate activation of follicular SCs and

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subsequent, premature depletion of this niche. CTIP2 interacts with the promoter region of *Lhx2* and *Nfatc1*, and positively regulates expression of the corresponding genes, suggesting that each is a *bona fide* target gene of this transcription factor. These findings are highly relevant because both LHX2 and NFATC1 are necessary for maintenance of hair cycling and HFSCs in adult skin.

Epithelial–mesenchymal interaction is required for formation of a mature hair follicle with hair shaft, root sheaths, and dermal papilla (Botchkarev and Paus, 2003; Millar, 2002). Loss of *Ctip2* from both epidermal and dermal compartment in the skin of *Ctip2-null* mice results in reduced numbers and reduced conversion of HF progenitors from one stage to another stage during morphogenesis. This finding implies that CTIP2 plays an important role in HF formation. The hair formation defect evident in *Ctip2-null* mice was due to the aberrant expression of components of signaling pathways that underlie embryonic hair development. CTIP2 interacts with the NOTCH1 and EGFR promoters and positively regulates expression of both genes in the epidermis at E14.5 and E16.5 (Zhang *et al*., 2012). Similarly, both NOTCH1 and EGFR are down-regulated in HFs of *Ctip2*$^{-/-}$ mice at E18.5, which may contribute to the HF developmental phenotype of these mice. Notably, *Egfr*$^{-/-}$ mice also exhibit delayed HF development (Doma *et al*., 2013) and the NOTCH pathway plays an important role in follicular patterning during embryogenesis (Millar, 2002). Mis-expression of DELTA1, a ligand of NOTCH pathway, in epidermis promotes expression of NOTCH1 and accelerates placode formation (Crowe *et al*., 1998; Crowe and Niswander, 1998). This may explain, at least in part, the link between down-regulation of NOTCH1 and the delay in HF morphogenesis in *Ctip2*$^{-/-}$ mice. We have also observed up-regulation of *Tcf3, Sox9 and Lhx2* expression in *Ctip2*$^{-/-}$ HFs. *Tcf3* induction in skin maintains an undifferentiated state and arrests downward growth of HF (Nguyen *et al*., 2006). Therefore, *Tcf3* overexpression may contribute in the delayed down-growth of HF progenitors in *Ctip2*$^{-/-}$ mice during development. However, the role(s) of over-expressed *Sox9* and *Lhx2* on the HF developmental phenotype in *Ctip2*$^{-/-}$ mice is unclear. SOX9 (Vidal *et al*., 2005) is thought to play no role in HF development. Although, we show that CTIP2 binds to *Nfatc1* and *Lhx2* promoter regions and increases expression of heterologous reporter constructs harboring these regions, *Nfatc1* transcript levels were unaltered, while *Lhx2* transcript was only modestly up-regulated in the embryonic *Ctip2*$^{-/-}$ skin, suggesting that other *cis*-acting regulatory elements or *trans*-acting factors may regulate expression of both genes during skin organogenesis and can compensate for loss of CTIP2. During osteoclastogenesis, it has been shown that co-stimulation of Fc receptor common γ chain (FcRγ) and DNAX-activating protein (DAP) 12 by RANKL drive phosphorylation of phospholipase Cγ (PLCγ) leading to calcium-mediated regulation of NFATC1 expression (Kim *et al*., 2012; Koga *et al*., 2004; Mao *et al*., 2006). Regulation of NFATC1 by these factors during hair formation and cycling has not been reported. It is possible that NFATC1 expression during embryogenesis can be predominantly regulated by these factors, without input from CTIP2.

To circumvent the postnatal lethality of *Ctip2-null* mice, we used *Ctip2*$^{ep-/-}$ mice to study the entire process of HF formation, establishment, and postnatal hair cycling. In contrast to the *Ctip2-null* mice, *Ctip2*$^{ep-/-}$ mice did not show differences in HF number or morphology during formation or the first postnatal hair cycling, suggesting that CTIP2 in dermis plays an

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essential role in HF morphogenesis through epithelial-mesenchymal interactions. *Ctip2*$^{ep-/-}$ mice exhibited a defect in the second phase of hair cycling with short telogen and early entry into anagen. This defect in hair cycling can be a synergy of the deletion of *Ctip2* in keratinocytes of hair follicles and also from the paracrine signaling produced by the infiltrating cells in dermis due to inflammation in *Ctip2*$^{ep-/-}$ mice. Therefore both both cell and non-cell autonomous effects can contribute in abnormal hair cycling in *Ctip2*$^{ep-/-}$ mice. Moreover, genetic disruption of the transcriptional coactivator complex *Med1* or of genes encoding transcription factors, such as *Foxp1, Lhx2* and *Nfatc1* generate phenotypically similar mice (Horsley *et al*., 2008; Leishman *et al*., 2013; Nakajima *et al*., 2013; Rhee *et al*., 2006; Tornqvist *et al*., 2010).These observations suggest that CTIP2 may genetically interact with these factors, regulate their expression and may also be involved in signaling pathway(s) which modulate hair cycling.

Activation of the bulge SCs is required for induction of anagen during normal hair cycling (Cotsarelis *et al*., 1990; Morris and Potten, 1999; Sun *et al*., 1991). Bulge SCs proliferate and give rise to transient amplifying cells, which in turn migrate towards secondary hair germ to initiate anagen (Cotsarelis *et al*., 1990; Sun *et al*., 1991).

Premature induction of anagen in *Ctip2*$^{ep-/-}$ mice is consistent with over-activation of bulge SCs and also an increase in HFSC differentiation. Enhanced SC activity appears to deplete the numbers of bulge SCs following ablation of *Ctip2*. This over-activation and eventual exhaustion of HFSCs may explain the loss of hair coat in *Ctip2*$^{ep-/-}$ mice at P120. In spite of the loss of hair coat, the number of HFs was not altered, suggesting that the loss of hair in mutant mice is most likely due to the loss of hair shaft or lack of shaft formation. Reduced expression of the bulge SC markers (CD34 and K15) in the *Ctip2*$^{ep-/-}$ HFs further indicates a role of CTIP2 for maintenance of the bulge stem cell quiescence and survival.

NFATC1 and LHX2 are known to regulate the switch between HFSC maintenance and activation (Horsley *et al*., 2008; Rhee *et al*., 2006). Loss of LHX2 leads to exhaustion of HFSCs and failure in anchorage of HFs (Folgueras *et al*., 2013). Moreover LHX2 plays a significant role in repithelialization after injury by regulating SOX9, TCF4 and LGR5 (Mardaryev *et al*., 2011). In our present study, we observed down-regulation of LHX2 and NFATC1 in *Ctip2*$^{ep-/-}$ HFs during hair cycling, and direct regulation of the corresponding promoters by CTIP2. Down-regulation of both of those genes may lead to enhanced SC activity and later depletion of HFSCs in *Ctip2*$^{ep-/-}$ mice. The difference in LHX2 and NFATC1 expression patterns observed between *Ctip2-null* embryonic skin and *Ctip2*$^{ep-/-}$ adult skin might be due to the temporal regulation of those genes by CTIP2 during development and in adulthood. These findings, along with our previously published data, demonstrate that CTIP2 is a top level transcription factor that performs a variety of functions, ranging from control of HF development, cycling and EPB formation, suppression of inflammation, maintenance of homeostasis and regeneration in skin. The pivotal role of CTIP2 in a variety of developmental processes makes it an ideal target for treatment of dermatological diseases, such as alopecia, chronic wounds and atopic dermatitis (eczema).

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Materials and Methods

Mice

Ctip2$^{-/-}$ and *Ctip2*$^{ep-/-}$ mice were previously described (Golonzhka *et al*., 2009; Liang *et al*., 2012; Wang *et al*., 2012). *Ctip2*$^{+/+}$ and *Ctip2*$^{L2/L2}$ mice were used as controls. Six to eight mice from multiple litters were used in each group and at each time point. Mice are maintained in a specific pathogen free environment with constant temperature control. Animal work was approved by the OSU Institutional Animal Care and Use Committee.

Hair cycle stages

Dorsal skin samples were harvested from mice from E14.5 to P0 for hair follicle morphogenesis studies as described (Paus *et al*., 1999). First hair cycle (P9, P16 and P19) and second hair cycle (P28, P42, P49 and P75) samples were similarly collected. Depilation was performed on 8-week old mice and skin biopsies were taken on day 7 (anagen), day 17 (catagen), day 21 (telogen) and day 28 after depilation.

A detailed description of histology, immunohistochemistry**,** BrdU labeling and detection, TUNEL assays, qRT PCR, ChIP, luciferase assays and statistics are available in the Supplementary Materials and Methods section.

Supplementary Material and Methods

Histology and Immunohistochemistry

Dorsal skin samples were fixed with 4% paraformaldehyde and embedded in paraffin. Deparaffinization and hematoxylin & eosin (H&E) staining was performed for histological analysis. For immunohistochemical (IHC) study, antigen unmasking with citrate buffer was performed as described (Golonzhka *et al*., 2009; Hyter *et al*., 2010; Liang *et al*., 2012; Zhang *et al*., 2012). Sections were blocked in 10% normal goat serum and primary antibody was added after blocking. Subsequently, sections were incubated with a secondary antibody conjugated with Cy3 or Cy2, and DAPI for nuclei staining. Images were taken at 20X and 40X magnification using Leica DMRA fluorescent microscope and Hamamatsu C4742-95 digital camera and were processed using Adobe Photoshop CS3. IHC data were quantified using Adobe Photoshop CS3 and Image J software. Table S1 shows the details of antibody used for immunohistochemistry.

BrdU labeling and detection

Groups of mice were injected with 100 μg/g BrdU (Sigma) and dorsal skin samples were collected after 24 hrs for short-pulse analyses. Long-chase animals were injected with 50 μg/g of BrdU every 12 hours for a total four injections and skin biopsies were taken after a 47-day chase period (Braun *et al*., 2003). Anti-BrdU staining (Serotec, Raleigh, NC, 1:200) was performed on paraffin sections to detect proliferating S-phase cells. Additional detail on BrdU staining is described in the Histology and Immunohistochemistry section.

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TUNEL assay

Dorsal skin samples were fixed in 4% PFA and deparaffinized slides were subjected to TUNEL staining using the fluorometric TUNEL system (Promega, no. TB235). The detailed protocol has been previously described in (Hyter *et al*., 2013).

Statistics

Statistical significance between the two groups was analyzed by Graphpad Prism software (Graphpad Software, La Jolla, CA) using unpaired Student t-test. The TUNEL- or BrdU-positive cells were counted and represented as a percentage of DAPI-positive cells. Multiple sections were analyzed for each genotype and for time point. Data from each group for each time point were combined for calculating the mean data and SEM and significance was determined using Student's unpaired *t*-test.

RT-qPCR

Total RNA was extracted from the epidermis of mouse skin and cDNA was synthesized from extracted RNA as described (Indra *et al*., 2005b). Real-time PCR was performed on an ABI 7500 Real-Time PCR system using SYBR Green methodology (Indra *et al*., 2005a; Indra *et al*., 2005b). Using HPRT as an internal control, relative gene expression of the RT-qPCR data was analyzed. The mean threshold cycle (Ct) for individual reactions was determined by using the ABI sequence analysis software. Primer sequences for qPCR are indicated Table S2. All assays were performed in triplicate.

Chromatin immunoprecipitation

ChIP studies were performed on epidermal tissue from newborn and adult mice. Cells from epidermis were isolated from newborn mice as described (Zhang *et al*., 2012) and from adult mice (Nowak and Fuchs, 2009). Successively cells were processed for ChIP as we previously described (Hyter et al., 2010) using appropriate antibodies. Recovered DNA was amplified by qPCR using primers specific for the distal or proximal regions of the promoters of *Lhx2* and *Nfatc1*, as well as primer sets for 3′ UTR (untranslated regions) of each gene. Proximal region is considered as 1.5 kb in and around the transcriptional start site (TSS). Distal region is indicated as sequences 2kb or beyond upstream of TSS. Primers used for individual ChIP assay are indicated in Table S3.

Dual luciferase reporter assay

Lhx2 and *Nfatc1* promoter reporter constructs were prepared by inserting respective sequences from region as described in Table S4 into the promoterless luciferase reporter plasmid pGL3-Basic (Promega). Correct insertions were verified by sequence analysis. Primary keratinocytes were isolated from P0 pup skin as described (Zhang *et. al*., 2012). Approximately 5×10^4 cells were individually transfected with 200ng of *Lhx2* and *Nfatc1* reporter constructs, 20ng of pcDNA3 containing *Ctip2* (or empty vector) and 8ng of renilla luciferase construct using the Neon transfection system (Invitrogen). Transfected cells were plated in collagen-coated, 96-well plate. Dual luciferase reporter assay was performed 48hrs after transfection using kit from Promega and synergy HT Multi-Mode microplate reader.

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Supplementary Material

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Abbreviations

CTIP2	Chicken ovalbumin upstream promoter transcription factor (COUP TF)-interacting protein 2, also known as BCL11B
EGFR	Epidermal growth factor receptor
NOTCH1	Neurogenic Locus Notch Homolog Protein 1
LHX2	LIM Homeobox 2
NFATC1	Nuclear factor of activated T cells cytoplasmic calcineurin-dependent 1
WNT	Wingless Type MMTV Integration Site Family
β-catenin	Beta catenin
LEF1	Lymphoid Enhancer-Binding Factor 1
TCF3	Transcription Factor 3
SHH	Sonic Hedgehog
BMP	Bone Morphogenetic Protein
Sox9	Sex Determining Region Y-Box 9
K14	Keratin 14
LoxP	locus of X-over P1
K15	Keratin15
HF	Hair Follicle
SC	Stem Cell
BrdU	Bromodeoxyuridine
qRT PCR	quantitative real time reverse transcription PCR
EPB	Epidermal Permeability Barrier
ChIP	Chromatin Immunoprecipitation

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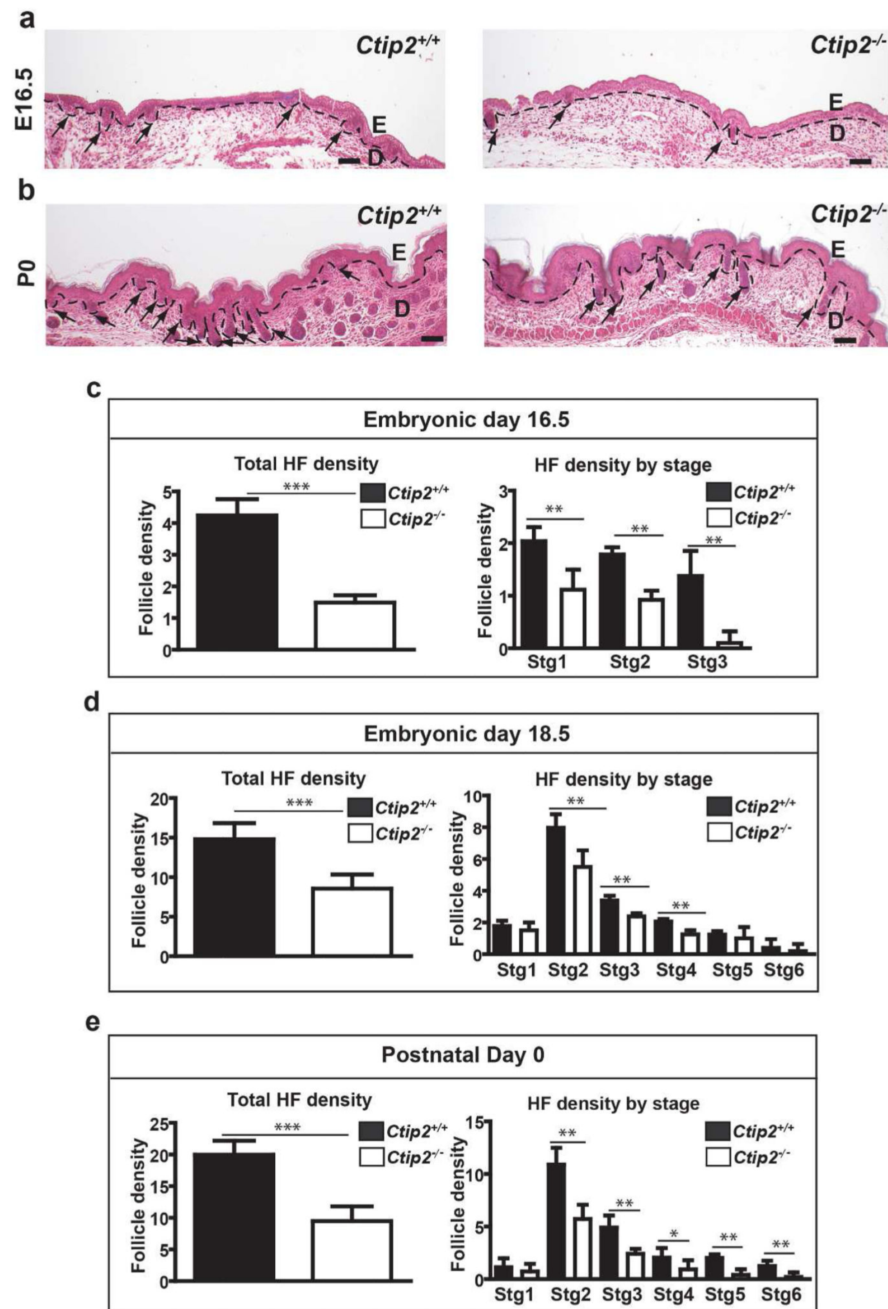

Figure 1. Impaired HF formation in *Ctip2-null* mice during development

(a-b) H&E stained skin sections of *Ctip2*$^{+/+}$ and *Ctip2*$^{-/-}$ mice at E16.5 **(a)** and P0 **(b)**. The dotted black line demarcates the epidermal-dermal boundary and the black arrows indicate HFs. E-Epidermis; D-Dermis. Scale Bar: 200μm. **(c)** Graph showing reduced number of HFs **(Left)** and number of HFs at each developmental stage **(Right)** at E16.5 in *Ctip2*$^{-/-}$ skin compared to *Ctip2*$^{+/+}$ skin. **(d)** Bar graph shows reduced number of the total HFs **(Left)** and number of follicles in stages 1-6 **(Right)** in E18.5 *Ctip2*$^{-/-}$ skin section. **(e)** Bar graph

representing total number of HFs and HFs in different stages at P0 skin. (*p< 0.05, ** p< 0.01, *** p<0.005)

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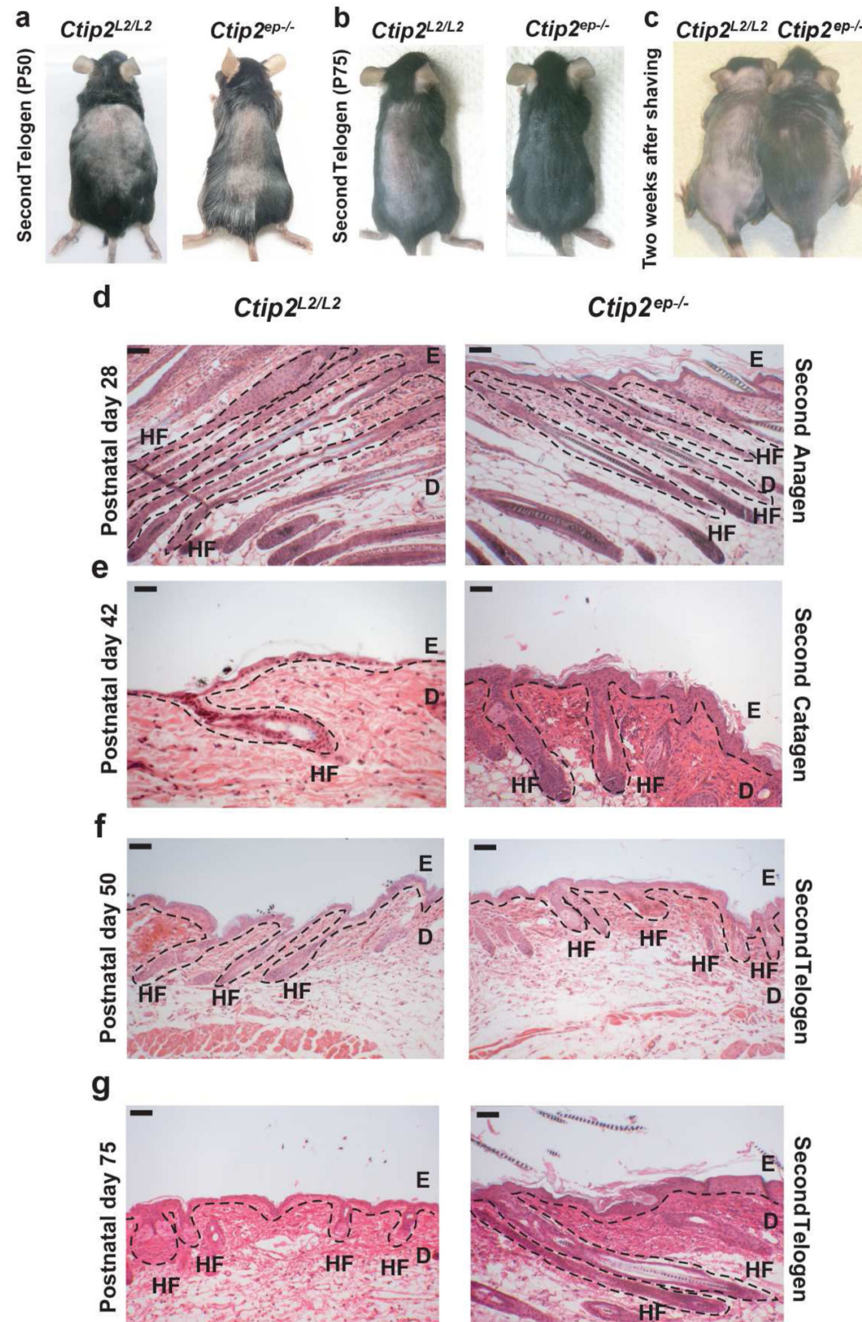

Figure 2. Epidermal-specific deletion of *Ctip2* alters hair cycling in adult skin

(a-b) Macroscopic images of *Ctip2*$^{L2/L2}$ and *Ctip2*$^{ep-/-}$ mice dorsal skin in second telogen at P50 (a) and P75 (b). Pink skin color in *Ctip2*$^{L2/L2}$ mice indicates telogen phase and black skin color in *Ctip2*$^{ep-/-}$ mice indicates anagen phase. **(c)** Rapid dorsal hair growth in 8 week old *Ctip2*$^{ep-/-}$ mice compared to the *Ctip2*$^{L2/L2}$ mice two weeks after shaving, **(d-g)** Hematoxylin and Eosin stained skin sections of *Ctip2*$^{L2/L2}$ and *Ctip2*$^{ep-/-}$ skin at different stages of hair cycling, **(d)** P28 (Second Anagen), **(e)** P42 (Second Catagen), **(f)** P50 (Early

Second Telogen) and **(g)** P75 (Late Second Telogen)**.** HF- Hair Follicle; E- Epidermis; D-Dermis**.** Scale Bar: 200 μm.

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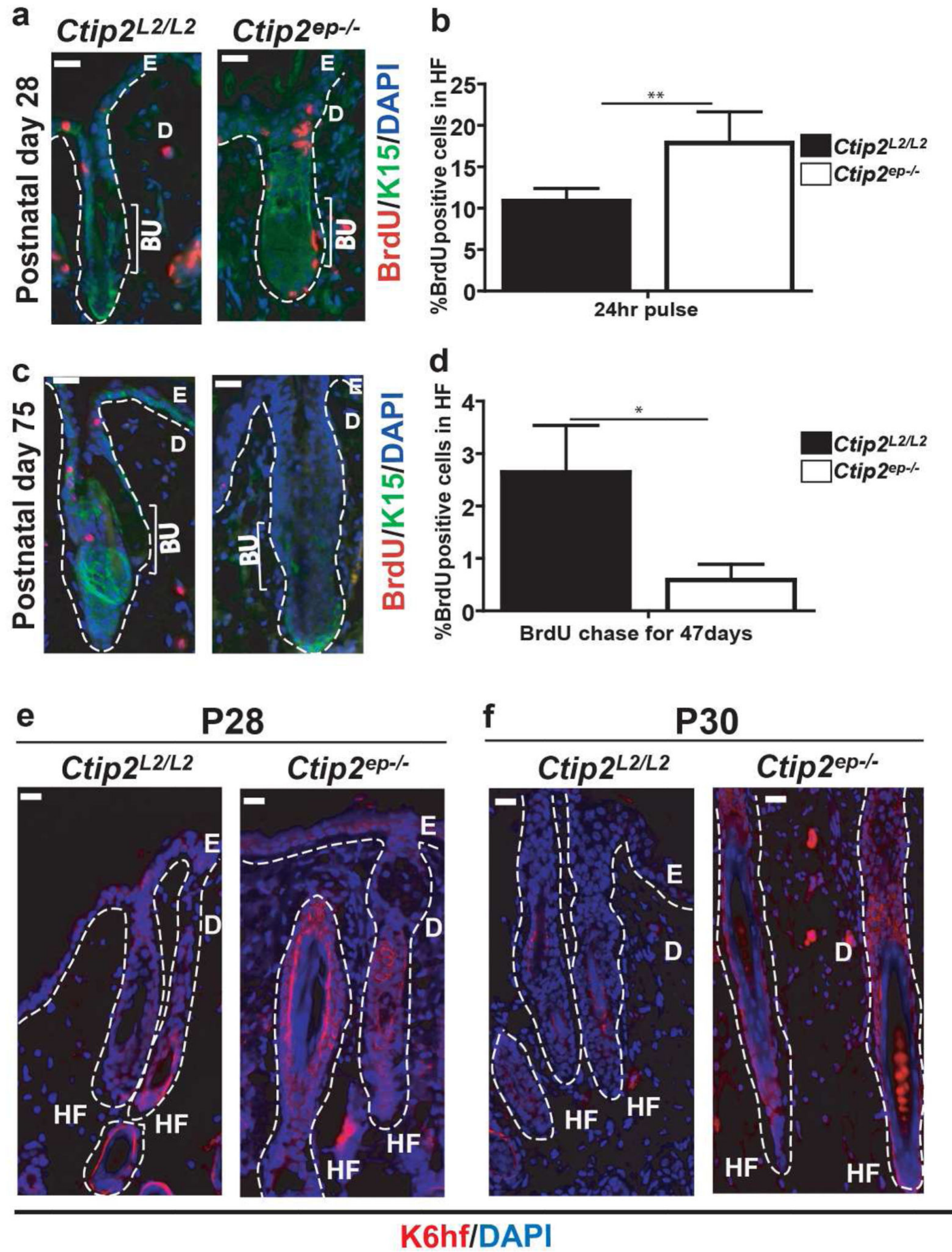

Figure 3. Loss of *Ctip2* in HF epithelium alters proliferation and differentiation in the HFs

(a) Anti-BrdU immunolabeling of P28 $Ctip2^{L2/L2}$ and $Ctip2^{ep-/-}$ skin 24 hrs after BrdU pulse. Sections were co-stained with K15, a bulge-specific marker. **(b)** Graph represents percentage of BrdU-positive cells in HFs. **(c)** Immunostaining after BrdU pulse and extended chase shows reduced number of BrdU-positive cells in P75 $Ctip2^{ep-/-}$ HFs. **(d)** Graphical representation of percentage of label retaining bulge cells in HFs. **(e-f)** Expression of K6hf was determined by immunostaining of $Ctip2^{L2/L2}$ and $Ctip2^{ep-/-}$ mice skin at stages

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P28 **(e)** and P30 **(f)** using anti-K6hf antibody. Bu-Bulge; HF- Hair Follicle; E- Epidermis; D- Dermis; Scale Bar: 100μm

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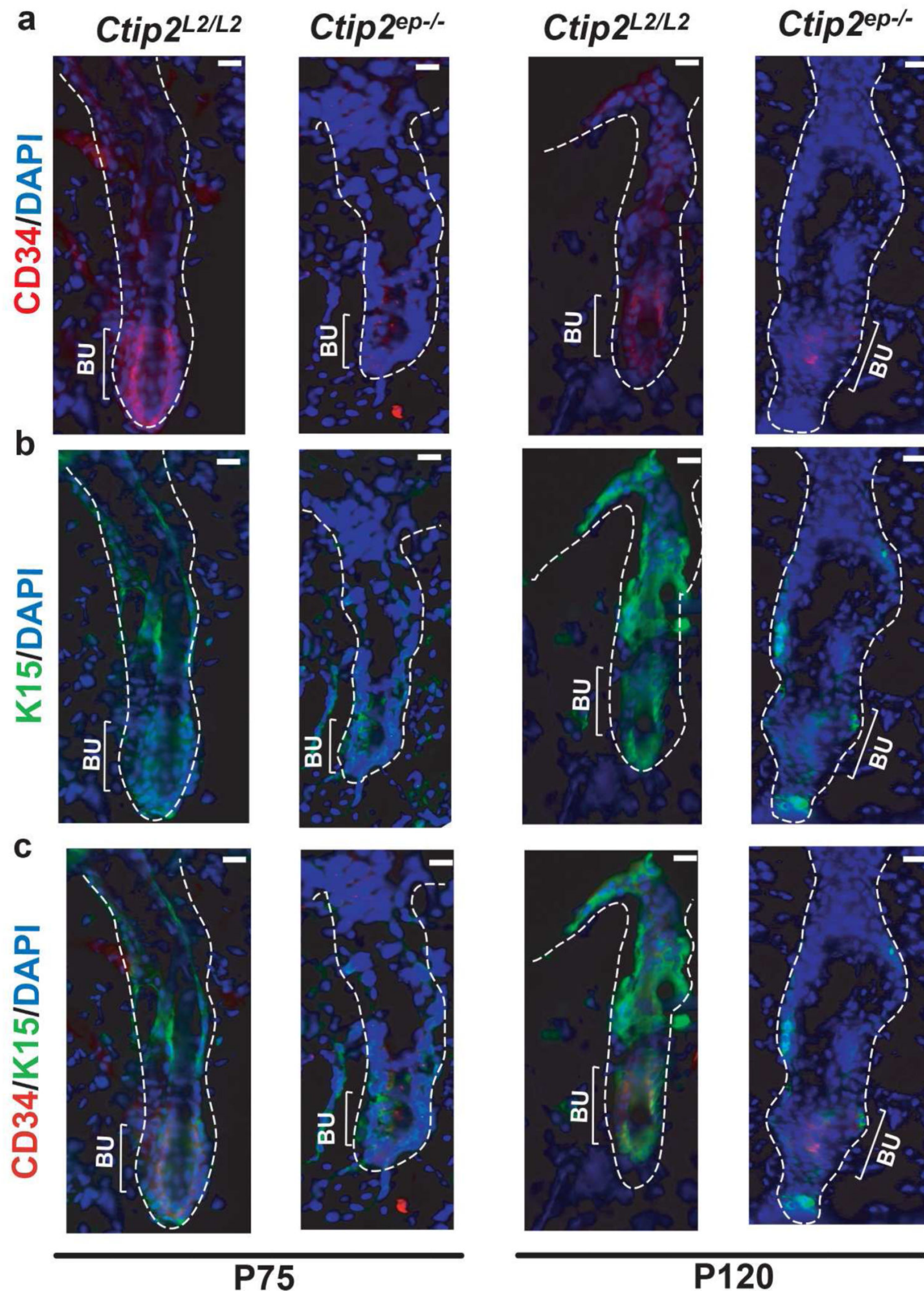

Figure 4. CTIP2 is essential for maintaining bulge stem cell pool

(a) Immunostaining with anti-CD34 antibody (red) shows a decrease in $CD34^+$ cells in HF bulge of *Ctip2*$^{ep-/-}$ dorsal skin compared to *Ctip2*$^{L2/L2}$ at P120. At P75, similar expression of CD34 in HFs of *Ctip2*$^{L2/L2}$ and *Ctip2*$^{ep-/-}$ skin was observed. **(b)** Staining with anti-K15 antibody (green) indicates reduced expression of K15 in hair follicle bulge region of *Ctip2*$^{ep-/-}$ dorsal skin compared to *Ctip2*$^{L2/L2}$ at P120 but not at P75. **(c)** Co-localization of CD34 and K15 expression at P75 and P120 in HFs from *Ctip2*$^{L2/L2}$ and *Ctip2*$^{ep-/-}$ mice skin. All sections are counterstained with DAPI (blue). Bu-Bulge; Scale Bar: 100μm

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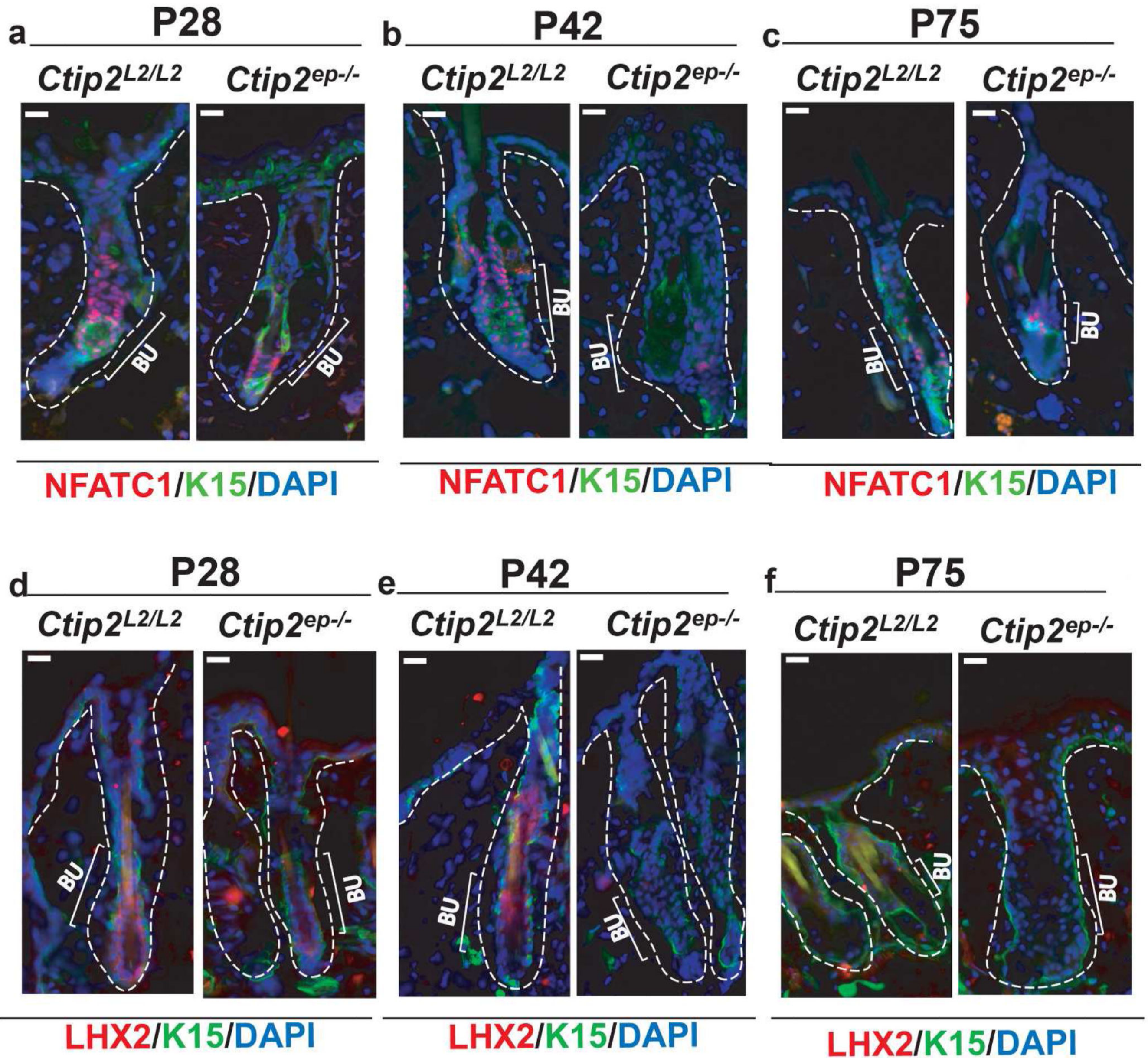

Figure 5. Conditional ablation of *Ctip2* in adult epidermis leads to altered expression of LHX2 and NFATC1

(a-c) NFATC1 (red) and K15 (green) co-immunostaining of **(a)** P28**, (b)** P42 and **(c)** P75 $Ctip2^{L2/L2}$ and $Ctip2^{ep-/-}$ dorsal skin was performed with anti-NFATC1 and -K15 antibodies **(d-e)** Immunohistochemical analysis of LHX2 expression in **(d)** P28 and **(e)** P42 HFs of $Ctip2^{L2/L2}$ and $Ctip2^{ep-/-}$ mice indicates reduced expression of LHX2 in $Ctip2^{ep-/-}$ HF bulge and hair germ. **(f)** Immunostaining for LHX2 (red) at P75 on $Ctip2^{L2/L2}$ and $Ctip2^{ep-/-}$ dorsal skin shows no detectable expression in bulge region in both mice. All sections are co-stained with the K15 antibody and DAPI (blue) to stain bulge cells and cell nuclei respectively. Bu-Bulge; Scale Bar: 100μm.

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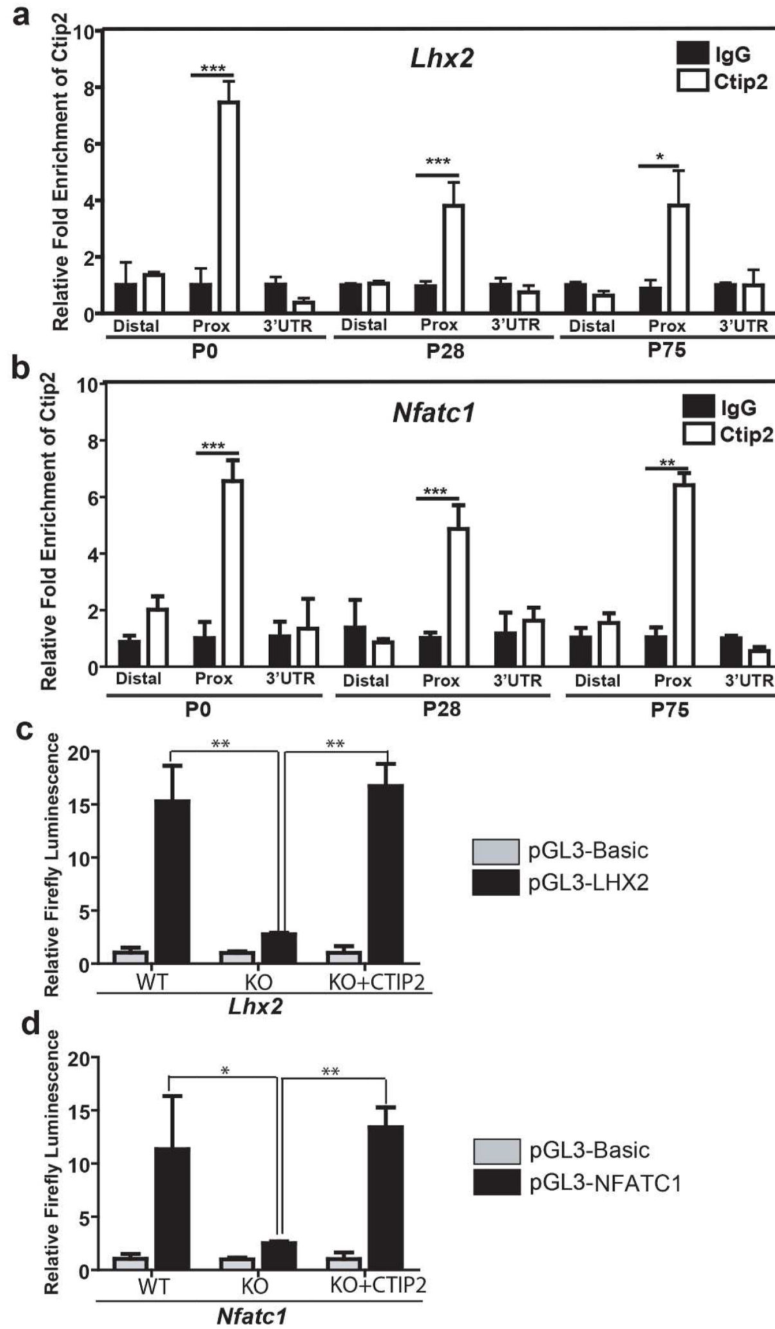

Figure 6. CTIP2 positively regulates *Lhx2* and *Nfatc1* expression in skin keratinocytes *in vitro*

(a and b) ChIP assays were performed on epidermal keratinocytes from P0, P28 and P75 mice using anti-CTIP2 antibody and the results were analyzed by primer sets indicated in Table S3. Rat IgG was used as the control. CTIP2 interacts with the proximal promoter regions of **(a)** *Lhx2* and **(b)** *Nfatc1*. **(c and d)** CTIP2 positively regulates *Lhx2* (a) and *Nfatc1* (b) promoter in cultured keratinocytes. $Ctip2^{+/+}$ and $Ctip2^{-/-}$ keratinocytes were transfected with either promoter-less pGL3-basic construct or pGL3-basic construct harboring respective promoter regions with or without CTIP2 expression vector. For

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normalization renilla luciferase was co-transfected. Bars represent relative expression levels of firefly luciferase. Statistical significance was determined by Student's un-paired *t*-test (*p<0.05;**p<0.01).