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Dlx3 is a crucial regulator of hair follicle differentiation and regeneration

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SUMMARY

Dlx homeobox transcription factors regulate epidermal, neural and osteogenic cellular differentiation. Here we demonstrate the central role of Dlx3 as a crucial transcriptional regulator of hair formation and regeneration. The selective ablation of Dlx3 in the epidermis results in complete alopecia due to failure of the hair shaft and inner root sheath to form, which is caused by the abnormal differentiation of the cortex. Significantly, we elucidate the regulatory cascade that positions Dlx3 downstream of Wnt signaling and as an upstream regulator of other transcription factors that regulate hf differentiation, such as Hoxc13 and Gata3. Co-localization of phospho-SMAD1/5/8 and Dlx3 is consistent with a regulatory role of BMP signaling of Dlx3 during hair morphogenesis. Importantly, mutant catagen follicles undergo delayed regression and display persistent proliferation. Moreover, ablation of Dlx3 expression in the telogen bulge stem cells is associated with a loss of BMP signaling, precluding re-initiation of the hf growth cycle. Taken together with hf abnormalities in humans with Tricho-Dento-Osseous (TDO) syndrome, an autosomal dominant ectodermal dysplasia linked to mutations in the *DLX3* gene, our results establish that Dlx3 is essential for hair morphogenesis, differentiation and cycling programs.

INTRODUCTION

Ectodermal appendages such as hair, feather, and tooth are attractive models for understanding the mechanisms underlying epithelial-mesenchymal interactions (Thesleff et al., 1995; Mikkola and Millar, 2006). A series of signaling molecules is involved in each step of primary hair development and differentiation. Wnt signaling is critical for the initiation of hair follicle (hf) development (Andl et al., 2002), and Shh controls proliferation of the epithelial hair germ (Chiang et al., 1999). Noggin, BMP and Ectodysplasin (Eda) signaling play important roles at early stages of hf placode development (Botchkarev et al., 1999; Mou et al., 2006; Schmidt-Ullrich et al., 2006; Pummila et al., 2007). The dermal papilla (DP) remains associated with the overlying epithelial matrix cells, which undergo an upward differentiation process to give rise to the different hf lineages such as the medulla, cortex and cuticle of the hair shaft and the inner root sheath (IRS) (Millar, 2002; Fuchs, 2007). The matrix is derived from epithelial stem cells located in the bulge region of the hf (Cotsarelis et al., 1990; Taylor et al., 2000; Oshima et al., 2001; Levy et al., 2005). Several important pathways and transcription factors that initiate and promote differentiation of the matrix cells have been determined, including Gata3 and Cutl that regulate IRS differentiation (Ellis et al., 2001; Kaufman et al., 2003; Kurek et al., 2007), and BMP signaling and the transcription factors such as Msx2, Foxn1, and Hoxc13 that are

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required for hair shaft differentiation (Godwin and Capecchi, 1998; Meier et al., 1999; Kulessa et al., 2000; Tkatchenko et al., 2001; Ma et al., 2003; Johns et al., 2005).

After early follicle morphogenesis, the hf undergoes cyclic transformations known as anagen (growth phase), catagen (regression) and telogen (resting phase), allowing the study of essential stages of proliferation and differentiation (Schmidt-Ullrich and Paus, 2005). Cyclical renewal of the hair is thought to recapitulate some of the signaling and control mechanisms found between the DP and overlying epithelial cells during the embryonic onset of hair formation (Oliver and Jahoda, 1988; Hardy, 1992; Schmidt-Ullrich and Paus, 2005).

Several of the transcription factors with distinct and important roles in the developing hf are homeobox-containing proteins such as Msx2, Lhx2 and Hoxc13 (Godwin and Capecchi, 1998; Tkatchenko et al., 2001; Ma et al., 2003; Rhee et al., 2006). Homeodomain transcription factors play crucial roles in many developmental processes, ranging from organization of the body plan to differentiation of individual tissues. Dlx3 belongs to the Dlx family of homeodomain transcription factors (Dlx1–6). In the genome, they are organized into three pairs of inverted, convergently transcribed genes, termed *Dlx1–2*, *Dlx3–4* and *Dlx5–6* (Morasso and Radoja, 2005).

Dlx3 has an essential role in epidermal, osteogenic and placental development (Morasso et al., 1996; Morasso et al., 1999; Beanan and Sargent, 2000; Hassan et al., 2004). Importantly, an autosomal dominant mutation in DLX3 is responsible for the ectodermal dysplasia termed Tricho-Dento-Osseous syndrome (TDO), characterized by defects in teeth, bone development and abnormalities in hair shaft morphology and diameter (Price et al., 1998; Wright et al., 2008). Despite strong evidence suggesting a major role for Dlx3 in epithelial appendage and hf development, early lethality of loss-of-function mutants have precluded the analysis of the specific function of Dlx3 in these processes (Morasso et al., 1999). Taking advantage of a $Dlx3^{Kin/+}$ line that has the *β-galactosidase* (*LacZ*) gene inserted into the *Dlx3* locus, we present a thorough analysis of the broad Dlx3 expression during the hair cycle. Using a K14cre line that expresses the Cre recombinase in epidermal cells and their derivatives (Andl et al., 2004) and a floxed Dlx3 line we determined the role of Dlx3 in hair development by epidermal-specific ablation. The most striking defects in the conditional knockout mice were complete alopecia due to a failure in hf development, concomitant with lack of expression of transcriptional regulators necessary for the differentiation of the IRS and hair shaft, and inability to undergo cyclic regeneration postnatally. Our results demonstrate that loss of ectodermal Dlx3 leads to altered morphogenesis, differentiation and cycling of the hfs. Taken together with the pathological conditions of TDO patients, these results establish Dlx3 as a crucial regulator of hair development.

MATERIALS AND METHODS

Gene targeting and generation of mutants

The targeting vector for the $Dlx3^{Kin/+}$ line was derived from the vector pZINI (nM) containing the *β-galactosidase* (*LacZ*) gene as a reporter. A 3.9-kb NotI–BamHI 5′ genomic flank, corresponding to the region directly upstream of Dlx3 exon1, was cloned upstream of *LacZ* gene (Fig. 1A). The 4.3-kb 3′ homologous flank was subcloned downstream of neomycin gene in pZINI. The targeting vector for the Dlx3 floxed ($Dlx3^{f/f}$) line contains 6.5 kb of Dlx3 genomic sequence from a 129/Sv strain in the pPNT vector (Fig. 3A). This construct was modified by inserting a loxP site immediately downstream of the neomycin gene (Neo). A second loxP site was inserted into the unique NotI site (N) between the first and second exons of the *Dlx3* gene.

Genotype of the $Dlx3^{Kin/+}$ line was determined by Southern blot analysis and PCR (Fig. 1A). Three oligonucleotides were used for the genotyping by PCR: PCRforward $Dlx3^{Kin/+}$ primer (GGGTCTTTGCCACTTTCTGTCTGTCATTTGCATAGA) is located 449 bp upstream from the transcription start site of Dlx3 gene; for determination of the wild-type allele we utilized PCRreverse1 $Dlx3^{Kin/+}$ (CCTGCGAGCCCATTGAGATTGAACTGGTGGTGGTAG) which is located 432 bp downstream from the transcription start site located on Exon 1, and generates a 880 bp fragment and for the determination of the LacZ knockin allele we utilized PCRforward $Dlx3^{Kin/+}$ primer (same as above) and PCRreverse2 $Dlx3^{Kin/+}$ (TGAAACGCTGGGCAATATCGCGGCTCAGTTCG) located 280 bp downstream from the transcription start site of *LacZ* gene and generates a 730 bp fragment. The cycling conditions were: 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min.

For the $Dlx3^{f/f}$ line, recombination was determined by Southern blot (Fig. 3A). For the epidermal-specific ablation of Dlx3, we utilized a K14cre line that has been previously characterized (Andl et al., 2004). The Cre-mediated deletion of Dlx3 to generate the K14cre;$Dlx3^{Kin/f}$ or K14cre;$Dlx3^{f/f}$ was assessed by PCR with the following oligonucleotides: PCRforward $Dlx3^{Kin/+}$ primer and PCRreverse-cre primer (TGTAAGGTGTGTCATTTTCCTCAACGGGTG) generating a 2.15Kb fragment (Fig. 3C). The cycling conditions were: 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 68°C for 4 min. Throughout this study, the K14cre;$Dlx3^{Kin/f}$ or K14cre;$Dlx3^{f/f}$ lines were analyzed obtaining similar results. All animal work was approved by the NIAMS Animal Care and Use Committee.

X-gal staining and treatment with benzyl-benzoate/benzyl alcohol

X-gal staining of $Dlx3^{Kin/+}$ whole embryos or individual dissected organs was performed with 1 mg/ml 5-Br-4-Cl-3-indolyl-β-D-galactosidase (X-gal), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM $MgCl_2$ in PBS and the detergent NP-40 (0.02%). Samples were fixed at 4°C in 4% paraformaldehyde/PBS. X-gal stained $Dlx3^{Kin/+}$ embryos were cleared by treatment with benzyl-benzoate/benzyl alcohol 2:1 mixture after dehydration in methanol.

Histology, immunofluorescence and confocal microscopy

Skin sections (10 μm thickness) were stained with either primary antibodies overnight at 4°C for immnunofluorescent staining or hematoxylin/eosin. The antibodies and dilutions used were anti-Dlx3 (1:250, Morasso Lab.; Bryan and Morasso, 2000), anti-β-galactosidase (1:250, Abcam), anti-PCNA (1:100, Calbiochem), anti-β-catenin (1:200, Sigma), anti-Phospho-SMAD1/5/8 (1:50, Cell Signaling Technology), anti-Lef1 (1:100, Cell Signaling Technology), anti-Hoxc13 (1:50, Novus Biologicals), anti-Gata3 (1:100, Santa Cruz), anti-AE13 (type I hair keratin, 1:10, gift from T.T. Sun), anti-AE15 (trichohyalin, 1:10, gift from T.T. Sun), anti-adipophilin (1:100, Fitzgerald), anti-K1 (1:500, Covance), anti-K10 (1:500, Covance), anti-K15 (1:100, Thermo Scientific), anti-K17 (1:1000, gift from P. Coulombe), anti-K35 (previous nomenclature Ha5) and anti-K85 (previous nomenclature Hb5) (1:50, Progen) and secondary antibodies: Alexa Fluor® 488 or Alexa Fluor® 546 goat anti-mouse, rabbit, chicken or guinea pig IgG (1:250, Molecular Probes). MOM immunodetection kit and antigen unmasking solutions (Vector Laboratories) were used to reduce background staining if applicable. The slides were mounted with Vector Shield (Vector Laboratories) and examined using laser scanning confocal microscope 510 Meta (Zeiss).

Hf cell preparation and Western blot analysis

Primary mouse hf cells were isolated from the dermis of mouse skins by Ficoll density gradient centrifugation after treatment with collagenase 0.35% and DNase 250 units/ml. Protein samples from hf cells were subjected to Western blot analysis. The antibodies and dilutions used: anti-

Dlx3 (1:1000, Morasso lab), anti-K35 (1:1000, Progen) and anti-α– tubulin (1:2000, Abcam). The immunoreactive proteins were detected using the horseradish peroxidase-linked secondary antibody (Vector Laboratories).

Cloning, cell culture, transfection and reporter assays

The −1055 to +134 bp DNA fragment of the K35 promoter was inserted into the pGL3-Basic vector (Promega). Site-directed mutations of putative Dlx3 binding sites on the *K35* promoter were performed using the ExSite™ PCR-based site-directed mutagenesis kit (Stratagene). The V5-tagged Dlx3 was cloned into the pCI-neo vector (Promega).

Transformed PAM212 mouse keratinocytes (Yuspa et al., 1980) were co-transfected with 1 μg of each construct using FuGENE 6 transfection reagent (Roche) (Hwang et al., 2007). Luciferase activity was measured 24–36 h after the transfection using the Dual-Luciferase® Reporter Assay System (Promega). The pRL-TK vector was also co-transfected as an internal control for the assay. Each transfection was done in duplicate and the experiment was repeated three times.

Chromatin immunoprecipitation (ChIP) assays

Primary mouse hf cells or transfected hf cells with pCI-neo-V5-Dlx3 construct were used for ChIP assays (Radoja et al., 2007). Chromatin was incubated with control anti-mouse IgG, anti-Lef1, anti-Dlx3 (Abnova) or anti-V5 antibody (Serotec) overnight at 4°C. The samples were eluted after washing and PCR reactions were performed by sets of specific primers: hair keratin K32 (previous nomenclature Ha2), F: GGCAACACAGGACAGGCTATGGCAG, R: CATGGGGGAGTGTTGATGTTTATACTTGGCCCC; hair keratin K35 F: ACGGGGCTTCTGTTTTACGAGGCCGG, R: CCCTAGCCCGACTTTATACTTCTGCCCCA; Hoxc13 F: GTTAGGGGAGGGGGGCAGAGAGGCTTAATTTGG, R: TACCGAAGTCTCTAAATTGGGGCTTGG; Dlx3, F: GTGTGTGTGTGTGTGTGTGTGTGTATTAGGGGTA, R: CGTGCCTCTCTCCGCGTCCCAAGCCACAGTCAAATG; GAPDH F: TACTAGCGGTTTTACGGGCG, R: TCGAACAGGAGGAGCAGAGAGCGA.

RESULTS

Dlx3 is expressed throughout the hair cycle

The *β-galactosidase* (*LacZ*) gene was inserted by homologous recombination into the *Dlx3* locus downstream of the endogenous *Dlx3* promoter to generate $Dlx3^{Kin/+}$ mice (Fig. 1A). The intergenic (*Dlx3–Dlx4*) region that contains important cis-regulatory elements such as transcriptional enhancers (Sumiyama et al., 2002;Sumiyama et al., 2003) was preserved with the targeting strategy utilized. Homozygous $Dlx3^{Kin/Kin}$ mice recapitulate the early lethality previously reported for ablation of Dlx3 function in Dlx3−/− mice (Morasso et al., 1999). Heterozygous $Dlx3^{Kin/+}$ mice were utilized for X-gal staining to reveal the temporal and spatial expression of Dlx3.

The hair cycle is an ideal system for studying the regulation of cell proliferation, differentiation, and apoptosis in regenerative tissues. The hf is a prominent site of Dlx3 expression during hair development, shown by in situ hybridization (see Fig. S1 in the supplementary material) and visualized by LacZ detection in whole mount and sections of embryos from $Dlx3^{Kin/+}$ mice (Fig. 1B). Detailed analysis of Dlx3 expression throughout the hair cycle using an anti-Dlx3 antibody revealed expression in the hf matrix (Fig. 2A, P1). By the anagen stage (P9), Dlx3 expression extended to the inner root sheath (IRS) and hair-forming compartments including cortex, medulla and cuticle. Nuclear Dlx3 expression persisted in the catagen follicle, and in

the resting, telogen bulge (magnified image in Fig. 2A, P20). The domain of Dlx3 expression is cyclically re-expanded in the first post-natal hf growth cycle (Fig. 2A, P30). To assess the validity of using LacZ expression as readout of endogenous Dlx3 expression, we performed immunohistochemistry with antibodies against Dlx3 and LacZ on sections of P1 of $Dlx3^{Kin/+}$ dorsal skin. As seen in Figure 2B (top panel), the pattern of Dlx3 expression is virtually identical by detection with either anti-Dlx3 or anti-LacZ antibodies. The anti-LacZ utilized in these studies was generated in chicken, which gave versatility in the colocalization studies performed utilizing antibodies raised in rabbit or mouse.

Colocalization studies were performed on histological sections to visualize the co-expression of hf differentiation markers with Dlx3. Keratin 17 (K17) was used as a marker for the ORS in early stages of anagen (Fig. 2B, P9). At this stage, Dlx3 expression is excluded from the ORS. Hair keratins K35 and K85 were used as differentiation markers for the matrix, cortex and cuticle in post-natal anagen phase follicles (Fig. 2B, P30) (Langbein and Schweizer, 2005). Dlx3 was expressed in the hair matrix cells, inner root sheath as well as in hair-forming compartments such as the cortex, medulla, and cuticle. The merged images confirmed that Dlx3 expression encompasses areas of K35 and K85 expression, and is also present in the IRS, which is K35/K85 negative (Fig. 2B).

Disrupted hf development in epidermal-Cre-mediated deletion of Dlx3

As Dlx3−/− mice die during embryogenesis, to understand better the role of Dlx3 during development we generated mice carrying a floxed Dlx3 allele ($Dlx3^{f/f}$) (Fig. 3A). To assess the specific functions of Dlx3 during epidermal development, we ablated Dlx3 expression in the skin by K14cre-mediated inactivation of the conditional Dlx3 allele generating K14cre;$Dlx3^{Kin/f}$ or K14cre;$Dlx3^{f/f}$ lines. The K14cre line utilized has been previously characterized as showing specific expression in the epidermis, hfs and oral epithelium (Andl et al., 2004). Specificity of expression was corroborated by crosses of the K14cre mice with mice from the reporter Rosa26 line, which allows identification of cells expressing Cre and their descendants by β-galactosidase staining (Soriano, 1999). In this study, mice from the $Dlx3^{f/f}$ and $Dlx3^{Kin/f}$ lines were indistinguishable from wild type mice. Mice from either K14cre;$Dlx3^{Kin/f}$ or K14cre;$Dlx3^{f/f}$ lines gave the same results. The role of Dlx3 was demonstrated by analyzing K14cre;$Dlx3^{Kin/f}$ or K14cre;$Dlx3^{f/f}$ mice, where the most noticeable deficiency was complete absence of hair (Fig. 3B). These mutant mice showed aberrant growth and weight loss, and complete and permanent alopecia.

The specificity of Cre-mediated recombination in skin was determined by PCR and Western blot analysis (Fig. 3C, D). Western blots were performed with hf extracts from P12 skin, and verified absence of Dlx3 protein in the K14cre;$Dlx3^{Kin/f}$ hf. Immunofluorescence with anti-Dlx3 demonstrated specific and complete recombination in K14cre;$Dlx3^{Kin/f}$ mice, where no expression of Dlx3 was detected (Fig. 3E), demonstrating efficient K14 Cre-mediated deletion of the Dlx3 floxed allele.

To perform a thorough analysis of the defects in hair development and cycling in the K14cre;$Dlx3^{Kin/f}$ mice, dorsal skin samples were collected from mutant and wild type littermates at 1, 5, 9, 12, 15, 18, 20 and 26 day(s) (P1 through P26), 13 weeks and 26 weeks after birth, sectioned and stained with hematoxylin/eosin (Fig. 4A; see Fig. S2A and Fig. S4 in the supplementary material, where the stages correspond as follows: P9–P12 late stages of postnatal hf morphogenesis, P15 catagen, P20 telogen, P26 first postnatal anagen). At P1, the K14cre;$Dlx3^{Kin/f}$ follicles appeared normal. Structural hf abnormalities were clearly observed by P5 in K14cre;$Dlx3^{Kin/f}$ mice (see Fig. S2A in the supplementary material). By P9, control hfs had formed large hair bulbs and differentiated hair shafts. In contrast, the mutant follicles had not developed hair bulbs and displayed morphologically abnormal undifferentiated shafts, with no apparent keratinized medulla. Dramatic defects in the inner root sheath and hair shaft

of K14cre;Dlx3$^{Kin/f}$ hfs were observed by the end of the anagen phase at P12. By stage P15, utricles (cyst-like structures opening to the epidermis) and enlarged sebaceous glands developed in the upper part of the mutant hfs (Fig. 4A), and were still present at P18 (see Fig. S2A in the supplementary material). At this stage the typical upward movement of the hair shaft and formation of the club structure in the upper hair bulb that characterized control follicles were not observed in the K14cre;Dlx3$^{Kin/f}$ follicles. Furthermore, the aberrant K14cre;Dlx3$^{Kin/f}$ hfs underwent an apparent regression by telogen phase P20, but no re-initiation of the hair cycle was observed by the first postnatal anagen at P26 or in samples from 13 and 26 week old mutant skin (Fig. S4 in the supplementary material)

To evaluate the proliferative capacity of hf through the hair cycle, we performed immunohistochemical analysis with anti-PCNA on sections of wild type and K14cre;Dlx3$^{Kin/f}$ skin (Fig. 4B). During P9–P12 late stages of postnatal hf morphogenesis, the hair matrix cells surrounding the DP and cells of the ORS were stained by anti-PCNA, indicating these cells were proliferating in both wild type and K14cre;Dlx3$^{Kin/f}$ follicles. By stages P15 and P18, PCNA staining was dramatically diminished in wild type follicles, but, by contrast, persisted in K14cre;Dlx3$^{Kin/f}$ hf, particularly in the bulge region and outer root sheath (Fig. 4B). This pattern of proliferation was maintained in K14cre;Dlx3$^{Kin/f}$ follicles at P26. At this stage, wild type follicles displayed elevated proliferation specifically localizing to epithelial matrix cells adjacent to the dermal papilla, whereas in K14cre;Dlx3$^{Kin/f}$ follicles such elevated localized proliferation was absent. A similar pattern of proliferation was observed when using anti-Ki67 (data not shown).

We also performed immunohistochemistry with anti-K15 antibody to follow the development of the bulge region, the location of the hf epithelial stem cells. K15 is preferentially expressed in mouse bulge cells, with variable expression in the immature mouse epidermis during the first weeks after birth, and anti-K15 antiserum may non-specifically label the sebaceous gland (Liu et al., 2003). We observe the predicted expression of K15 in wild type hfs through the hair cycle, where K15 is prominently detected in the hf bulge of the telogen follicles (see Fig. S2B in the supplementary material). The expression pattern of K15 is present in the K14cre;Dlx3$^{Kin/f}$ follicles through the hair cycle, and at stages P15, P18 and P20 it is localized to the enlarged cyst-like structures. However, K15 levels were somewhat diminished in the P26 bulge region of K14cre;Dlx3$^{Kin/f}$ follicles when compared to wild type littermates.

In a few instances, abnormal detection of K1 and K10 expression was observed in the mutant follicles, suggesting a possible ‘epidermalization’ of the hfs in the K14cre;Dlx3$^{Kin/f}$ mice. The expression of sebaceous cell marker, adipophilin was still maintained in mutant follicles although the area of staining became enlarged compared to controls (see Fig. S2C in the supplementary material).

Dlx3 is normally expressed in the differentiated layers of the epidermis and we have previously shown that misexpression of Dlx3 in the basal proliferative layer of the epidermis led to cessation of proliferation and premature induction of terminal differentiation (Morasso et al., 1996). Histological analysis of K14cre;Dlx3$^{Kin/f}$ or K14cre;Dlx3$^{f/f}$ stratified epidermis revealed that the epidermis appears hyperplastic (Fig. 4C). Immunochemical analysis using antibodies against PCNA and epidermal differentiation markers such as K14 and K1 shows an increase in the number of proliferative cells and thickening of the entire stratified epithelium, with the underlying dermis also appearing thickened and hypercellular (Fig. 4C). Taken together with our previous report on Dlx3 misexpressing skin, these findings indicate that the absence of a Dlx3-regulated differentiation signal might lead to epidermal hyperplasia.

Dlx3 is an essential regulatory factor in the signaling pathway controlling hair development

To determine the role of Dlx3 in IRS and hair shaft differentiation, we assessed by immunofluorescence the expression levels of established signaling molecules and mediators such as Lef1, β-catenin and phospho-SMAD1/5/8 in K14cre;Dlx3$^{Kin/f}$ and control littermates (Fig. 5A). The transcriptional regulator Lef-1, and β-catenin, which localizes to the nucleus and complexes with Lef1 to activate target genes, were analyzed as effectors of the Wnt pathway. BMP signaling, assayed by staining for phospho-SMAD1/5/8, is normally expressed in differentiating hair matrix cells that give rise to the IRS and hair shaft medulla, cortex and cuticle (Andl et al., 2004). At P1, detection of these factors was reduced although it was present in the same areas in mutant and control follicles. By P9, the expression of all factors was reduced and altered. The expression of Lef1, while still present in the K14cre;Dlx3$^{Kin/f}$ underdeveloped bulb matrix cells, was highly diminished when compared to the control littermate hfs. This could be a consequence of the underdevelopment of the hair bulb in the K14cre;Dlx3$^{Kin/f}$ follicles (Fig. 5A). For β-catenin, expression was also reduced, although in some cells still localized to the nucleus. The detection of phospho-SMAD1/5/8 was seen in the differentiating matrix cells in the upper part of the follicle, but no detection was found in the lower section of the underdeveloped bulb region in the K14cre;Dlx3$^{Kin/f}$ follicles.

Co-localization of Lef1 and phospho-SMAD1/5/8 with Dlx3 was visualized in the hair forming compartment and IRS at stage P1 by immunofluorescent staining. Lef1 expression is also detected in the dermal region, where Dlx3 is not expressed (Fig. 5B). It is established that Lef1-positive hair matrix cells respond to Wnt signaling by activating hair-specific keratin genes (Merrill et al., 2001). Analysis of the Dlx3 proximal promoter led to the identification of a putative Lef1 binding motif (Fig. 5C). To further investigate if Dlx3 is a direct target of Lef1 regulation, we performed Chromatin immunoprecipitation (ChIP) with anti-Lef1 antibodies on genomic DNA from primary mouse hfs. The results were normalized to IgG, and a set of PCR primers for GAPDH was used to confirm the specificity of immunoprecipitated genomic DNA (Fig. 5C). The PCR results verified that Lef1 binds the Dlx3 promoter in vivo, demonstrating that Dlx3 is a potential immediate target of Lef1.

Dlx3 is a crucial regulator of transcriptional factors and hair keratin genes in hf development

To establish if epidermal-specific Dlx3 deletion affects the expression of hf differentiation markers and transcriptional regulators necessary for the formation of the IRS and hair shaft, we studied the expression of the following markers in skin samples at P1 and P9: the homeobox transcription factor Hoxc13, that is expressed in the precortex, cuticle, cortex and medulla; the transcriptional regulator Gata3 normally expressed in the inner root sheath; the hair keratin marker AE13, expressed in the hair shaft cortex and cuticle; and AE15 (trichohyalin), expressed in the IRS and medulla. No detection of these proteins was found in the K14cre;Dlx3$^{Kin/f}$ hfs during early stages of hair development at P1, with significantly reduced expression at anagen stage P9 when compared to wt littermates (Fig. 6A). Similar results were obtained by western blot using hf extracts, where hair keratin *K35* levels were highly decreased in the K14cre;Dlx3$^{Kin/f}$ hfs at P10 (see Fig. S3A in supplementary material). Our data indicate that Dlx3 is an essential regulator of Hoxc13, Gata3, AE13 and AE15, and plays an indispensable role during normal hf differentiation.

A conserved TAAT-motif was identified as a high-affinity binding site for numerous homeodomain proteins (Desplan et al., 1988). The Hoxc13 homeodomain protein is a regulator that binds to TAAT-motifs in the promoter regions of the hair keratin *K32 (*previous nomenclature *Ha2), K35 (Ha5)* and *K37* (*Ha7*) genes (Jave-Suarez et al., 2002). The Dlx3 consensus-binding motif has been established as TAATT (Feledy et al., 1999), sharing the preferred DNA-recognition core of many homeodomain proteins. Previous studies have reported that the surrounding 5′ and 3′ base pairs of the motif are important for selecting in

vitro binding sites (Svingen and Tonissen, 2003). Competition binding assays (EMSA) were performed to compare specificity of the binding of Dlx3 versus Hoxc13 in the motifs previously identified in the hair keratin promoters (see Fig. S3B in the supplementary material). The sequence and location of each putative binding site analyzed are indicated in Supplemental Figure S3C. The optimal consensus DNA binding sequence for Dlx3 (Feledy et al., 1999) was ^{32}P-end-labeled, while putative Dlx3 binding sites derived from the promoters of hair keratins and *Hoxc13* genes were used as unlabeled competitors. The consensus (Con) binding sequence for Dlx3 and unspecific (Un) DNA were used as control competitors. Specific binding sites for Dlx3 were identified in the promoter regions of hair keratins *K32*, *K35* and *K37* genes. Several of these sites in the hair keratin promoters had been previously described as Hoxc13 binding sites. We also examined the ability of Dlx3 to bind to TAAT-motifs present in the proximal promoter of *Hoxc13* gene. EMSA and competition assays indicated that Dlx3 strongly bound to a motif present approximately 600 bp upstream of the transcriptional start site of Hoxc13 (between −564 and −599bp). These in vitro results support the role of Dlx3 as an immediate upstream regulator of Hoxc13 and hair keratins.

The ability of Dlx3 to transactivate hair keratin genes was confirmed by reporter assays. A 1.2-kb DNA fragment of *K35* promoter was cloned into a luciferase reporter construct and mutations were introduced specifically into binding sites numbers 2, 6 and 7 of the *K35* promoter (Fig. 6B, C). These sites were shown in EMSA competition assays (see Fig. S3B in the supplementary material), to compete (sites 2 and 7) or not compete (site 6) for optimal Dlx3 binding. Wild-type (wt) and mutant constructs (K35mut2/Luc, K35mut6/Luc and K35mut7/Luc) were co-transfected with a Dlx3 expression vector into a PAM212 keratinocyte cell line. In the presence of Dlx3, the luciferase activity of the wild-type K35/Luc construct increased 4 fold when compared to baseline in absence of Dlx3. For the mutant constructs K35mut2/Luc and K35mut7/Luc, the luciferase activity was 40% and 48% of wild type levels, respectively. However, a mutation in site 6 of the *K35* promoter, K35mut6/Luc, did not significantly affect reporter gene expression when compared to the wild type construct (Fig. 6B). When performing EMSA with the motifs 2 and 7, a complex was formed, and supershift assays with anti-Dlx3 antibody corroborate the presence of Dlx3 in the complex (see Fig. S3D in the supplementary material). No complex or supershift was detected when performing the assays with binding site 6. These results support the hypothesis that Dlx3 regulates the transcriptional activity of genes involved in hair development by binding to one or more TAAT-motifs within their promoter region.

To examine if Dlx3 bound directly in vivo to the regulatory sequences of Hoxc13 and hair keratins, chromatin immunoprecipitation (ChIP) assays were performed on genomic DNA from hf cells using anti-Dlx3 and anti-V5 antibodies (Fig. 6C). The numbers in the triangles correspond to the binding sites confirmed as binding motifs by EMSA (see Fig. S3B in the supplementary material). The ChIP results verify that Dlx3 binds the promoter of hair keratin genes and *Hoxc13* in vivo. The results were normalized to control IgG and their specificity was verified using PCR for GAPDH. Altogether, these results demonstrate that Dlx3 is an immediate upstream regulator of the hair keratin genes as well as of the transcriptional regulator Hoxc13.

Dlx3 is essential for the cyclic postnatal regeneration of the hf

An important finding in our studies on K14cre;Dlx3$^{Kin/f}$ mutants is that ablation of epidermal Dlx3 leads to inability to re-initiate the hf growth cycle postnatally (Fig. 4), where the inability to initiate the first post-natal anagen might be due to the lack of Dlx3 function and, in addition, to altered responsiveness of the abnormal hf.

Stimulation of hf stem cell proliferation at anagen onset is thought to involve suppression of BMP signaling, and activation of the Wnt/beta-catenin pathway (Plikus et al., 2008). Consistent

with this report, deletion of Bmpr1a causes upregulated stem cell proliferation, as well as failure of hf differentiation, while activation of beta-catenin signaling promotes anagen onset (Kobielak et al., 2003; Van Mater et al., 2003; Andl et al., 2004; Kobielak et al., 2007). To address the underlying mechanisms responsible for the failure of anagen onset in the Dlx3 mutant, we performed immunohistochemical analysis with antibodies against the Wnt transcriptional effector Lef1 and phospho-SMAD1/5/8 on skin sections at P18, P20 and P26 (Fig. 7A). Mutant hf epithelial cells displayed nuclear Lef1 expression, but a complete absence of phospho-SMAD1/5/8 at all of these stages. These data suggest that Dlx3 might be required for BMP signaling in telogen as well as early anagen, and that the continual proliferation observed in mutant ORS cells in catagen and telogen may be due in part to absence of BMP signaling.

DISCUSSION

Hf development and Dlx3: Dlx3 controls inner root sheath and hair shaft differentiation

It has recently been proposed that the different follicular cell lineages derive from a stem cell-harboring region to generate distinct follicular compartments (Langbein and Schweizer, 2005). In this model, cells from the upper internal ORS directly give rise to the medulla and cortex, while the keratin-free germinal matrix compartment (GMC) contains amplifying cells that differentiate into the IRS and cuticle (Langbein and Schweizer, 2005). As embryonic hf morphogenesis reaches completion (P9), expression of the homeobox transcription factor Hoxc13 is detected in the hair-shaft forming compartments of the matrix, cuticle, cortex and medulla (Godwin and Capecchi, 1998; Langbein and Schweizer, 2005; Potter et al., 2006). Hoxc13 directly regulates expression of the hair keratin genes such as *K32*, *K35* and *K37* and of the transcription factor Foxq1 (Jave-Suarez et al., 2002; Potter et al., 2006), with its role in hair development being supported by the findings that ablation or overexpression of Hoxc13 in differentiating keratinocytes results in a hairless phenotype (Godwin and Capecchi, 1998; Tkatchenko et al., 2001). However, little is known about upstream regulation of Hoxc13 expression. Here we demonstrate that Hoxc13 is a target of Dlx3 in the transcriptional control of hair shaft development. Furthermore, microarray analysis of RNA derived from mouse skin samples has shown that Dlx3 is part of a gene expression network that includes the transcription factor Hoxc13 and many of the hf keratin genes (A. Balmain, personal communication). Other transcription factors such as Gata3 are required for proper differentiation of the IRS (Kaufman et al., 2003; Kurek et al., 2007). We find that the mutant K14cre;$Dlx3^{Kin/f}$ hfs have significantly reduced levels of Gata3.

Based on Dlx3 expression in the IRS and hair-shaft forming compartments of the matrix, cuticle, cortex and medulla, on the ability of Dlx3 to directly regulate hair keratins and Hoxc13, and the reduced expression of Gata3 in the mutant IRS, we propose a model in which Dlx3 acts as a crucial controller of follicle differentiation (Fig. 7B).

How far upstream in the follicular signaling pathway is Dlx3 function required?

BMP and WNT have been determined as crucial signaling pathways during early hair morphogenesis and later in the differentiation of the inner root sheath and hair shaft (Fuchs, 2007). The Wnt/β-catenin pathway is required for hf morphogenesis and differentiation (Gat et al., 1998; Millar et al., 1999; Huelsken and Birchmeier, 2001; Huelsken et al., 2001; Andl et al., 2002; Narhi et al., 2008), and Smad7-induced β-catenin degradation disrupts hf development (Han et al., 2006).

In this study, we show that Dlx3 is a direct target of Lef1 regulation. During hair morphogenesis, Lef1 and Dlx3 expression overlap in the precortical region at early anagen (Fig. 5B). By full anagen, Dlx3 is expressed primarily in the differentiating parts of hf such as

hair shaft and inner root sheath, whereas Lef1 expression is closer to the dermal papilla in the developed hair bulb (Fig. 5A). Our results show that mutant K14cre;Dlx3$^{Kin/f}$ hfs have diminished and aberrant Lef1 expression, which is maintained in the differentiating matrix and IRS cells, but is completely abolished in the GMC of the underdeveloped bulbs. We propose that as a direct target of Lef1, Dlx3 is an early effector of the Wnt signaling pathway in matrix cells that have migrated from the GMC and started differentiating to form the IRS and hair shaft lineages. In the absence of Dlx3, this differentiation process does not proceed, consistent with known roles of Dlx3 in directing cells to differentiate.

Our results also show co-localization of phospho-SMAD1/5/8 and Dlx3, which is consistent with a regulatory role of BMP signaling of Dlx3. Previous results have shown Dlx3 to be a direct target of BMP signaling (Luo et al., 2001; Park and Morasso, 2002; Hassan et al., 2004). However, by stages P18, P20 and P26, phospho-SMAD1/5/8 staining is absent from the hair matrix and bulge region of the Dlx3 mutant follicles, indicating that BMP signaling is disrupted during hair regression and regeneration (Fig. 7).

Essential role of Dlx3 during re-initiation for cycling in first postnatal anagen

Regenerating hfs are derived from a reservoir of stem cells in the bulge, whose progeny migrate and will generate the distinct hf cell lineages. A recent study has demonstrated that cyclic dermal BMPs control hf epithelial stem cell activation, with high levels of BMP signaling being associated with the resting, non-proliferative phase of hf growth cycle (Plikus et al., 2008). Furthermore, BMP signaling within the DP appears to be important in instructing the bulge stem cells to initiate the next cycle of hf formation (Rendl et al., 2008). An important finding in our study is that epithelial Dlx3 ablation leads to persistent proliferation in the regressing hf and in the inability of the hf to reinitiate the first postnatal anagen despite expression of Lef1 and K15. Significantly, active BMP signaling is completely absent in mutant follicles at these stages, suggesting that persistent proliferation may be due in part to lack of BMP signaling. Altogether, our data support a model where Dlx3 and BMP may act in a positive feedback loop in a certain subset of follicle cells. This is suggestive of the feedback loop between BMP4 and the Msx1 homeobox gene during early tooth development (Tucker and Sharpe, 2004). Another homeodomain Lhx2 is expressed in the postnatal bulge compartment, and studies show that Lhx2 functions to specify and maintain the hf stem cell character but not their differentiation (Rhee et al., 2006). It is plausible that Dlx3 expression in the telogen bulge region plays an important role in the stem cell differentiation in contrast to the role of Lhx2 in maintaining the undifferentiated state. Since the follicles do not appear to have entered a normal telogen phase, subsequent phenotypic abnormalities might reflect not only any effects of Dlx3 deficiency on anagen induction, but also the altered responsiveness of an abnormal hf. Altogether these important findings show that in the absence of Dlx3-coordinated differentiation the new anagen phase cannot be initiated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard abbreviations used

BMP
bone morphogenetic protein

ChIP
chromatin immunoprecipitation

DP
dermal papilla

Dlx3
Distal-less 3

hf
hair follicle

hs
hair shaft

IRS
inner root sheath

ORS
outer root sheath

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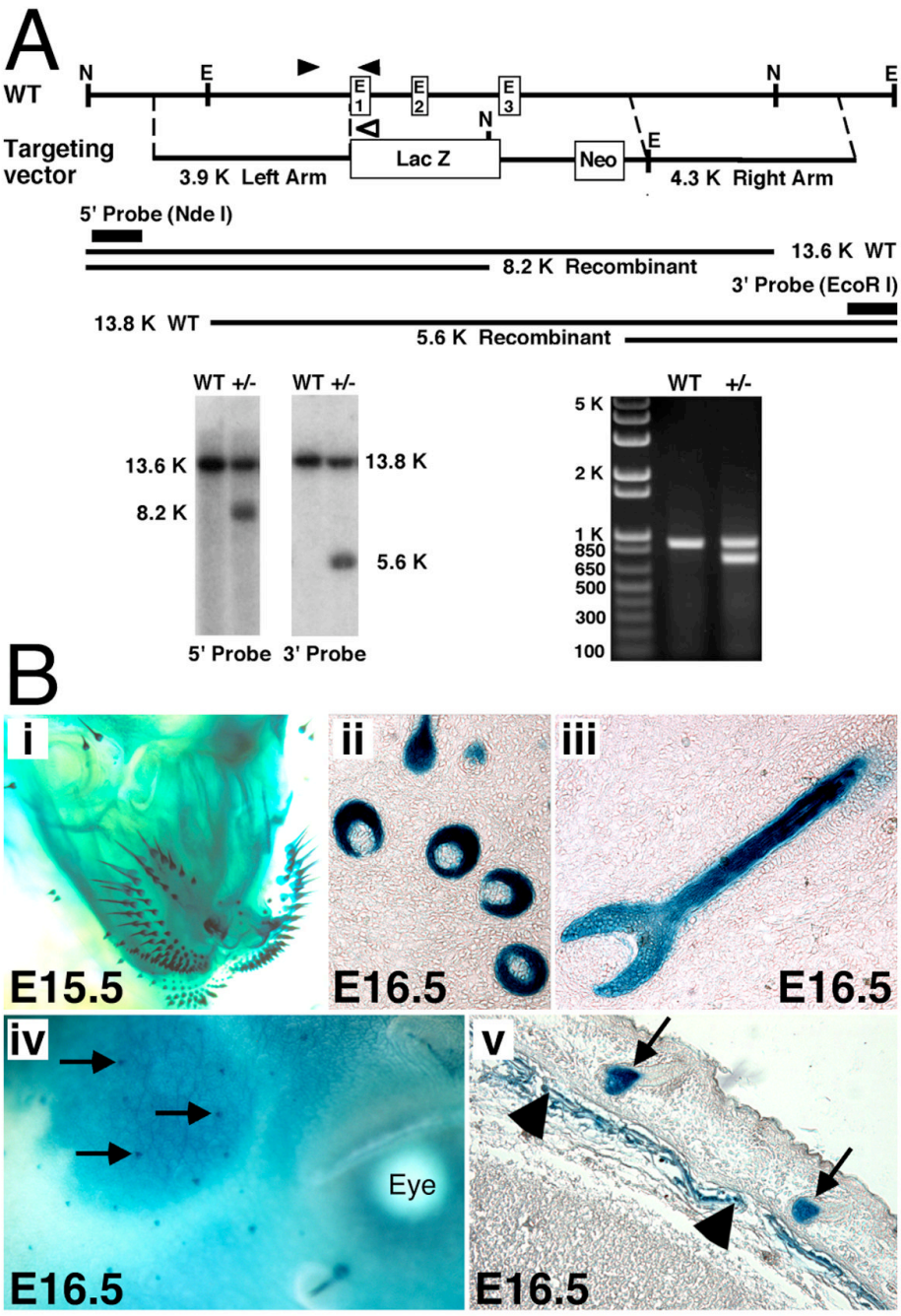

Fig. 1. *LacZ* knockin into the *Dlx3* locus and Dlx3 expression pattern

(**A**) Exon-intron organization and enzyme restriction patterns of murine *Dlx3* locus (N, NdeI and E, EcoRI), before and after homologous recombination (Upper panel). Genotyping by Southern blot (lower panel, left) and PCR (lower panel, right) of wild type (WT) and LacZ knockin (+/−; $Dlx3^{Kin/+}$) mice. Open and black arrowheads indicate the localization of the oligonucleotides used in the PCR reactions. (**B**) Whole-mount X-gal staining shows Dlx3/LacZ expression patterns in the $Dlx3^{Kin/+}$ mice. i) clarified head of embryological stage, E15.5 $Dlx3^{Kin/+}$ embryo by benzyl-benzoate/benzyl alcohol after X-gal staining showing expression in the vibrissae; ii) cross and iii) sagittal section of vibrissae at E16.5. iv) Dlx3/LacZ expression

in hfs in the epidermis of E16.5 head (arrows) and v) sagittal section through the head, showing expression in the intramembranous bone of the cranium (arrowheads) and the hf (arrows).

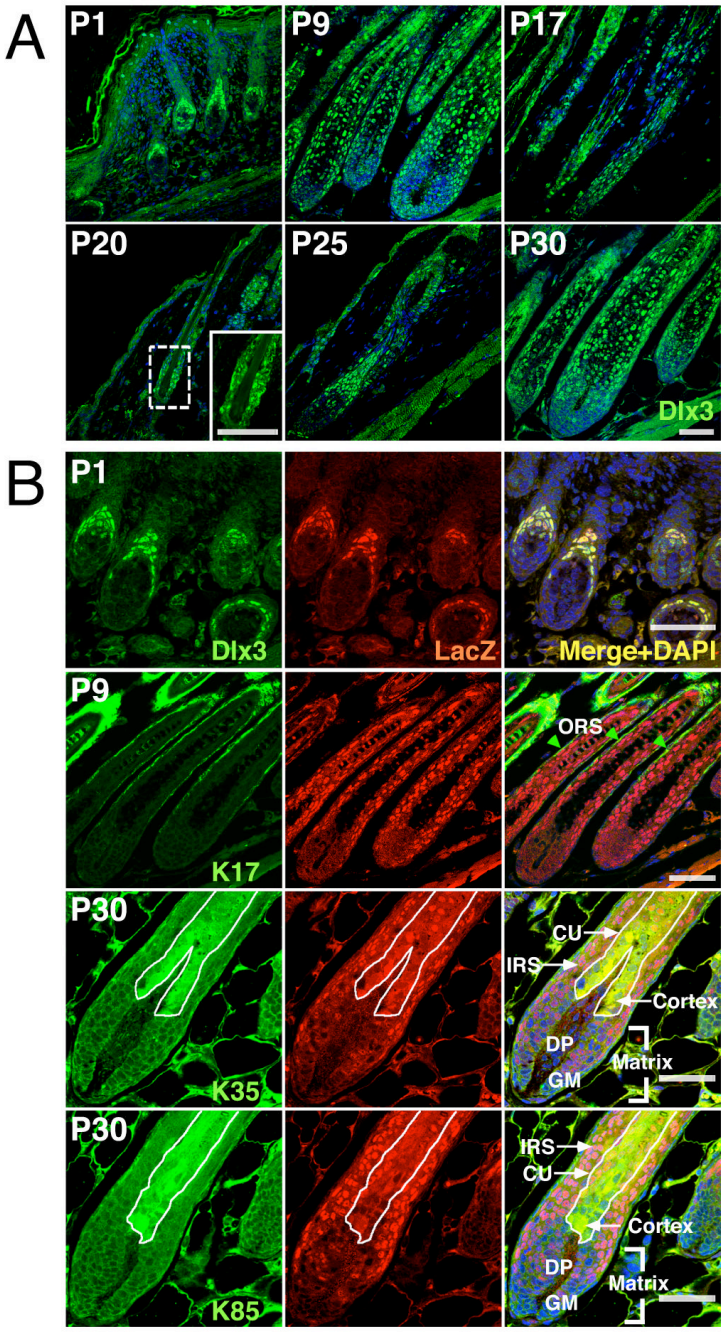

Fig. 2. Dlx3 expression during hf development

(**A**) Dlx3 expression was detected using an anti-Dlx3 antibody in the hair matrix cells at postnatal day 1 (P1, early anagen), P9 (full anagen), P17 (catagen), in the telogen bulge cells at P20 (higher magnification of bulge area in box), P25 (the onset of first postnatal anagen) and P30 (first postnatal anagen). (**B**) Immunofluorescence studies on P1 hf using anti-Dlx3 (green) and anti-β-galactosidase (red) to demonstrate co-localization of Dlx3 and LacZ expression (top panel). Immunofluorescence with anti-keratin antibodies (green): anti-K17 at P9, and anti-K35 and anti-K85 at P30. Merged images are shown with DAPI staining at the right panels. ORS, outer root sheath; IRS, inner root sheath; CU, cuticle; DP, dermal papilla. Scale bar, 50 μm.

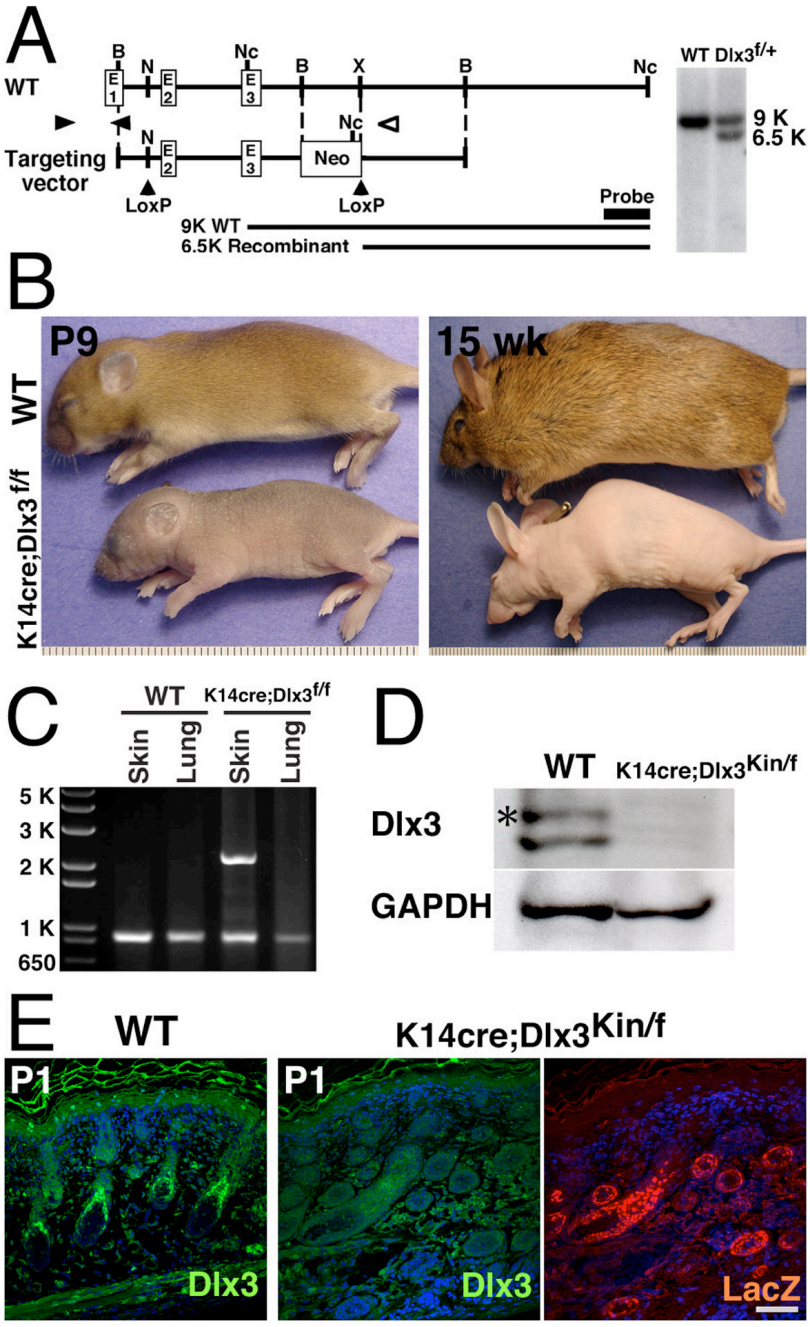

Fig. 3. Conditional deletion of Dlx3 in the epidermis using K14cre

(**A**) Gene targeting to generate the floxed-Dlx3 mice. Left panel, LoxP sites were inserted into the NotI site (N) between the first and second exons of the Dlx3 gene and immediately downstream of the neomycin gene (Neo). Open and black arrowheads indicate the localization of the oligonucleotides used in the PCR reactions. Right panel, recombination in the ES cells was assessed by Southern blot analysis, WT, wild type and Dlx3$^{f/+}$, heterozygote for floxed Dlx3 allele. (**B**) Gross phenotype of wild type (top) and K14cre;Dlx3$^{f/f}$ (bottom) mice at P9 (left) and 15 wk (right). (**C**) Genotype by PCR for wild type (WT) and K14cre;Dlx3$^{f/f}$ mice showing epidermal specific recombination of the Dlx3 allele in the K14cre;Dlx3$^{f/f}$ skin. (**D**) The specific deletion of Dlx3 expression was detected in the K14cre;Dlx3$^{Kin/f}$ hf extracts by

Western blot analysis. Asterisk (*) indicates Dlx3 specific band. (**E**) The specificity of Cre-mediated recombination was also determined by immunohistochemistry on skin sections from WT and K14cre;Dlx3$^{Kin/f}$ littermates. Dlx3 (green) expression is shown in the matrix cells of P1 hf in the WT skin, however only the LacZ (red) expression is detected in the K14cre;Dlx3$^{Kin/f}$ skin. Images are shown with DAPI staining to denote nuclear staining. Scale bar, 50 μm.

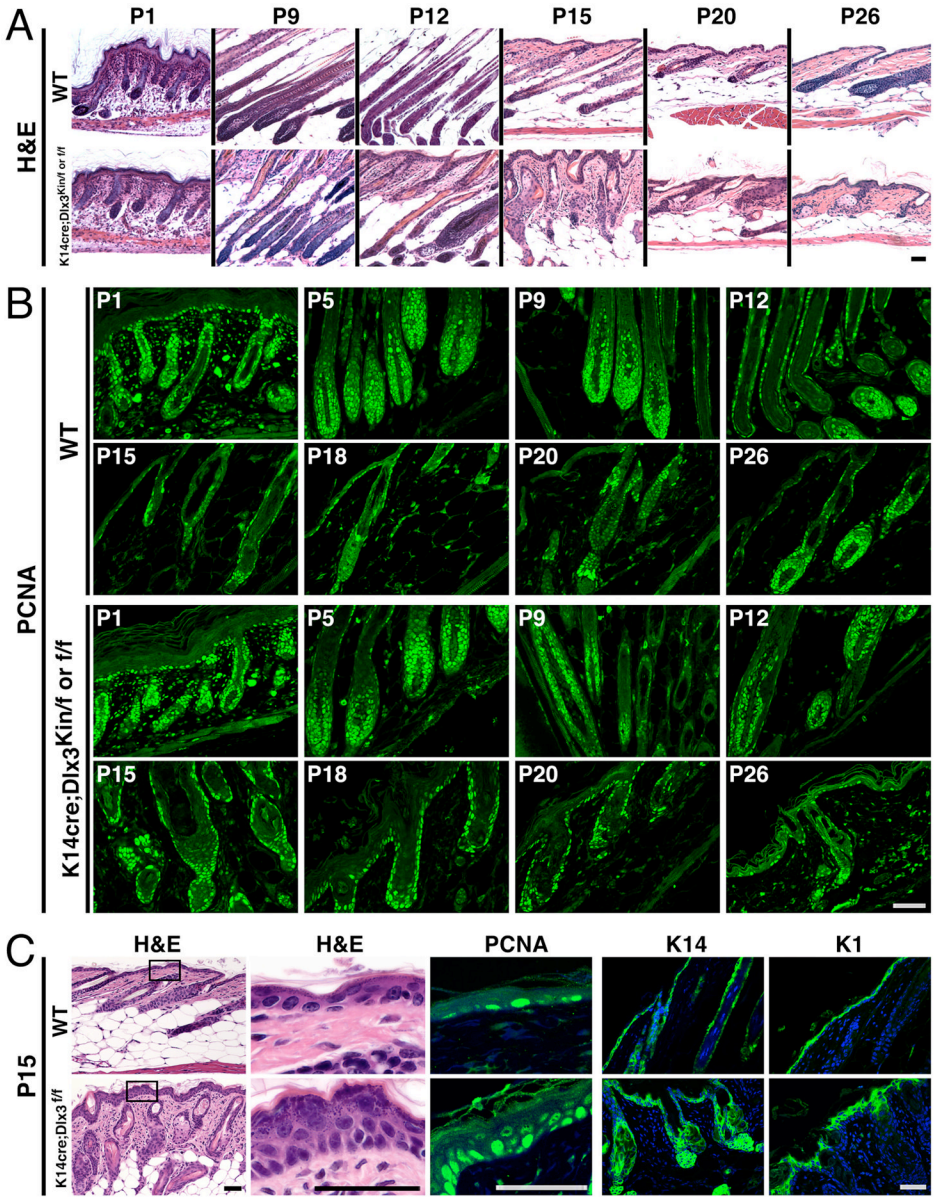

Fig. 4. Abnormalities in the hair growth and cycling in Cre-mediated conditional Dlx3 knockout mice at late stages of postnatal hf morphogenesis

(**A–B**) Skin samples were obtained from both wild type (WT) and mutant (K14Cre;Dlx3$^{Kin/f}$ or K14Cre;Dlx3$^{f/f}$) littermates at P1, P9, P12, P15, P20, and P26, and the deparaffinized sections were stained with hematoxylin/eosin (A) and anti-PCNA antibody (B, green). (**C**) Skin sections of wild type (WT) and mutant K14Cre;Dlx3$^{f/f}$ at P15 were stained with hematoxylin/eosin and antibodies against PCNA, K14 and K1. Scale bar, 50 μm.

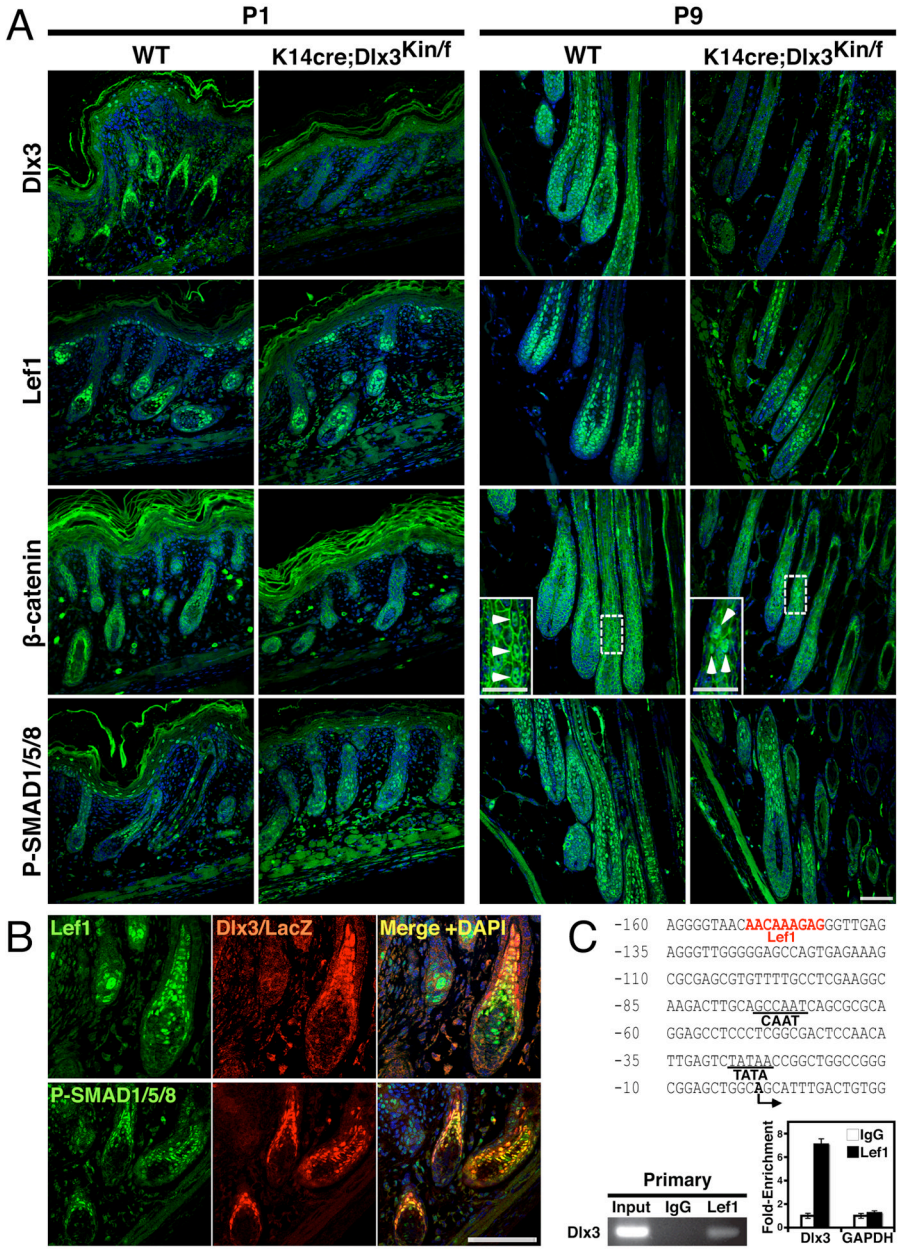

Fig. 5. Follicular signaling in the regulation of Dlx3 expression

(**A**) Expression of Lef1, β catenin and SMAD1/5/8 were determined in control and conditional knockout skin to analyze effects of absence of epithelial Dlx3 at P1 and P9 anagen stages. (**B**) Co-localization of Dlx3/LacZ with Lef1. The skin sections of wild type ($Dlx3^{Kin/+}$) at P1 were stained with by anti-Lef1, anti-phospho SMAD1/5/8, and anti-β-galactosidase antibodies (red, middle), and the merged images are shown with DAPI staining. (**C**) Lef1 direct binding in vivo to the *Dlx3* promoter was demonstrated by ChIP assays using Lef1 antibody. Putative Lef1 binding site in the mouse *Dlx3* promoter sequence (−160 to +15bp) is indicated in red letters. CCAAT box and TATA box are underlined. The transcriptional start site is indicated by an arrow. Bottom left shows gel image of ChIP assay and right panel shows the specificity of the ChIP assays, which was determined by both control IgG antibody and a set of PCR primers for GAPDH. Scale bar, 50μm.

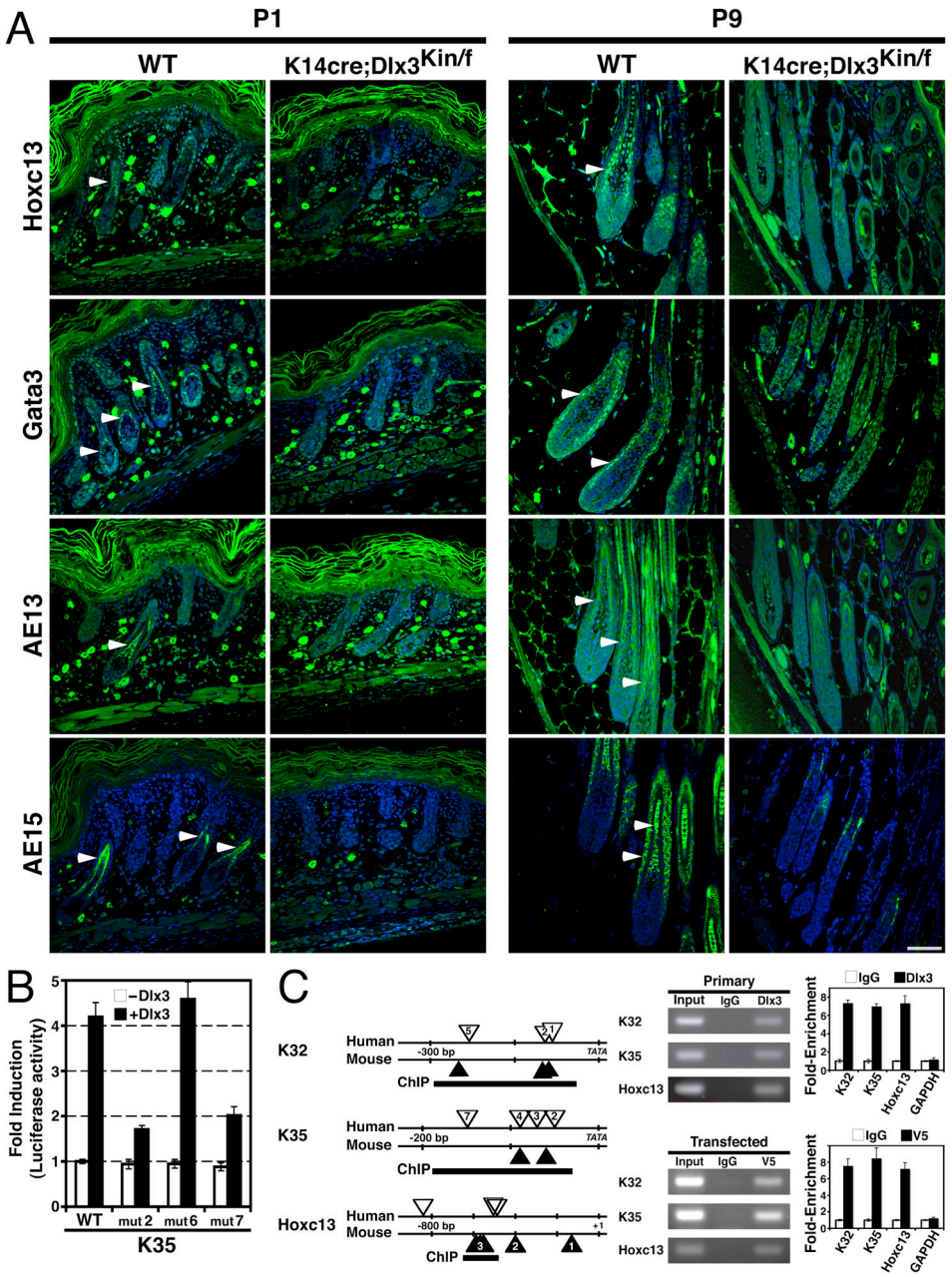

Fig. 6. Dlx3 is an indispensable regulator for the expression of follicular-specific structural and transcription factors

(**A**) Expression of Hoxc13, Gata3 and A13 and A15 hair keratins were significantly reduced in the K14Cre;Dlx3$^{Kin/f}$ follicles at P1 and P9 anagen stages. White arrowheads point to expression areas of each factor. Scale bar, 50 μm. (**B**) Luciferase reporter assays of the 1.2-kb *K35* promoter and mutants no.2, 6 and 7 sites in the absence and presence of Dlx3 co-expression. (**C**) ChIP assays were performed in primary and transfected mouse hf cells using anti-Dlx3 and anti-V5 antibodies respectively. Triangles indicate the putative binding sites in the promoters for hair keratins (*K32* and *K35*) and *Hoxc13*, which are conserved between human (white) and mouse (black). Each number in the triangles correspond to the sequences analyzed by EMSA (see Fig. S3B in the supplementary material). The PCR amplified areas

are indicated by black lines in the mouse promoters for hair keratins (*K32* and *K35*) and *Hoxc13*. The gel images for each ChIP assays are shown in middle panels and the specificity of the amplified fragments was verified by sequencing. Right panels show the specificity of the ChIP assays, which was determined by both control IgG antibody and a set of PCR primers for GAPDH.

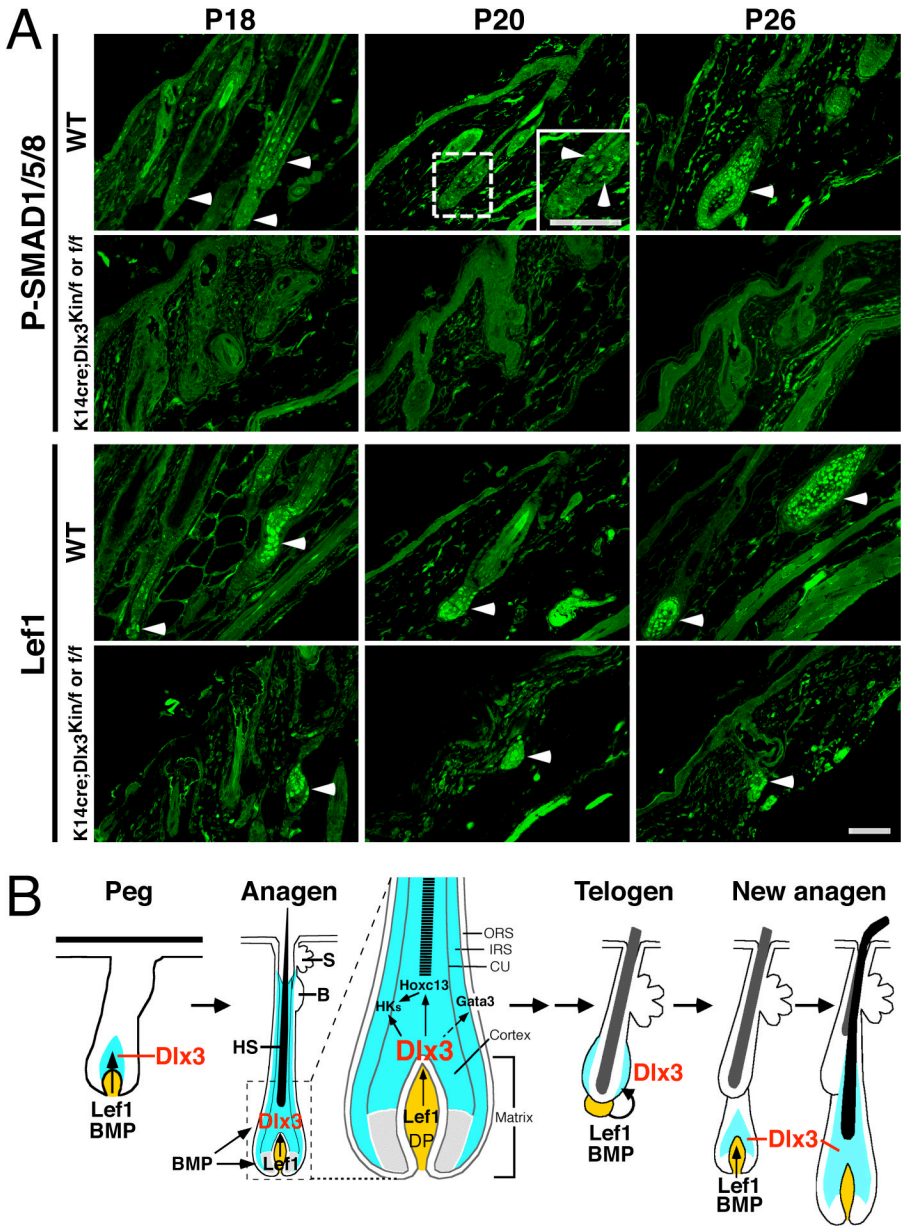

Fig. 7. Role of Dlx3 during hair cycling

(A) phospho-SMAD1/5/8 and Lef1 expression in K14Cre;Dlx3$^{Kin/f}$ and K14Cre;Dlx3$^{f/f}$ at P18, P20 and P26. White arrowheads indicate specific expression. Scale bar, 50 μm. (**B**) Schematic diagram of Hf morphogenesis and onset of a new hair cycle. The area of Dlx3 expression is indicated in blue; dermal papilla in yellow, keratin-free germinal matrix compartment in shaded gray. Dlx3 expression is first detected in the hair matrix; by anagen stage Dlx3 is present in all differentiating hair compartments (matrix, cortex, cuticle and IRS) and later is detected in the telogen bulge. Our results demonstrate that Dlx3 is a target of Wnt, and co-localization of phospho-SMAD1/5/8 and Dlx3 is consistent with a regulatory role of BMP signaling of Dlx3. Our results determine that Dlx3 is a direct transcriptional regulator of Hoxc13, Gata3 and hair keratins and that Dlx3 expression is necessary for the re-initiation of the hair cycle. HS, hair shaft; S, sebaceous gland; B, bulge; ORS, outer root sheath; IRS, inner root sheath; CU, cuticle; DP, dermal papilla.