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Distinct Functions for Wnt/β-Catenin in Hair Follicle Stem Cell Proliferation and Survival and Interfollicular Epidermal Homeostasis

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SUMMARY

Wnt/β-catenin signaling is a central regulator of adult stem cells. Variable sensitivity of Wnt reporter transgenes, β-catenin's dual roles in adhesion and signaling, and hair follicle degradation and inflammation resulting from broad deletion of epithelial β-catenin have precluded clear understanding of Wnt/β-catenin's functions in adult skin stem cells. By inducibly deleting β-catenin globally in skin epithelia, only in hair follicle stem cells, or only in interfollicular epidermis and comparing the phenotypes with those caused by ectopic expression of the Wnt/β-catenin inhibitor *Dkk1*, we show that this pathway is necessary for hair follicle stem cell proliferation. However, β-catenin is not required within hair follicle stem cells for their maintenance, and follicles resume proliferating after ectopic *Dkk1* has been removed, indicating persistence of functional progenitors. We further unexpectedly discovered a broader role for Wnt/β-catenin signaling in contributing to progenitor cell proliferation in nonhairy epithelia and interfollicular epidermis under homeostatic, but not inflammatory, conditions.

INTRODUCTION

The Wnt/β-catenin signaling pathway is broadly utilized in development and controls the activity of embryonic and adult stem

β-catenin for degradation. As a consequence, β-catenin accumulates in the cytoplasm and translocates to the nucleus where it partners with members of the LEF/TCF family of DNA binding factors to activate target gene expression (McNeill and Woodgett, 2010). Members of the Dickkopf (DKK) family of secreted Wnt inhibitors specifically inhibit Wnt/LRP signaling by forming a complex with LRP and Kremen (KRM) receptors that is internalized, removing LRP from the membrane (Mao et al., 2002).

Certain Wnt ligands and Wnt-FZD pairings initiate signaling through noncanonical pathways that are independent of both LRP and β-catenin. Furthermore, the β-catenin pathway can be activated by non-Wnt ligands (McNeill and Woodgett, 2010). Adding to the complexity of its biological functions, β-catenin is a component of adherens junctions and plays dual roles in adhesion and signaling (Incassati et al., 2010). Thus manipulation of β-catenin may lead to phenotypes that are independent of Wnt ligands and LRP.

Hair follicles (HFs) regenerate periodically throughout life, providing an accessible system for delineating molecular mechanisms controlling adult stem cell proliferation and maintenance. In mice, HF morphogenesis is completed in the 2 weeks following birth, when rapidly proliferating epithelial matrix cells almost completely surround the mesenchymal component of the HF, known as the dermal papilla (DP), and differentiate to produce the hair shaft and an inner root sheath (IRS) that molds the hair shaft as it emerges from the follicle. The follicle is bounded by an outer root sheath (ORS) that is contiguous with the epidermis and contains a population of rarely cycling epithelial stem cells in a specialized niche known as the bulge (Myung and Ito, 2012) (Figure 1A).

At the end of the growth phase (anagen), the lower two-thirds of the follicle, including the matrix and lower part of the IRS,

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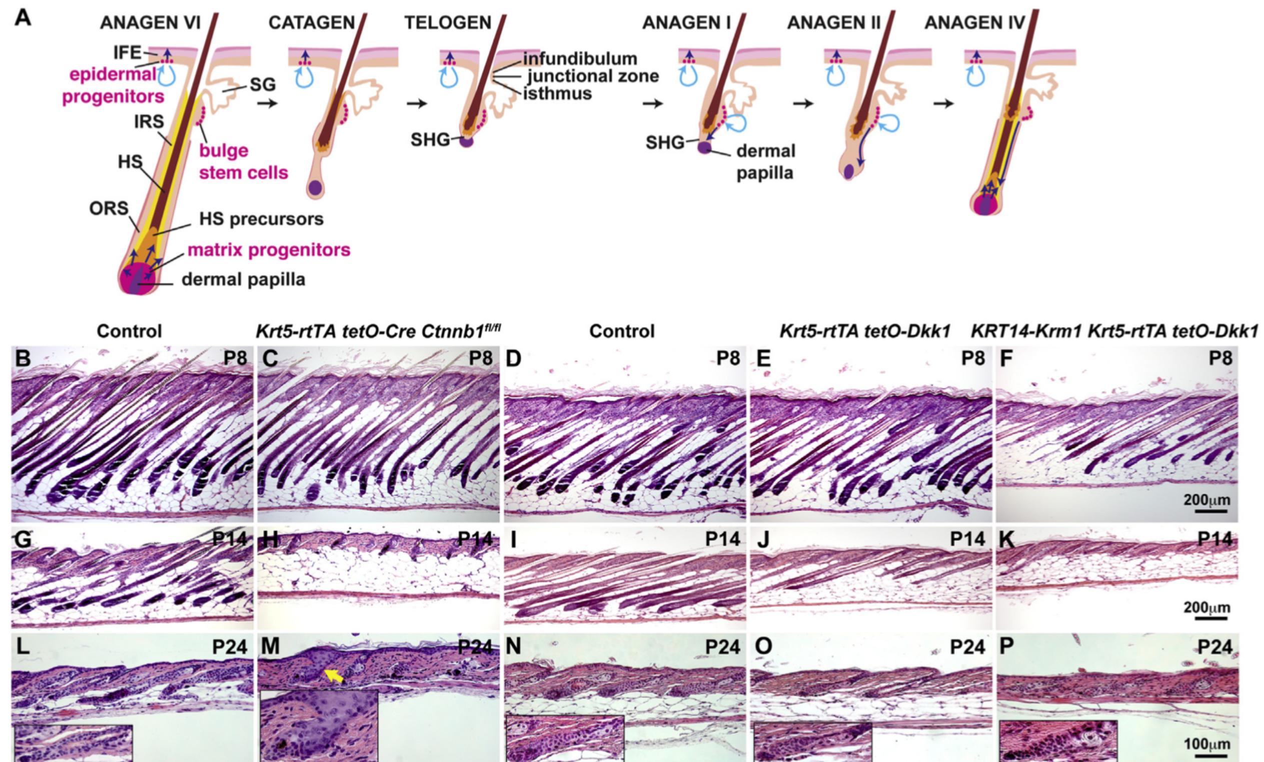

Figure 1. Inducible Postnatal Ectopic Expression of *Dkk1* or Deletion of Epithelial β-Catenin Causes Rapid Regression of Anagen HFs

(A) Schematic of HF growth cycle and structure. Light blue arrows indicate stem cell self-renewal. Dark blue arrows indicate cell movements.

(B–P) *Krt5-rtTA tetO-Cre Ctnnb1$^{fl/fl}$* mutants, *Dkk1* transgenics and *Dkk1*/*Krm1* transgenics, and their respective littermate controls were induced from P4 (B–K) or P7 (L–P) and H&E stained skin sections were photographed at the stages indicated. Arrow (M) indicates structural abnormalities in β-catenin mutant HF. Insets in (L–P) represent higher magnification photographs.

Scale bars: (B)–(K), 200 μm; (L)–(P), 100 μm. See also Figure S1.

(SHG) cells that abut the DP. SHG cells possess lower proliferative potential than bulge cells in vitro, but in vivo they can replenish the bulge following its destruction, indicating that they hold stem cell potential (Myung and Ito, 2012). Onset of a new anagen growth phase is preceded by proliferation of SHG cells, which begin to populate a new matrix, while transient proliferation of bulge cells occurs in very early anagen (Myung and Ito, 2012). Additional stem cell populations in the HF include Lrig1-expressing cells in the junctional zone between the bulge and the infundibulum that can contribute to adjacent interfollicular epidermis (IFE) but do not give rise to the bulge or lower follicle and Lgr6-positive cells in the isthmus that can contribute to sebaceous gland and IFE (Myung and Ito, 2012). Despite intense investigation, the molecular signals regulating HF proliferation and maintenance of the bulge stem cell population are not fully understood.

Wnt/LRP/β-catenin signaling is required for embryonic HF morphogenesis but is dispensable for development of IFE (Andl et al., 2002; Huelsken et al., 2001). Forced activation of β-catenin signaling converts embryonic ectoderm to an HF-like

high levels of β-catenin signaling direct acquisition of appendage identity.

Nuclear-localized β-catenin and/or Wnt reporter transgene activity have been described in HF SHG at anagen onset and in the matrix, DP, and hair shaft precursor cells during anagen but are low or undetectable in telogen HFs (DasGupta and Fuchs, 1999; Maretto et al., 2003). Loss of β-catenin in postnatal DP or epithelial deletion of Wntless (WLS), a protein required for efficient secretion of both canonical and noncanonical Wnt ligands, causes failure of matrix cell proliferation and premature catagen (Enshell-Seijffers et al., 2010; Myung et al., 2013). It is not clear whether the effects of *Wls* deletion are mediated primarily through the DP or HF epithelia or reflect contributions of noncanonical Wnt signaling. However, proliferation of progenitor cells in response to forced expression of stabilized β-catenin, and the effects of injection of recombinant DKK1 on hair follicle growth, suggest functions for Wnt/β-catenin signaling in HF epithelial cells during anagen (Kwack et al., 2012; Lowry et al., 2005; Van Mater et al., 2003). Global deletion of epithelial β-catenin in telogen causes stem cell depletion (Lowry et al.,

and the consequences of specifically inhibiting canonical Wnt signaling upstream of β-catenin, have not been systematically investigated.

Unlike the HF, which proliferates periodically, basal IFE is active throughout life, both renewing itself and generating cells that differentiate to form a cornified layer that is continuously shed. While expression of the TOPGAL Wnt reporter transgene is undetectable in the IFE (DasGupta and Fuchs, 1999), expression of other, more sensitive reporters, and possible functions of β-catenin signaling in adult IFE in vivo, have not been examined.

Here we show, using two independent, sensitive in vivo reporters, that Wnt/β-catenin signaling is active in IFE and specialized nonhairy epithelia as well as in anagen HFs. Using multiple genetic approaches to manipulate signaling in specific cell types, we demonstrate that epithelial β-catenin signaling is required for maintenance of proliferation in anagen HFs and contributes to proliferation of footpad and tongue but is not required within the HF bulge and SHG for stem cell survival. Consistent with this, hair regrowth occurs spontaneously after removal of Wnt/β-catenin signaling inhibition. To analyze the role of β-catenin in the IFE of hairy skin, we developed a system that permits gene deletion specifically in IFE while sparing the hair follicle bulge, SHG, and DP, allowing analysis of IFE phenotypes in the absence of inflammatory reactions associated with HF degradation. These experiments revealed a previously unknown role for β-catenin signaling in contributing to proliferation of IFE in vivo.

RESULTS

Wnt/β-Catenin Signaling Is Active in Basal IFE, Nonhairy Epithelia, and Anagen HFs

To assay for Wnt/β-catenin signaling in postnatal skin, we utilized *Axin2*^{lacZ}, in which *lacZ* is inserted into the endogenous *Axin2* locus, a ubiquitous Wnt target (Lustig et al., 2002; Yu et al., 2005), and *TCF/Lef:H2B-GFP* (*TL-GFP*), in which six copies of a TCF/LEF responsive element and an *hsp68* minimal promoter drive expression of an H2B-GFP fusion protein (Ferrer-Vaquer et al., 2010). Sensitivity of the *TL-GFP* reporter is supported by its activity at sites of Wnt/β-catenin signaling not documented with other reporters but confirmed through genetic analysis (Ferrer-Vaquer et al., 2010). Expression of *Axin2*^{lacZ} and *TL-GFP* was low or undetectable in the bulge, SHG, and DP of telogen HFs (Figures S1D and S1H available online). Both reporters were expressed in the DP, SHG, and matrix in early and mid-anagen and very strongly in hair shaft precursor cells at mid-anagen (Figures S1A–S1C, S1E, and S1G). Unexpectedly, both *Axin2*^{lacZ} and *TL-GFP* were expressed at low levels in IFE (Figures S1B, S1D, S1E, S1F, and S1H). *TL-GFP*-expressing IFE cells were observed in basal and suprabasal layers. *Axin2*^{lacZ}-positive cells were confined to basal cells, suggesting that suprabasal expression of *TL-GFP* may be due to perdurance of H2B-GFP. The number of positive cells and the intensity of reporter expression in IFE increased with age (Figures S1F and S1H). *Axin2*^{lacZ} and *TL-GFP* were also expressed in stratified tongue epithelia (Figures S1I and S1K). *Axin2*^{lacZ},

heterozygous for loss of *Axin2* function. Consistent with Wnt/β-catenin signaling in IFE, several Wnt ligands and FZD receptors are expressed in embryonic and adult IFE as well as in HFs (Reddy et al., 2001, 2004) (Figures S1M and S1N).

Epithelial β-Catenin Deletion or Ectopic *Dkk1* Expression Induced during Embryonic Anagen Causes Rapid HF Regression

To delineate the requirements for Wnt/β-catenin signaling at successive stages of the embryonic hair growth cycle, we compared the effects of doxycycline-inducible deletion of β-catenin and inducible ectopic expression of DKK1, which inhibits signaling at the level of the LRP coreceptor. Mice carrying a *Krt5-rtTA* transgene (Diamond et al., 2000) in which a reverse tet transactivator is expressed in basal epidermis and HF ORS including bulge stem cells were mated either to mice carrying *tetO-Cre* (Mucenski et al., 2003) and a conditional null allele of *Ctnnb1* (Brault et al., 2001) or to *tetO-Dkk1* mice (Chu et al., 2004) (Figures S1O–S1S). Inducible *Dkk1* was expressed at higher levels in HFs than in IFE (Figure S1S). In contrast with published data (Kwack et al., 2012), we were not able to detect significant levels of expression of endogenous *Dkk1* by in situ hybridization at any stage of the adult HF growth cycle. The Wnt/LRP inhibitory actions of DKK1 require interaction with KRM (Mao et al., 2002), which is expressed at low levels in postnatal skin (Figures S1T and S1U). Because limiting levels of KRM may restrict the effectiveness of DKK1-mediated inhibition, we generated *KRT14-Krm1* mice that constitutively expressed high levels of *Krm1* in epithelial cells (Figure S1V) and assayed the effects of coexpressed *Krm1* and *Dkk1* on hair growth. *KRT14-Krm1* mice did not display detectable abnormalities in skin histology or in timing of the hair growth cycle in the absence of coexpressed *Dkk1* (Figures S1W and S1X).

Experimental *Krt5-rtTA tetO-Cre Ctnnb1*$^{fl/fl}$ (β-catenin mutant), *Krt5-rtTA tetO-Dkk1* (*Dkk1* transgenic), or *KRT14-Krm1 Krt5-rtTA tetO-Dkk1* (*Dkk1/Krm1* transgenic) mice and their respective control littermates were doxycycline treated from postnatal day (P) 4 (Figures 1B–1K) or 7 (Figures 1L–1P), and dorsal skin was harvested at P8, P14, and P24. Loss of epithelial β-catenin or forced expression of *Dkk1* caused rapid cessation of anagen and entry into a premature regression phase compared with controls (Figures 1B–1E, 1G–1J, and 1L–1O). The effects of *Dkk1* were enhanced by coexpression with *Krm1* (Figures 1F, 1K, and 1P). As HF regression is caused by either epithelial β-catenin deletion or LRP signaling inhibition, these data indicate that canonical Wnt/LRP/β-catenin pathway activity is required within epithelial cells to maintain anagen. At P24, HFs in epithelial-β-catenin-deleted, but not *Dkk1*- or *Dkk1/Krm1*-expressing skin, displayed structural defects including a widened infundibulum and loss of a clearly distinguishable SHG (Figure 1M, arrow; see inset), suggesting additional, LRP-independent roles for β-catenin in telogen (Figures 1L–1P, see insets).

Rapid Regression of Mutant HFs Is Associated with Inhibition of Cell Proliferation, but Bulge Stem Cell Markers Are Maintained

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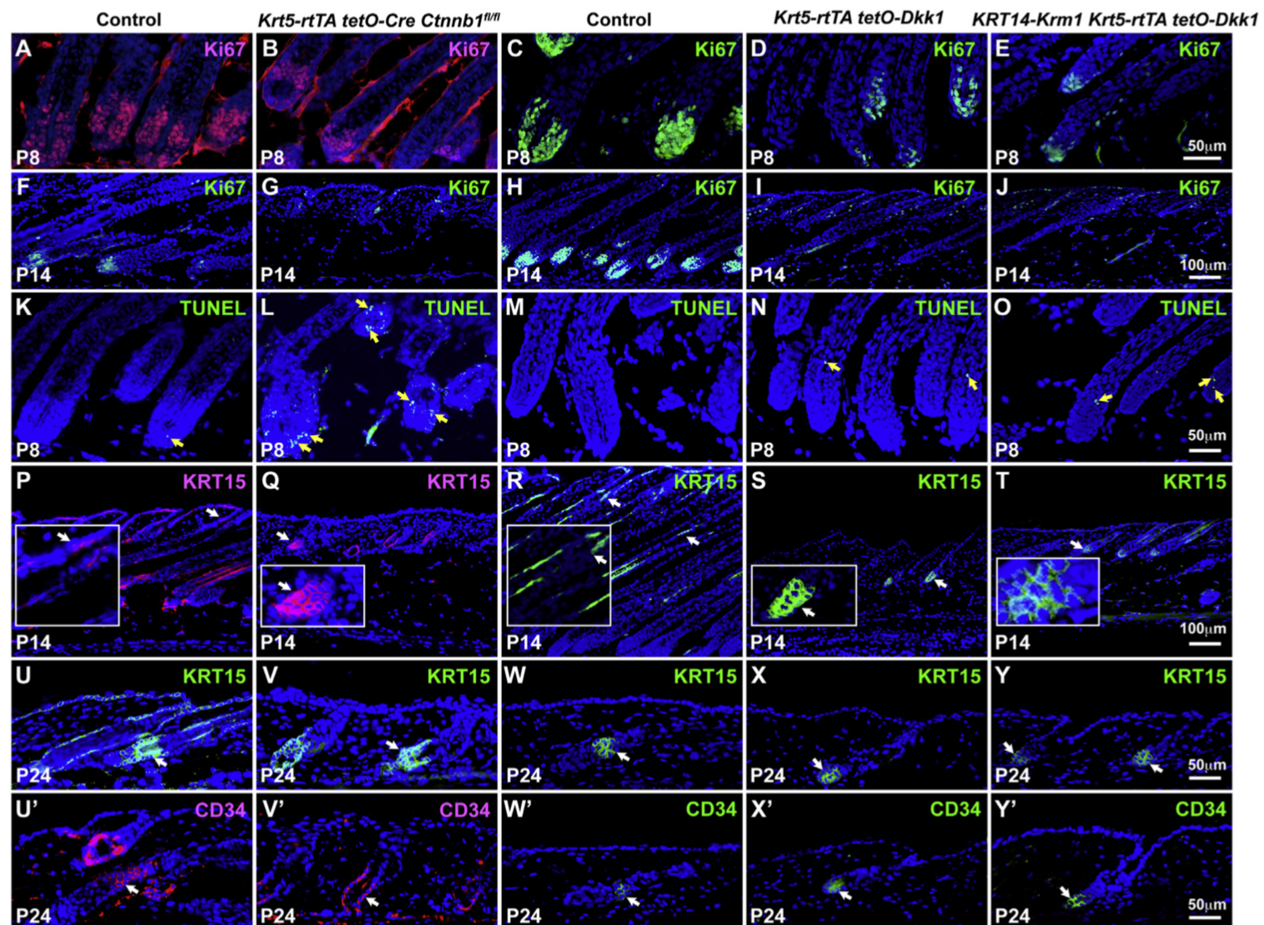

Figure 2. Inducible β-Catenin Deletion or *Dkk1* Expression in Anagen Inhibits HF Matrix Proliferation without Loss of Stem Cells
Krt5-rtTA tetO-Cre Ctnnb1$^{fl/fl}$ mutants, *Dkk1* transgenics and *Dkk1/Krm1* transgenics, and their respective littermate controls mice were induced from P4 (A–T) or P7 (U–Y′) and skin samples were harvested at the stages indicated. (A–J) Ki67 immunofluorescence (A, B, red; C–J, green). (K–O) TUNEL staining (green) (yellow arrows). (P–Y) KRT15 immunofluorescence (P, Q, red; R–Y, green) (white arrows). Insets in (P)–(T) represent higher magnification photographs of regions indicated by arrows. (U′–Y′) CD34 immunofluorescence (U′, V′, red; W′–Y′, green) (white arrows). Scale bars: (A)–(E), (K)–(O), (U)–(Y′), 50 μm; (F)–(J), (P)–(T), 100 μm. See also Figure S2.

P8 or P14 revealed greatly reduced proliferation in both β-catenin mutant (Figures 2B and 2G) and *Dkk1* or *Dkk1/Krm1* transgenic (Figures 2D, 2E, 2I, and 2J) follicles compared with controls (Figures 2A, 2C, 2F, and 2H). Similarly, *Dkk1*-expressing hair bulb sections contained a mean of 16% ± 3% BrdU-positive nuclei after 4 days of induction at P8, compared with 36% ± 5% in controls (n = 17 HFs from two control mice and 13 HFs from two transgenic mice) (Figures S2A–S2C). β-catenin-deleted and *Dkk1*-expressing HFs showed greatly diminished expression of cyclin D1, a direct Wnt/β-catenin target gene that helps initiate transition from late G1 to S phase of the cell cycle (Kobielak et al., 2003; Tetsu and McCormick, 1999), likely contributing to decreased HF matrix proliferation (Figures S2D–S2G).

Consistent with accelerated entry into catagen, P8 *Dkk1*- and *Dkk1/Krm1*-expressing follicles contained slightly increased numbers of apoptotic cells compared with controls (Figures 2K, 2M, and 2N), and many more TUNEL-positive cells were observed in β-catenin mutant HF matrix (Figures 2L and 2O).

Interestingly, expression of both KRT15, a marker for epithelial stem cells in the HF bulge and SHG (Morris et al., 2004), and CD34, which specifically marks bulge stem cells (Trempus et al., 2003), was readily detected at P14 and P24 in β-catenin mutants and *Dkk1* transgenics induced from P4 and P7, respectively (Figures 2P–2Y′), indicating that cessation of anagen was not due to immediate loss of the stem cell compartment.

β-Catenin Deletion or Ectopic *Dkk1* Block Both Plucking-Induced and Spontaneous Anagen

To determine whether adult anagen onset requires β-catenin or signaling through LRP, we doxycycline-treated *Krt5-rtTA tetO-Cre Ctnnb1$^{fl/fl}$* and *Dkk1* or *Dkk1/Krm1* transgenic mice and their respective littermate controls from P50 (telogen) and plucked

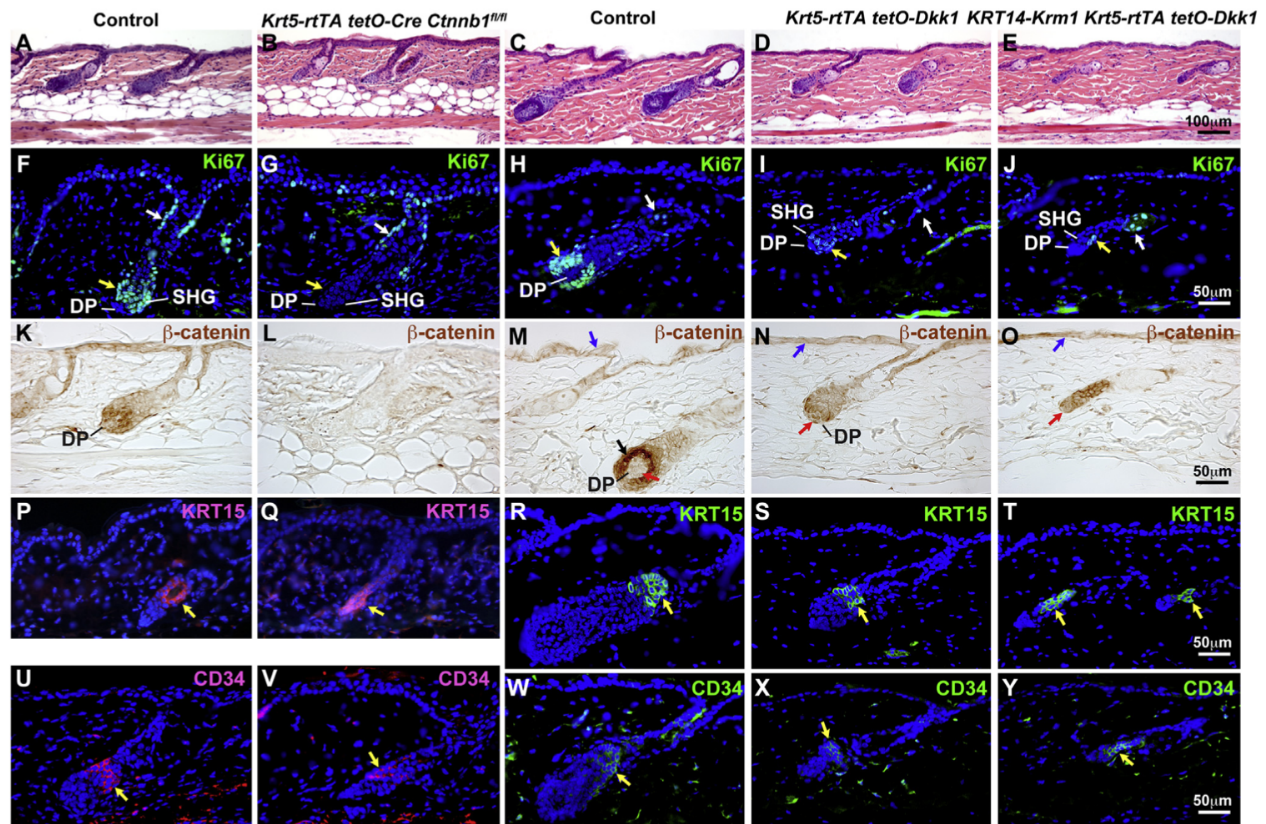

Figure 3. Inducible β-Catenin Deletion or Ectopic *Dkk1* Expression Blocks Plucking-Induced Anagen

Krt5-rtTA tetO-Cre Ctnnb1$^{fl/fl}$ mutants, *Dkk1* transgenics and *Dkk1/Krm1* transgenics, and their respective littermate controls were induced from P50, their hair was plucked at P54, and their dorsal skin was analyzed at P57. Paraffin sections were stained with H&E (A–E) or immunostained for Ki67 (green) (F–J), β-catenin (brown) (K–O), KRT15 (P, Q, red; R–T, green), or CD34 (U, V, red; W–Y, green). In (F) and (G), yellow arrows indicate SHG; white arrows indicate upper ORS. In (M)–(O), black arrow indicates SHG, blue arrows indicate IFE, and red arrows indicate DP. Yellow arrows in (P)–(Y) indicate positive signals. Scale bars: (A)–(E), 100 μm; (F)–(Y), 50 μm. See also Figures S2 and S3.

matrix that began to surround the DP (Figures 3A and 3C). High levels of proliferation were observed in the developing matrix and in the upper ORS (Figures 3F and 3H), and nuclear localized β-catenin was prominently present in the matrix and at lower levels in the DP (Figures 3K and 3M). Deletion of epithelial β-catenin caused failure of matrix cells to migrate around the DP (Figure 3B). Upper regions of the mutant HFs proliferated in response to plucking; however, proliferation of the SHG was markedly decreased or absent (Figure 3G).

Dkk1 double transgenic and *Dkk1/Krm1* triple transgenic follicles displayed signs of very early anagen at 3DPP with limited migration of epithelial cells around the DP (Figures 3C–3E). Proliferation of the SHG was severely reduced compared with controls, but not completely absent (Figures 3H–3J). Presence of β-catenin at the cell membrane was unaffected by ectopic *Dkk1* (Figures 3M–3O, blue arrows); however, nuclear β-catenin was dramatically reduced in *Dkk1*-expressing matrix cells (Figures 3M–3O, black arrow; Figures S3A and S3B), and expression of the Wnt target gene cyclin D1 was almost completely absent (Figures S3C and S3D). *Dkk1*-expressing HF failed to ... 2004; Kobielak et al., 2003) (Figures S3I and S3J), and absence of markers for IRS and hair shaft differentiation (Figures S3K–S3N). Nuclear β-catenin and *Axin2^{lacZ}* reporter expression were reduced in the epithelium but still present in the DP of most *Dkk1*-expressing follicles (Figures 3M–3O; Figures S3A, S3B, and S3E–S3H, red arrows), suggesting that *Dkk1*-mediated inhibition did not extend effectively to DP cells.

Because plucking-induced anagen may induce a wound-like response in the skin, we also tested whether epithelial β-catenin deletion or ectopic *Dkk1* or *Dkk1/Krm1* blocked entry of HFs into spontaneous anagen. In mice induced from P17 (catagen), control HFs entered early anagen by P25 and were in full anagen at P30. By contrast, β-catenin mutant follicles failed to enter anagen (Figures S2H–S2K). *Dkk1*-expressing follicles were arrested in early anagen at P30; this effect was more pronounced in follicles coexpressing *Dkk1* and *Krm1* (Figures S3O–S3T).

β-Catenin Is Required within Bulge and SHG Cells for Anagen Initiation

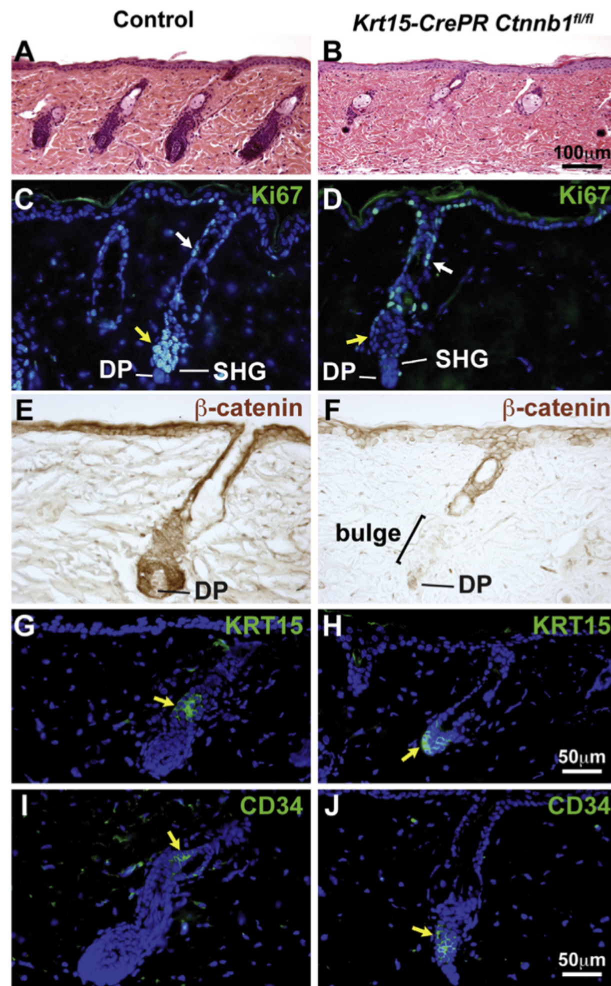

Figure 4. β-Catenin Is Required in HF Stem Cells for Anagen Onset
Krt15-CrePR1 Ctnnb1$^{fl/fl}$ mice were induced for 5 days prior to plucking at P54 and skin was harvested at P59. *Krt15-CrePR1 Ctnnb1$^{fl/fl}$* mutant HFs fail to enter anagen (A and B). The upper ORS proliferates in mutants (white arrows), but proliferation of the SHG (yellow arrows) is severely reduced or absent compared with that of controls (C and D). β-catenin protein (brown) is absent in mutant HF SHG and bulge, but not in IFE or HF epithelia above the bulge (E and F). KRT15 (G and H) or CD34 (I and J) immunofluorescence (green) (yellow arrows) reveals stem cell persistence in control (G and I) and *Krt15-CrePR1 Ctnnb1$^{fl/fl}$* mutant (H and J) HFs at 5DPP. Scale bars: (A) and (B), 100 μm; (C)–(J), 50 μm. See also Figure S2.

specifically in *Krt15* promoter-active bulge and SHG cells by topical treatment with mifepristone (RU486). *Krt15-CrePR1 Ctnnb1$^{fl/fl}$* mice were induced for 5 days prior to hair plucking at P54. β-catenin was efficiently deleted in CD34-positive HF bulge and SHG cells in mutant skin at 5DPP but was retained in the upper HF above the bulge (Figures S2L and S2M; Figures 4E and 4F). Unlike littermate controls, which displayed robust hair regrowth by 14DPP, *Krt15-CrePR1 Ctnnb1$^{fl/fl}$* mutant HFs did not regrow their hair coats (Figure S2N). Expression of KRT15 and CD34 was maintained at early stages after hair plucking in adult *Krt5-rtTA tetO-Cre Ctnnb1$^{fl/fl}$*, *Krt15-CrePR1 Ctnnb1$^{fl/fl}$*, *Dkk1*-expressing, and *Dkk1/Krm1*-expressing mice (Figures 3P–3Y; Figures 4G–4J). Thus, short-term deletion of epithelial β-catenin or inhibition of LRP signaling produced dramatic defects in SHG and matrix proliferation without loss of the stem cell compartment.

HF Stem Cells Are Maintained during Long-Term Induction of *Dkk1*, but Are Eventually Lost following Broad Deletion of β-Catenin

External hair was almost completely absent in epithelial β-catenin mutants following long periods of deletion (Figures S4A and S4B). To ask whether maintenance of HF structures and their associated stem cells was affected by long-term loss of β-catenin, we induced deletion from P4 and examined skin histology and expression of KRT15 and CD34 at P60. HF density was greatly reduced at this stage compared with controls, and remaining structures appeared degraded or formed cysts (Figures 5A and 5B). Expression of KRT15 and CD34 was robust in control littermate HFs but completely absent from β-catenin-deficient skin (Figures 5K, 5K′, 5L, and 5L′). Similarly, in β-catenin mutants induced from P30 and examined at P100 or P180, HFs were degraded and/or had formed utricles, and expression of KRT15 and CD34 was absent (Figures S4A′, S4B′, S4F–S4G′, S4K, S4L, S4P–S4Q′, S4U, and S4V). Thus, epithelial β-catenin is required for long-term maintenance of HF structures and their associated stem cells. DP cells were frequently noted stranded in the dermis or at the tail end of “streamers” of degenerating HFs (Figures S4W and S4X), suggesting that follicular degradation may have been caused in part by loss of contact of HF epithelia with the DP.

Similar to epithelial β-catenin mutants, mice coexpressing ectopic *Dkk1* and *Krm1* had completely lost external hair by P103, following doxycycline treatment from P4 (Figure S4E). However, analysis of skin histology at P60 revealed maintenance of HF structures that were apparently arrested in early anagen (Figures 5C and 5D), and KRT15 and CD34 stem cell markers were expressed at similar levels to those observed in control littermate HFs (Figures 5M, 5M′, 5N, and 5N′). Remarkably, HF structures and associated KRT15 and CD34-positive stem cells were still maintained at P102 and P185 in mice induced to express *Dkk1* or both *Dkk1* and *Krm1* from P1 (Figures S4C′–S4E′, S4H–S4J′, S4M–S4O, and S4R–S4T′). Therefore, over long periods of time, broad loss of epithelial β-catenin, but not ectopic *Dkk1*-mediated inhibition of Wnt signaling, causes loss of HF structures and stem cells.

To determine whether long-term stem cell maintenance requires epithelial Wnt ligands, we directly compared the effects of deletion of epithelial WLS on maintenance of HF stem cells at stages when these are lost in induced β-catenin mutants. Consistent with published data (Myung et al., 2013), broad deletion of epithelial *Wls* in *Krt5-rtTA tetO-Cre Wls$^{fl/fl}$* mutants induced from P4 resulted in premature regression of HFs by P14 without loss of bulge stem cell markers (Fig-

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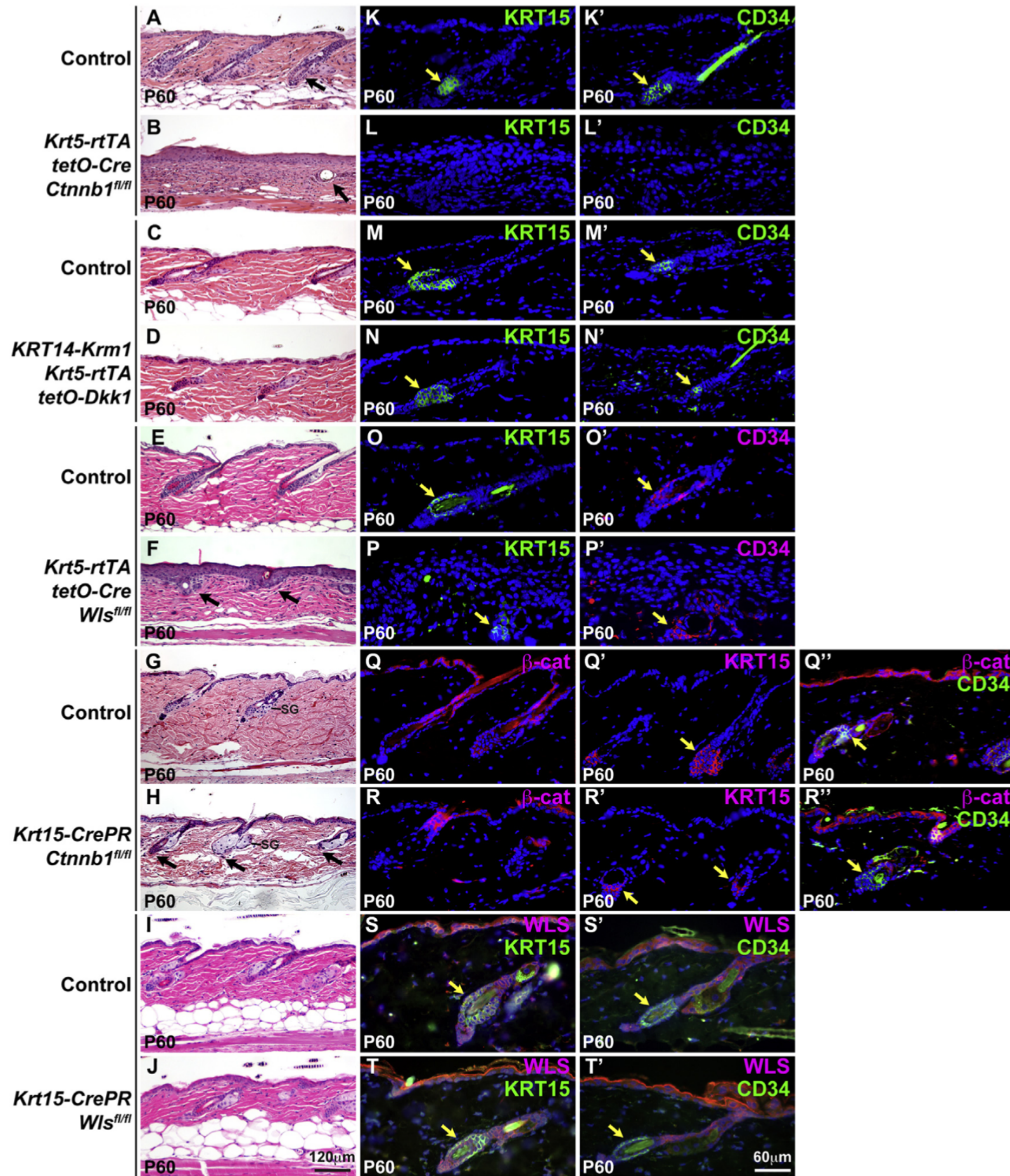

Figure 5. HF Stem Cells Disappear after Long-Term Broad Deletion of Epithelial β-Catenin but Persist when β-Catenin Deletion Is Restricted to the Bulge and SHG

Krt5-rtTA tetO-Cre Ctnnb1$^{fl/fl}$ mutants, *Dkk1/Krm1* triple transgenics, and control littermates were doxycycline treated from P4 to P60 and *Krt5-rtTA tetO-Cre Wls*$^{fl/fl}$ and control mice, from P4 to P18. *Krt15-CrePR1 Wls*$^{fl/fl}$ and control littermates were treated topically with 1% mifepristone from P19 to P25. *Krt15-CrePR1 Ctnnb1*$^{fl/fl}$ mice and control littermates were treated topically with 1% mifepristone from P20 to P27. All skin samples were analyzed at P60 (telogen) by H&E staining (A–J) or immunofluorescence for KRT15 (K–P, S, T, green; Q′, R′, red), CD34 (K′–N′, Q′′, R′′, S′, T′, green; O′, P′, red), β-catenin (Q, R, Q′′, R′′, red), or WLS (S, T, S′, T′, red). Yellow arrows indicate positive staining for KRT15 or CD34. Scale bars: (A)–(J), 120 μm; (K)–(T′), (Q′′), and (R′′), 60 μm. See also Figures S2, S4, S6, and S7.

HF structures were retained but showed marked abnormalities including the formation of cysts (Figures 5E and 5F). Despite

deletion of WLS in the bulge and SHG of *Krt15-CrePR1 Wls*$^{fl/fl}$ mutant HFs (Figures S2Q and S2R; Figures 5S, 5S′, 5T, and

Stem Cells Are Maintained following Specific Deletion of β-Catenin in the Bulge and SHG

To determine whether eventual loss of HF stem cell markers in epithelial β-catenin mutants was due to a requirement for β-catenin within bulge stem cells and SHG, we examined their expression in P60 *Krt15-CrePR1 Ctnnb1$^{fl/fl}$* mice, following topical 1% mifepristone treatment from P20–27. Double immunofluorescence for β-catenin and CD34 revealed maintenance of CD34 immunoreactivity in β-catenin-deleted bulge cells at this stage (Figures 5Q″ and 5R″). Staining of serial sections for β-catenin and KRT15 indicated that expression of KRT15 persisted in β-catenin-deleted HFs (Figures 5Q, Q′, R, and R′). Similar results were obtained with another bulge stem cell marker, S100A4 (data not shown). Despite persistence of bulge stem cell marker expression, the SHG of *Krt15-CrePR1 Ctnnb1$^{fl/fl}$* HF appeared abnormal or even absent in some HFs; however, the upper follicles and sebaceous glands (SGs) remained intact (Figures 5G and 5H). These data, and the slow time course of bulge stem cell disappearance in *Krt5-rtTA tetO-Cre Ctnnb1$^{fl/fl}$* mice, suggested that bulge cell loss in *Krt5-rtTA tetO-Cre Ctnnb1$^{fl/fl}$* mutants occurred secondary to follicular degradation resulting from combined absence of β-catenin in the HF bulge/SHG and KRT15-negative cell populations, such as those in the junctional zone and isthmus (Myung and Ito, 2012).

Long-Term *Dkk1*-Mediated Inhibition of HF Growth Is Reversible

Our observation that KRT15 and CD34 expression persisted even following very long periods of *Dkk1* induction raised the possibility that functional stem cells were maintained in *Dkk1*-expressing follicles. To test this, we asked whether hair growth was reversible following removal of the inhibitor. Control and *Dkk1* transgenic mice were maintained on doxycycline from postnatal day 21 until 15.5 months of age. At this stage, the experimental mice completely lacked external hair (Figures 6A and 6B), but maintained HF structures (Figures 6C and 6D) with associated stem cells (Figures 6E–6H). Ectopic expression of *Dkk1* was readily detected in induced double transgenic skin by in situ hybridization (Figure 6I). Mice were sacrificed for analysis 14 days after doxycycline withdrawal. At this time point, expression of *Dkk1* was no longer detected (Figure 6J) and, remarkably, growth of numerous follicles had occurred spontaneously (Figures 6K and 6L). HF growth following doxycycline withdrawal was accompanied by resumption of Wnt/β-catenin signaling, indicated by accumulation of nuclear β-catenin in the HF matrix and precortical regions (Figures 6M and 6N), expression of cyclin D1 (Figures 6O and 6P), and high levels of matrix cell proliferation (Figures 6Q and 6R). To avoid multiple survival biopsies, we did not test whether external hair growth occurred following removal of the inhibitor. However, the reactivated matrix cells were capable of differentiating, as indicated by positive staining for hair shaft precursor cells with the hair keratin marker AE13 (Figures 6S and 6T). These data suggested persistence in *Dkk1*-inhibited follicles of Wnt ligands capable of reactivating HF growth following removal of the inhibitor. Consistent with this, *Wnt10b*, a ligand specifically ex-

To test whether HFs coexpressing *Dkk1* and *Krm1* were also capable of reinitiating follicular growth following removal of the inhibitor, control littermate, *Dkk1* transgenic, and *Dkk1/Krm1* transgenic mice were induced from birth until P100. Analysis of histology and immunostaining for KRT15, CD34, β-catenin, cyclin D1, Ki67, and AE13 confirmed maintenance of HF structures and stem cell marker expression in *Dkk1/Krm1* and *Dkk1* transgenic and control mice at this stage, with lack of nuclear β-catenin or cyclin D1 expression, absent or low levels of proliferation, and lack of hair shaft differentiation (Figures S5A–S5Q). Following 15 days of doxycycline withdrawal, both *Dkk1* and *Dkk1/Krm1* skin contained numerous HFs that had spontaneously entered anagen, compared with control littermate follicles that remained in telogen (Figures S5A′–S5C′). Hair follicle regrowth was less synchronous in *Dkk1/Krm1* skin than in skin that had expressed *Dkk1* alone, consistent with the stronger inhibitory effects of *Dkk1/Krm1*. *Dkk1* and *Dkk1/Krm1* transgenic follicles displayed an extended pattern of KRT15 and CD34 staining that is characteristic of anagen, rather than the compact staining pattern seen in control telogen stage follicles (Figures S5D′–S5I′). This was distinct from the staining observed in *Dkk1* and *Dkk1/Krm1* transgenics prior to doxycycline removal, which was intermediate between telogen and full anagen patterns, consistent with arrest in early anagen (Figures S5E, S5F, S5H, and S5I). Both *Dkk1* and *Dkk1/Krm1* transgenic samples displayed accumulation of nuclear β-catenin and expression of cyclin D1 in HF matrix regions following doxycycline withdrawal (Figures S5J′–S5M′), as well as robust matrix proliferation (Figures S5N′ and S5O′) and expression of hair shaft keratins (Figures S5P′ and S5Q′).

These data indicated functional persistence of HF bulge and/or SHG cells and reversibility of the effects of LRP inhibition even following very long-term *Dkk1* induction, with or without coexpressed *Krm1*. Histological analyses, the intermediate expression pattern of KRT15, persistent expression of *Wnt10b*, and the ability of HFs to spontaneously resume growth immediately following removal of the inhibitor supported the interpretation that *Dkk1*-inhibited HFs were arrested in early anagen and primed for hair regrowth rather than being maintained in a normal telogen resting phase.

β-Catenin Contributes to Proliferation of the IFE during Homeostasis

The epidermis of mice with long-term broad deletion of epithelial β-catenin or *Wls* displayed marked thickening and hyperproliferation, expansion of p63-positive basal and KRT10-positive suprabasal layers, and ectopic expression of hyperproliferation markers including KRT6 and KRT17 (Figures 5A, 5B, 5E, and 5F; Figures S4A′, S4B′, S6A, and S6C). Induced *Krt14-CreERT2 Ctnnb1$^{fl/fl}$* and *Krt5-rtTA tetO-Cre Ctnnb1$^{fl/fl}$* mutants did not display overt blistering, and α-catenin and E-cadherin were present at cell membranes in β-catenin mutant as well as control epidermis (Figures S7A–S7D). Transmission electron micrography (TEM) revealed the presence of enlarged intercellular spaces in β-catenin mutant epidermis following long periods of broad depletion of epithelial β-catenin. In some cases, mutant keratino-

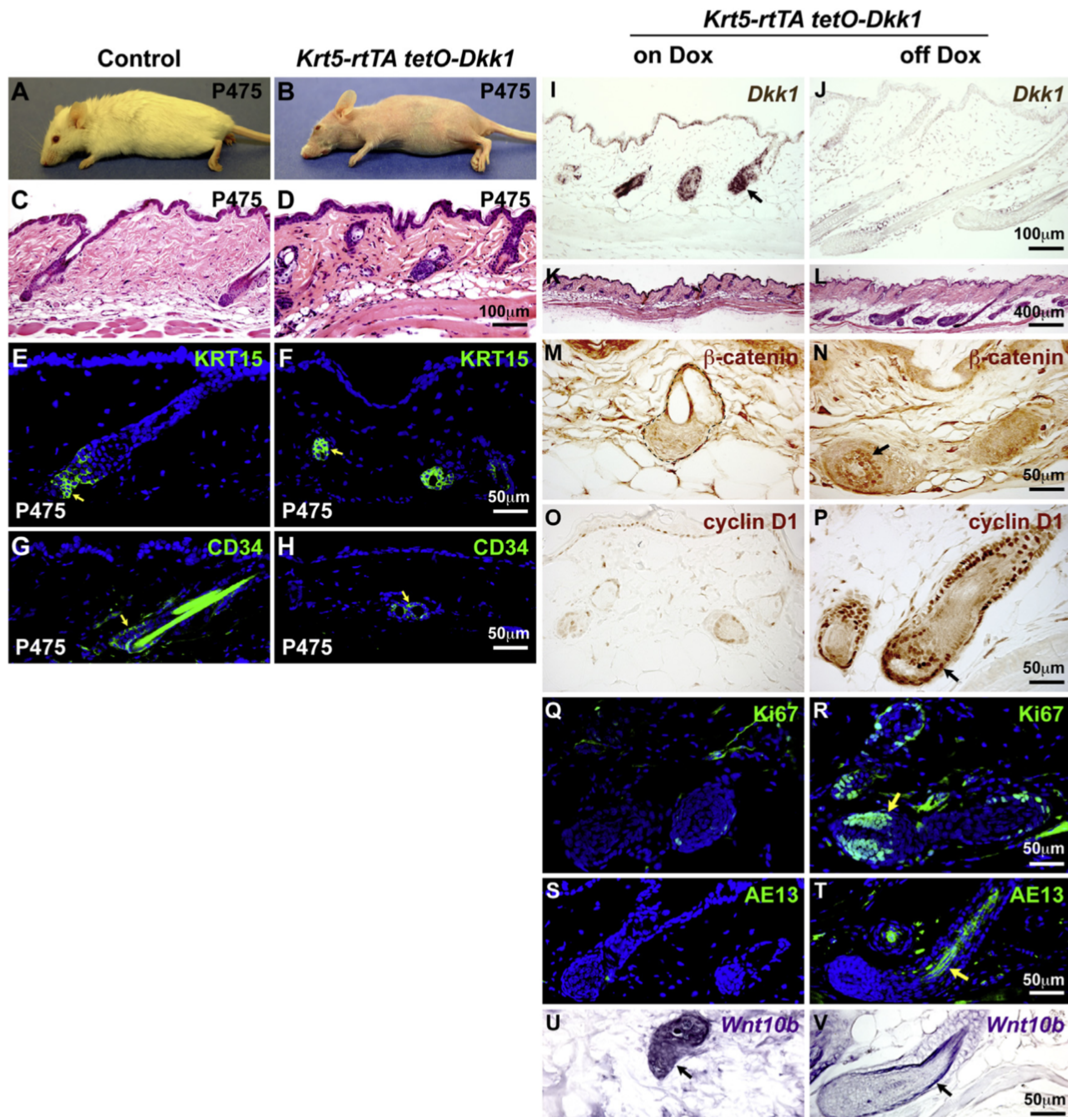

Figure 6. *Dkk1*-Mediated Hair Growth Inhibition Is Reversible

Control and *Dkk1* double transgenic mice were induced from P21 to P475, and skin biopsies were performed at P475 (on Dox) (A–H, I, K, M, O, Q, S, and U) and at P489, 14 days after doxycycline withdrawal (off Dox) (J, L, N, P, R, T, and V). *Dkk1* transgenic mice completely lacked external hair at P475 (A and B) but HF structures (C and D) and expression of KRT15 (E and F) and CD34 (G and H) (green, arrows) were maintained. (I and J) In situ hybridization reveals *Dkk1* expression (brown) in IFE and HFs (arrow) of *Dkk1* transgenic skin on Dox (I) and absence of *Dkk1* expression following Dox withdrawal (J). (K and L) HFs were arrested in early anagen prior to Dox withdrawal (K) and progressed into full anagen following Dox removal (L). (M)–(R) Immunostaining reveals absence of nuclear-localized β-catenin (M, brown), cyclin D1 (O, brown), Ki67 (Q, green), and AE13 (S, green) expression in HFs prior to Dox withdrawal and the presence of these markers in HFs following Dox withdrawal (N, P, R, and T). (U and V) In situ hybridization for *Wnt10b* shows its persistent expression (purple) (black arrows) in HFs of *Dkk1* transgenic skin on Dox (U) and after Dox withdrawal (V). Arrows indicate positive signals. Scale bars: (K) and (L), 400 μm; (C), (D), (I), and (J), 100 μm; (E)–(H) and (M)–(V), 50 μm. See also Figure S5.

following bulge-cell-restricted deletion of β-catenin (not shown). Interestingly, however, IFE from induced *Krt5-rtTA tetO-Cre Wls*$^{fl/fl}$ mutants displayed similar abnormalities at the ultrastructural level (Figures S7I and S7J) but retained β-catenin

Because degrading HFs elicit inflammation (Kirkham, 2009), epidermal hyperproliferation could be a consequence of an inflammatory response to HF disintegration (Augustin et al., 2013), or it might reflect a direct requirement for β-catenin

KRT14-Krm1 Krt5-rtTA tetO-Dkk1 mice, which express lower levels of *Dkk1* in IFE than in HFs (Figures S6B, S6D, S6E, and S1S). However, these mutants also lacked overt HF structural defects and displayed lower levels of inflammatory cells than those seen in *Krt5-rtTA tetO-Cre*-driven mutants (Figures S7K–S7T).

To test directly whether β-catenin is required for IFE homeostasis, we generated a *Axin2CreERT2/tdT* knockin line by inserting a CreERT2/tdTomato fusion cDNA downstream of the first codon of the endogenous mouse *Axin2* gene (Figure S7U). This line expresses cytoplasmic tandem dimer Tomato (tdT) and tamoxifen-inducible Cre-recombinase (CreERT2) in *Axin2*-promoter-active cells (not shown). To assess the efficiency of inducible deletion, we bred *Axin2CreERT2/tdT* mice with the $R26R^{mTmG}$ Cre reporter line that expresses membrane-targeted tandem dimer Tomato (mT) prior to Cre-mediated excision and membrane-targeted GFP (mG) after excision (Muzumdar et al., 2007). Following induction of Cre activity by treatment with tamoxifen during telogen, GFP-positive cells were present in the IFE and HF infundibulum, but no excision was observed in the HF bulge, SHG, or DP (Figure 7A). Clones of GFP-positive deleted cells were also observed in tongue and footpad epidermis (Figures 7B and 7C), consistent with $Axin2^{lacZ}$ expression in basal progenitor cells of these tissues. To determine whether *Axin2*-promoter-active cells give rise to clones in the IFE, we crossed *Axin2CreERT2/tdT* mice with *R26R-Confetti* mice that function as a stochastic multicolor Cre reporter, permitting clonal analysis of cells expressing GFP, YFP, RFP, or CFP under the control of a CAG promoter (Snippert et al., 2010). RFP was expressed much more strongly than *Axin2*-promoter-driven tdT, allowing us to easily identify RFP-positive and other fluorescently marked clones. Following tamoxifen induction in telogen, we observed clones of fluorescently marked cells originating in the basal progenitor layer of the IFE (Figures 7D and 7D′).

Because efficient *Axin2CreERT2/tdT*-mediated deletion of β-catenin may cause systemic defects, we used conditions under which mice exhibited mosaic deletion. *Axin2CreERT2/tdT* $Ctnnb1^{fl/fl}$ mice and *Axin2CreERT2/tdT* $Ctnnb1^{fl/+}$ control littermates were injected with 200 mg/kg tamoxifen daily at P43, P44, and P45 and tissue was harvested 4 weeks later. Immunofluorescence for β-catenin revealed mosaic absence of β-catenin in IFE, but not in HF bulge or SHG (Figures 7E–7H). Histological analysis did not reveal gross HF structural defects in these animals (not shown). We quantified proliferation by assaying for Ki67 immunofluorescence in nonserial sections of IFE, choosing regions where β-catenin was either efficiently deleted in continuous strips of at least 20 cells (n = 75 nonadjacent strips of 22–67 cells, total of 2,965 cells analyzed) or retained in continuous strips of at least 20 cells (n = 23 nonadjacent strips of 20–65 cells, total of 903 cells analyzed), and compared proliferation rates with those seen in littermate control epidermis (n = 52 nonadjacent strips of 38–85 cells, total of 2,780 cells analyzed). Surprisingly, rather than exhibiting hyperproliferation, regions of β-catenin-deleted epidermis showed a statistically significant decrease in proliferation rate of more than 40%

epidermis was similar to that of control epidermis, indicating that decreased proliferation of deleted cells was not due to systemic defects (Figure 7Y).

Nonhairy epithelia of the footpad and tongue lacked obvious inflammation; however, filiform papillae were reduced or absent in induced *Krt5-rtTA tetO-Cre* $Ctnnb^{fl/fl}$ and *Krt5-rtTA tetO-Dkk1 KRT14-Krm1* mice compared with controls (Figures 7I, 7J, 7Q, and 7R). We assayed for epithelial proliferation in n = 10 nonadjacent samples of footpad and dorsal tongue from each littermate control and mutant or transgenic, with each sample containing 60 DAPI-stained basal nuclei. Proliferation was significantly reduced in footpad and tongue of *Krt5-rtTA tetO-Cre* $Ctnnb^{fl/fl}$ mutants (Figures 7K–7P and 7Y); similar though less pronounced, defects were observed in the footpad and tongue of induced *Krt5-rtTA tetO-Dkk1 KRT14-Krm1* mice (Figures 7S–7Y), indicating that they were caused by decreased Wnt/LRP/β-catenin signaling, rather than being directly due to loss of β-catenin's functions in adhesion. Thus, in addition to controlling HF matrix proliferation, Wnt/β-catenin signaling contributes to proliferation of IFE and specialized nonhairy epithelia.

In summary, our data suggest a model in which high levels of Wnt/β-catenin signaling in the HF matrix drive proliferation. As cells exit the matrix compartment, a further elevation of Wnt/β-catenin signaling levels causes them to terminally differentiate (Zhang et al., 2008; Zhou et al., 1995). Ectopic Dkk1, or deletion of *Wls* or β-catenin, inhibits these high levels of signaling, preventing matrix proliferation and differentiation. Unlike broad loss of epithelial β-catenin, bulge/SHG-restricted β-catenin deletion does not cause loss of stem cells, indicating that β-catenin is not required within bulge/SHG cells for their maintenance. Unexpectedly, our data also reveal low level Wnt/β-catenin signaling in IFE that is required to maintain normal levels of proliferation under homeostatic conditions (Figure 7Z) and demonstrate that Wnt/β-catenin signaling contributes to proliferation in specialized nonhairy epithelia. Despite these functions, complete loss of epithelial β-catenin does not prevent long-term maintenance of IFE or footpad and tongue epithelia, and β-catenin-deleted IFE can still mount a hyperproliferative response to inflammation associated with HF degradation.

DISCUSSION

Here we describe the sites and levels of Wnt/β-catenin signaling in postnatal skin using two independent, sensitive in vivo reporter assays. High levels of signaling were observed in the HF DP, SHG, and matrix at early stages of anagen. As matrix cells differentiated, they showed a further increase in signaling levels. Reporter gene expression was not observed in telogen HF bulge, SHG, or DP, but it was detected at low levels in the infundibulum and IFE. Reporter expression was also detected in nonhairy basal epithelia of footpad and tongue.

To delineate the functions of high- and low-level signaling in different epithelial cell types, we directly compared the effects of three independent genetic manipulations: deletion of epithelial β-catenin, which abolishes all signaling through the Wnt/β-catenin pathway; deletion of epithelial *Wls*, which dramatically

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Figure 7. β-Catenin Contributes to Proliferation of IFE and Nonhairy Epithelia

the levels of DKK1 expression and availability of the DKK1 receptor KRM.

During an established anagen phase, either deletion of epithelial β-catenin or expression of *Dkk1* caused HFs to rapidly cease proliferating and enter premature catagen. The similar phenotypes resulting from these two independent manipulations indicate that maintenance of proliferation during anagen requires signaling within epithelial cells through the canonical Wnt/LRP/β-catenin pathway. The KRT15-positive stem cell compartment was maintained during short-term deletion of β-catenin or expression of *Dkk1*. This observation, together with the strong localization of nuclear β-catenin and Wnt reporter gene expression in normal matrix cells, suggests that the immediate effects of Wnt pathway inhibition are manifest directly in the matrix, rather than via acute loss of bulge stem cells.

Because β-catenin is cell autonomous, the block to proliferation caused by deletion of epithelial β-catenin in anagen was caused by its loss within epithelial cells. Interestingly, however, the observed effects on proliferation and catagen induction are similar to those noted when β-catenin is specifically deleted in the anagen DP (Enshell-Seijffers et al., 2010). Thus β-catenin is required in both these HF compartments for continued hair growth. Our results further suggest that antagonism of Wnt signaling in the epithelium and DP could constitute part of the normal mechanism of catagen induction and prompt study of endogenous Wnt inhibitors that might promote the anagen-catagen transition.

Epithelial β-catenin mutants and mice expressing *Dkk1* displayed subtle differences in response to hair plucking: in β-catenin mutants cells proliferated in the ORS of plucked HFs, but not in the SHG. By contrast, expression of ectopic *Dkk1*, even when concomitant with forced expression of the DKK1 receptor Kremen1, permitted some degree of proliferation of SHG as well as ORS cells. Similarly, following deletion of epithelial *Wls*, many follicles enter anagen but are arrested in anagen I (Myung et al., 2013). These observations provide circumstantial evidence that the initial burst of β-catenin-dependent proliferation at anagen onset is controlled by mechanisms other than Wnt/LRP signaling, consistent with prior data indicating that (1) HF stem cells are initially activated by downregulation of dermal BMP signaling (Plikus et al., 2008) and (2) impaired BMP signaling stabilizes β-catenin through a Wnt-ligand-independent pathway (Kobielak et al., 2007).

In mice expressing epithelial *Dkk1*, with or without forced expression of *Krm1*, HFs and their associated stem cells were maintained for more than 1 year of *Dkk1* expression. Stem cells were also maintained following long-term deletion of epithelial *Wls*, either throughout skin epithelia or specifically in the bulge/SHG stem cell compartment, and following specific deletion of β-catenin in bulge/SHG stem cells. These observations suggest that the degradation of HF structures and complete loss of bulge stem cells seen following broad deletion of β-catenin results from combined absence of β-catenin in the HF bulge/SHG, junctional zone, isthmus, and/or infundibulum. Furthermore, long-term maintenance of follicular structures and their associated stem cells occurs via a mechanism distinct from that controlling proliferation and either requires very low levels of Wnt/LRP/β-catenin signaling that persist in the presence of ectopic *Dkk1* or absence of epithelial *Wls* or is independent of LRP and epithelial Wnt ligands.

Remarkably, removal of the inducing agent in aged *Dkk1* transgenic mice following long periods of *Dkk1* expression lead to spontaneous resumption of hair germ proliferation, formation of a new matrix, and differentiation of hair shaft and IRS cells, providing evidence that functional bulge/SHG stem cells were maintained in the absence of Wnt/β-catenin signaling. These data suggest graded inhibition of Wnt/LRP signaling as a promising method for reversibly inhibiting the growth of unwanted hair without causing permanent follicle destruction and skin damage. Small molecule Wnt inhibitors have been identified (Voronkov and Krauss, 2013) and could potentially be tested as topical agents for prevention of growth of unwanted hair in humans. Use of such approaches would require tight control of the levels of applied Wnt inhibitor to avoid causing hair follicle abnormalities.

Unexpectedly, we detected activity of Wnt reporter genes in adult IFE and in specialized nonhairy epithelia. We discovered that specific loss of β-catenin in the IFE of hairy skin, sparing the HF bulge and SHG, caused significantly reduced IFE proliferation. Similarly, β-catenin deletion or forced expression of *Dkk1*/*Krm1* decreased the thickness and proliferation of specialized nonhairy epithelia of the footpad and dorsal tongue. In line with these observations, human patients with mutations in the canonical Wnt gene *WNT10A* display epidermal defects, smooth tongues, and palmoplantar keratoderma in addition to hair growth defects (Adaimy et al., 2007; Petrof et al., 2011).

(D and D′) Cytoplasmic YFP- and RFP-marked clones originating in whisker pad basal IFE of *Axin2CreERT2/tdT R26R-Confetti* mice tamoxifen-treated for 3 days at 16 weeks and analyzed 5 weeks later.

(E–H) *Axin2CreERT2/tdT Ctnnb1$^{fl/fl}$* (F and H) and control littermate *Axin2CreERT2/tdT Ctnnb1$^{fl/+}$* (E and G) mice were tamoxifen treated at P43–P45 and skin sections were analyzed at P71 by immunofluorescence for β-catenin (green) and Ki67 (red) after microwave pretreatment to remove tdT fluorescence. β-catenin was mosaically deleted in mutant IFE (E–H) and upper HF (E and F) but was retained in HF bulge and SHG. β-catenin-deleted IFE displayed fewer Ki67-positive cells than controls.

(I–P) Absent tongue filiform papillae (I and J), decreased footpad epidermal thickness (M and N), and decreased proliferation of tongue (K and L) and footpad (O and P) epithelia in P100 *Krt5-rtTA tetO-Cre Ctnnb$^{fl/fl}$* mice doxycycline-treated from P20 compared with control littermates.

(Q–X) Defective tongue filiform papillae (Q and R), mildly decreased footpad epidermal thickness (U and V), and decreased proliferation of dorsal tongue (S and T) and footpad (W and X) epithelia in P360 *KRT14-Krm1 Krt5-rtTA tetO-Dkk1* mice doxycycline-treated from P20 compared with control littermates.

(Y) Quantification reveals statistically significant reductions in proliferation of β-catenin-deleted and *Dkk1*/*Krm1*-expressing IFE, footpad, and dorsal tongue epithelia. Blue bars represent littermate controls; orange and green bars represent regions of β-catenin-deleted and -undeleted epidermis, respectively, in *Axin2CreERT2/tdT Ctnnb1$^{fl/fl}$* mutants; red bars represent *Krt5-rtTA tetO-Cre Ctnnb$^{fl/fl}$* or *KRT14-Krm1 Krt5-rtTA tetO-Dkk1* samples as indicated. Results are

Furthermore, inducible deletion of epithelial β-catenin causes regression of squamous cell carcinoma (SCC) in mice (Malanchi et al., 2008). By contrast, broad epithelial deletion of β-catenin in IFE and HFs was associated with HF disintegration, an inflammatory response, and IFE hyperproliferation. Taken together, these data indicate that Wnt/β-catenin signaling contributes to adult IFE proliferation under homeostatic conditions and in SCC but is not required for long-term maintenance of the IFE and is bypassed during hyperproliferative responses to inflammation. The approaches described here will be generally useful for determining the effects of gene deletion in adult IFE in cases where HF degradation and inflammation resulting from broad epithelial deletion complicate analysis of IFE phenotypes.

EXPERIMENTAL PROCEDURES

Mouse Strains, Transgene Induction, Depilation, Skin Biopsies, and Genotyping

Krt5-rtTA tetO-Dkk1 mice were generated as described previously (Chu et al., 2004). A *KRT14-Krm1* transgene was constructed by cloning full-length mouse *Krm1* cDNA (NM_032396) into KRT14 promoter vector (Saitou et al., 1995). *Ctnnb1*$^{fl/fl}$, *Wls*$^{fl/fl}$, *Krt15-CrePR1*, *Krt14-CreER, R26R-Confetti*, and *R26R*^{mTmG} mice were obtained from Jackson Labs (Bar Harbor, ME). *Axin2CreERT2/tdT* mice were generated by insertion of a *CreERT2/tdTomato* fusion cDNA downstream of the first ATG of the mouse *Axin2* gene using homologous recombination in mouse ESCs. Transgene induction was performed as described previously (Chu et al., 2004; Ito et al., 2005). Skin biopsies were taken from euthanized mice or under anesthesia. Detailed methods are provided in the Supplemental Information. The IACUC committees of the University of Pennsylvania and the University of Cincinnati approved all experimental procedures involving mice.

Histology, Staining Procedures, and TEM

Preparation of paraffin sectioned skin, histological analysis, BrdU incorporation assays, TUNEL assays, immunostaining, in situ hybridization, X-gal staining, alkaline phosphatase staining, and TEM were performed according to published protocols (Andl et al., 2006; Zhang et al., 2009). Details are provided in the Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.10.003.

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