Epithelial Wnt Ligand Secretion Is Required for Adult Hair Follicle Growth and Regeneration

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β-Catenin, a key transducer molecule of Wnt signaling, is required for adult hair follicle growth and regeneration. However, the cellular source of Wnt ligands required for Wnt/β-catenin activation during anagen induction is unknown. In this study, we genetically deleted *Wntless (Wls)*, a gene required for Wnt ligand secretion by Wnt-producing cells, specifically in the hair follicle epithelium during telogen phase. We show that epithelial Wnt ligands are required for anagen, as loss of *Wls* in the follicular epithelium resulted in a profound hair cycle arrest. Both the follicular epithelium and dermal papilla showed markedly decreased Wnt/β-catenin signaling during anagen induction compared with control hair follicles. Surprisingly, hair follicle stem cells that are responsible for hair regeneration maintained expression of stem cell markers but exhibited significantly reduced proliferation. Finally, we demonstrate that epidermal Wnt ligands are critical for adult wound-induced *de novo* hair formation. Collectively, these data show that Wnt ligands secreted by the hair follicle epithelium are required for adult hair follicle regeneration and provide new insight into potential cellular targets for the treatment of hair disorders such as alopecia.

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INTRODUCTION

Hair follicle growth in adult mammals occurs through cycles of growth (anagen), regression (catagen), and rest (telogen). The ability to continuously cycle through these phases is dependent on hair follicle stem cells (HFSCs), which reside in a specialized niche of the telogen hair follicle that consists of the bulge and secondary hair germ (sHG) (Cotsarelis *et al.*, 1990; Greco *et al.*, 2009; Myung and Ito, 2012). Remarkably, hair follicles can also regenerate *de novo* in adult mice following wound repair in a manner that recapitulates embryonic hair follicle development (Ito *et al.*, 2007). Similar to embryonic hair development, signals that coordinate the growth and activation of follicular epithelial cells during both anagen onset and adult hair neogenesis involve interactions between follicular epithelial cells, as well as their heterotypic interactions with adjacent mesenchymal cells in the dermis (Millar, 2002; Schmidt-Ullrich and Paus, 2005). Characterization of the molecular signals that govern adult hair follicle growth and regeneration has been the focus of recent efforts to understand both the regulation of hair growth by somatic stem cells and how intercellular interactions are coordinated to promote adult organ regeneration.

Wnt/β-catenin signaling is a central signaling pathway that regulates embryonic and adult hair follicle growth and is mediated by the intracellular molecule β-catenin, which functions in both the regulation of cell–cell adhesion and Wnt-dependent signal transduction (Huelsken *et al.*, 2001; Lowry *et al.*, 2005; Zhang *et al.*, 2009). Secreted extracellular Wnt ligands activate Wnt signaling, leading to stabilization of β-catenin and its translocation to the nucleus, where it binds T-cell transcription factor/lymphoid enhancer-binding factor proteins. This complex regulates transcription of downstream target genes responsible for the pleiomorphic functions of Wnt signaling in proliferation, differentiation, and migration (van Amerongen and Nusse, 2009). Previous studies showed that Wnt/β-catenin signaling is active in the hair follicle during both embryonic hair morphogenesis and postnatal anagen phase. During initiation of hair follicle development, Wnt/β-catenin signaling is first upregulated uniformly in the upper dermis and then focally in both the epithelial hair follicle placode and underlying dermal condensate (DasGupta and Fuchs, 1999; Zhang *et al.*, 2009; Chen *et al.*, 2012). During the adult hair cycle, Wnt activity is observed in the sHG during anagen onset and in the precortex of anagen follicles during hair shaft differentiation (DasGupta and Fuchs, 1999; Greco *et al.*, 2009; Rabbani *et al.*, 2011). How this dynamic pattern of Wnt signaling is regulated during hair follicle development and the adult hair cycle is still unclear.

Several studies have demonstrated a required role for Wnt/β-catenin activation in hair follicle growth. Functional studies have shown that the activation of Wnt/β-catenin in the epidermis is critical for the initiation of embryonic hair

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Abbreviations: DP, dermal papilla; HFSC, hair follicle stem cell; RU486, mifepristone; sHG, secondary hair germ; WIHN, wound-induced hair neogenesis; Wls, Wntless

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follicle development and for upregulation of Wnt signaling in the dermal condensate (Huelsken *et al.*, 2001; Andl *et al.*, 2002; Nguyen *et al.*, 2009; Zhang *et al.*, 2009). Similarly, wound-induced hair neogenesis (WIHN) is also dependent upon Wnt/β-catenin signaling, lending support to the theory that embryonic programs of morphogenesis are reactivated to promote regeneration in adult mammals (Ito *et al.*, 2007).

In the adult hair cycle, progressive deletion of β-catenin in the epidermis resulted in loss of contact between the follicular epithelium and dermal papilla (DP) with formation of epithelial cysts that expressed markers of interfollicular epidermal differentiation (Huelsken *et al.*, 2001). In another study, deletion of epidermal β-catenin specifically at the first telogen phase resulted in hyperproliferation of HFSCs concomitant with their depletion from the niche and failure to enter anagen, suggesting that Wnt/β-catenin signaling is necessary for HFSC quiescence and maintenance (Lowry *et al.*, 2005). Conversely, forced expression of a constitutively active β-catenin in the epidermis is sufficient to induce anagen onset and *de novo* hair follicle formation in adult mice (Gat *et al.*, 1998; Van Mater *et al.*, 2003; Lo Celso *et al.*, 2004; Lowry *et al.*, 2005). These studies established a critical role for epithelial β-catenin in hair growth. More recently, a cell-autonomous requirement for β-catenin in DP cells to support the growth of follicular epithelial cells during anagen was also demonstrated, revealing an essential role for Wnt activation in both the hair follicle epithelium and mesenchyme during the hair cycle (Enshell-Seijffers *et al.*, 2010).

Despite evidence demonstrating the importance of Wnt/β-catenin signaling in adult hair follicle biology, some questions remain unresolved. First, the requirement for Wnt signaling during anagen onset has not been tested independently of β-catenin's function in adherens junctions or independently of T-cell transcription factor/lymphoid enhancer-binding factor that have Wnt-dependent and Wnt-independent functions (Huelsken *et al.*, 2001; Merrill *et al.*, 2001; Niemann *et al.*, 2002; Lowry *et al.*, 2005; Nguyen *et al.*, 2009). In particular, a Wnt signaling mediator upstream of β-catenin has not been formally examined during anagen induction in the adult hair cycle. In addition, it is unclear how Wnt signaling is initiated during anagen onset. Several Wnt ligands are upregulated in the sHG and less prominently in the DP during anagen (Reddy *et al.*, 2001; Greco *et al.*, 2009; Rabbani *et al.*, 2011). Partly owing to functional redundancy between these Wnt ligands, no study has dissected the specific requirement for Wnt ligands secreted by the hair follicle epithelium to activate Wnt signaling in both the follicular epithelium and DP during anagen onset.

To address these questions, we deleted *Wntless* (*Wls*), a gene required for the secretion of Wnt ligands by Wnt-producing cells (Banziger *et al.*, 2006; Bartscherer *et al.*, 2006; Goodman *et al.*, 2006). In contrast to overexpression of Dickkopf-related protein 1 (DKK1), a diffusible protein that inhibits Wnt/β-catenin activation transdominantly, deletion of *Wls* provides a tool to inhibit Wnt-responsive cells that are physiologically activated by epithelial Wnt ligands. We show that loss of *Wls* specifically in HFSCs results in the inhibition of Wnt/β-catenin activity in the sHG and DP, with concomitant hair cycle arrest at telogen/early anagen during both spontaneous and depilation-induced anagen. In contrast to results obtained from previous studies in which β-catenin is depleted (Huelsken *et al.*, 2001; Lowry *et al.*, 2005; Nguyen *et al.*, 2009), loss of follicular epithelial Wnt ligands leads to markedly decreased HFSC proliferation, whereas the expression of HFSC markers is maintained. In addition, WIHN fails to occur in the absence of epidermal Wnt ligands. Altogether, these data suggest that Wnt ligands secreted by the hair follicle epithelium are essential to drive early events in adult hair follicle growth and regeneration.

RESULTS

Wls is upregulated in the hair follicle epithelium and DP during anagen

To begin to address the requirement for epidermal Wls in promoting anagen onset, we first characterized the expression of Wls in the hair follicle during telogen and anagen phases.

During telogen, the hair follicle epithelium and DP express Wls at very low or undetectable levels (Figure 1a). During depilation-induced early anagen phases (anagen I/II) induced by depilation, Wls expression became markedly upregulated initially in the sHG and then in both the sHG and DP. By anagen III, Wls was highly expressed in the DP, epithelial cells surrounding the DP, and outer root sheath cells. By anagen VI, elevated levels of Wls were maintained in the outer root sheath, differentiating cells of the inner root sheath and precortex, and the DP.

We sought to correlate the location of Wls expression with Wnt activity during the resting and growth phases of the hair cycle. A recent study showed that nuclear β-catenin is first detected in the sHG at the telogen–anagen transition, suggesting that Wnt activation initially occurs in the epithelium early during anagen onset (Greco *et al.*, 2009). We further characterized changes in nuclear β-catenin immunoreactivity throughout anagen. During depilation-induced anagen, nuclear β-catenin was detected in both the sHG and DP during early anagen (Figure 1b). Consistent with previous studies, by anagen III, Wnt-activated cells became localized to cells adjacent to the DP, corresponding to the forming matrix/precortex (DasGupta and Fuchs, 1999). At this stage, nuclear β-catenin became downregulated in the DP compared with early anagen DP cells. By anagen VI, nuclear β-catenin immunoreactivity was most prominent in the precortex and differentiating cells of the hair shaft, whereas the DP continued to show lower and more heterogeneous levels of nuclear β-catenin. *In situ* hybridization detection of *Axin2* mRNA, a direct downstream transcriptional target and marker of Wnt signaling (Jho *et al.*, 2002), showed that *Axin2* expression was first evident in the sHG during early anagen and in both the DP and sHG by anagen II (Figure 1c). Similar expression patterns of Wls and nuclear β-catenin were also seen during spontaneous anagen (Supplementary Figure S1 online). These results suggest that Wnt ligands are secreted by the follicular epithelium during anagen onset and then by both epithelial and mesenchymal components of the hair follicle during later stages of anagen. This expression pattern overlaps with the timing and location of Wnt activity in the hair follicle (Figure 1d).

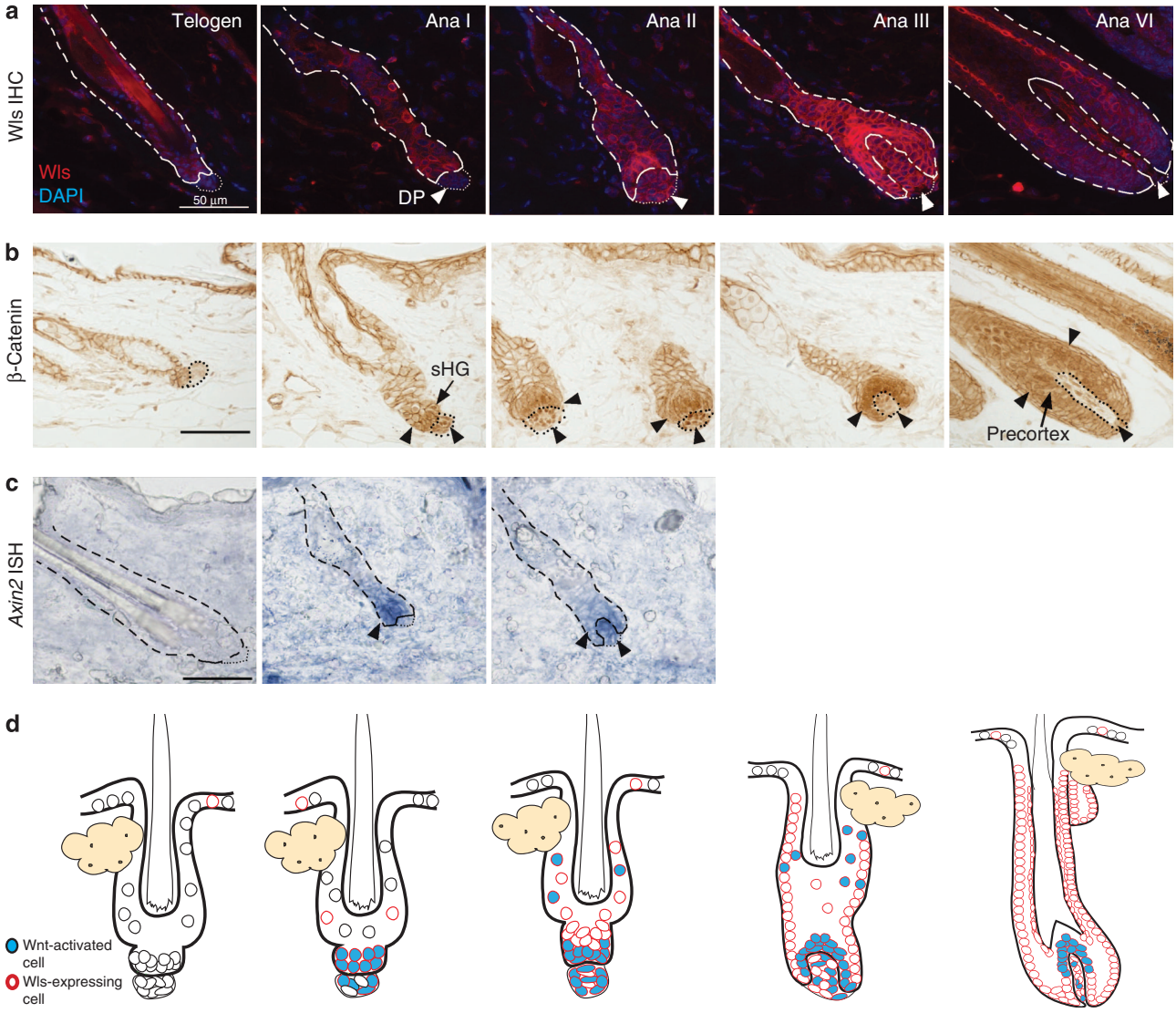
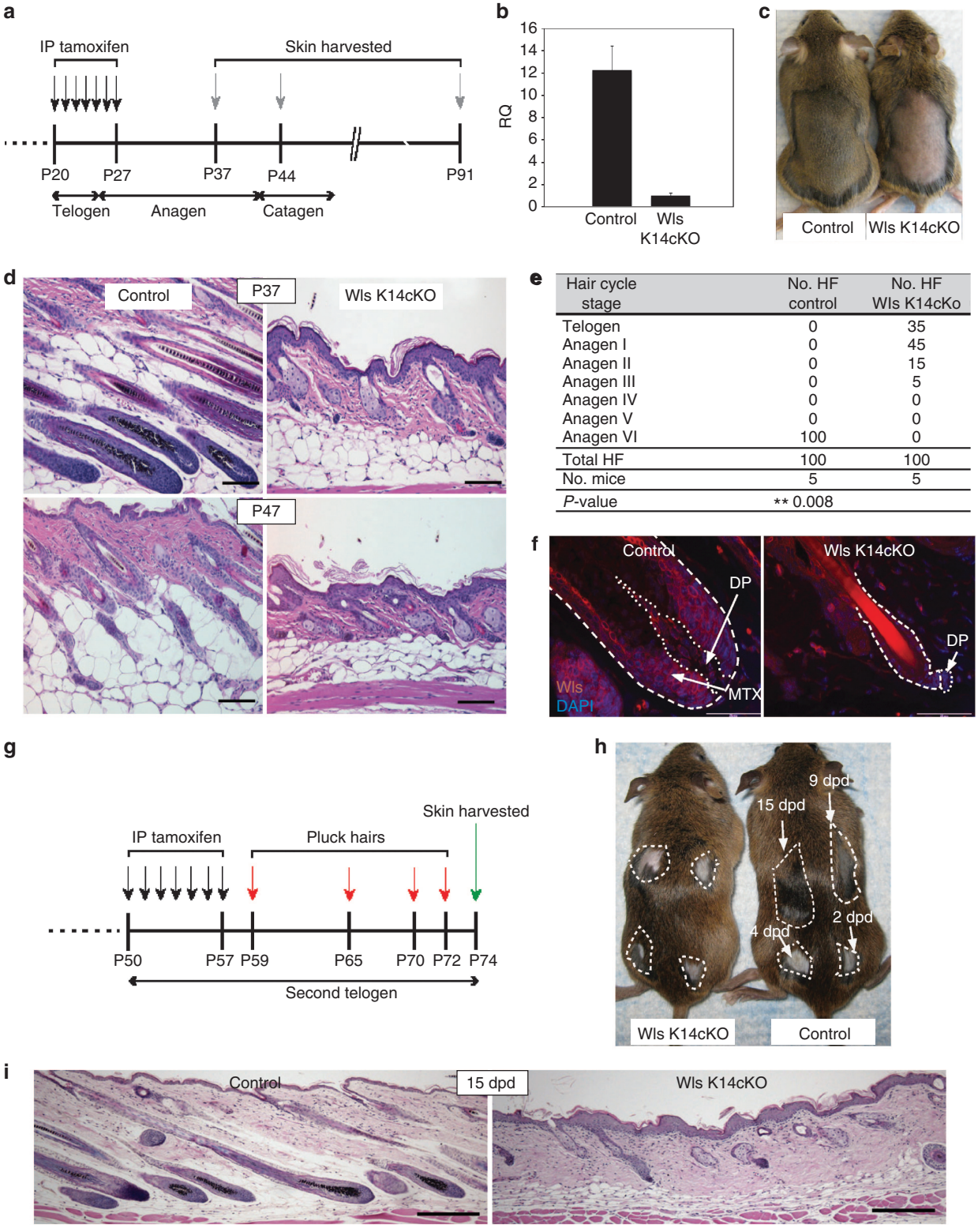


Figure 1. Wntless (Wls) expression correlates with Wnt activation during anagen. (**a**) Wild-type (P60) club hairs were either left unplucked (telogen) or plucked to induce anagen and harvested at either 2 days (Ana I), 3 days (Ana II), 4 days (Ana III), or 9 days (Ana VI) post depilation. Sections were analyzed for Wls expression by immunofluorescence. (**b**) Brightfield immunohistochemical (IHC) detection of β-catenin during telogen and anagen phases as described above. Cells positive for nuclear β-catenin (arrowheads) in the epithelium and DP. (**c**) *In situ* hybridization detection of *Axin2* mRNA transcripts was used to detect Wnt activation on cryosections of telogen and depilation-induced early anagen phase hair follicles. (**d**) Illustrations of the temporospatial distribution of Wls expression and Wnt activation during telogen and anagen phases. DAPI, 4′,6-diamidino-2-phenylindole; DP, dermal papilla; sHG, secondary hair germ. Bar = 50 μm.

Epidermal *Wls* is required for anagen phase

To determine whether epidermal Wnt ligands are required for the hair cycle growth phase, we deleted *Wls* expression specifically in the basal layer of the epidermis and hair follicle, using *K14-CreER;Wls*$^{fl/fl}$ (Wls K14cKO) mice (Vasioukhin *et al.*, 1999; Carpenter *et al.*, 2010). Cre-mediated recombination of the *Wls*$^{fl/fl}$ allele was induced during the first telogen phase (Figure 2a). Quantitative PCR analysis of epidermal preparations showed significantly decreased *Wls* mRNA in induced Wls K14cKO skin compared with control skin (Figure 2b).

To determine whether the deletion of epidermal *Wls* expression affects anagen onset, skin from Wls K14cKO and control mice was examined 10–14 days after induction. At P37, control mice showed darker skin from new hair growth, whereas skin of Wls K14cKO mice remained pink, reflecting lack of hair growth (Figure 2c). Histologically, control littermate hair follicles had entered anagen VI by P37, whereas most Wls K14cKO hair follicles were conspicuously arrested at telogen or early anagen phases (Figure 2d). This arrest was still apparent by P47 when control hair follicles had entered catagen. Overall, 80% of hair follicles from P35–37 Wls K14cKO mice were arrested in telogen and anagen I phases, whereas 100% of hair follicles from littermate controls progressed to anagen VI (Figure 2e). Consistent with quantitative PCR results, mutant hair follicles



Hair cycle stage	No. HF control	No. HF Wls K14cKo
Telogen	0	35
Anagen I	0	45
Anagen II	0	15
Anagen III	0	5
Anagen IV	0	0
Anagen V	0	0
Anagen VI	100	0
Total HF	100	100
No. mice	5	5
P-value	** 0.008	

Figure 2. Epidermal Wntless (Wls) is required for anagen. (**a**) Tamoxifen-mediated Cre induction regimen. (**b**) Relative quantities of *Wls* messenger RNA determined by quantitative PCR (qPCR) from RNA isolated from dorsal skin epidermis of control and Wls K14cKO mice 5 days after induction (P32, $N = 5$ mice). (**c**) Images of P37 mice shaved after induction. (**d**) Hematoxylin and eosin (H&E) sections from control mice during anagen (P37) and catagen (P47; bar = 100 μm). Wls K14cKO hair follicles at the same time points remained arrested in telogen or anagen I/II. (**e**) Hair cycle distribution of control and mutant mice at P37–40. (**f**) Wls expression in P37 control and mutant hair follicles (bar = 50 μm). Scattered Wls immunoreactive cells were noted throughout the dermis, but were similar between control and mutant mice. (**g, h**) Tamoxifen was administered during second telogen before depilation at indicated times. (**i**) H&E sections from skin plucked 15 days post depilation (15 dpd; bar = 200 μm). IP, intraperitoneal; RQ, relative quantitation.

showed markedly lower Wls expression immunohistochemically compared with controls (Figure 2f).

Histologically, mutant hair follicles showed a club hair surrounded by a two-layer epithelial sac corresponding to the bulge. Those in telogen exhibited a compact cluster of cells forming the sHG, which rested adjacent to the DP. Those that had progressed to early anagen showed elongation and widening of the sHG, with those in anagen II beginning to encase the DP. There was apparent hyperplasia of both the epidermis and sebaceous glands in the mutant mice, although the interfollicular epidermis of mutant mice showed normal differentiation by hematoxylin and eosin and marker analyses (Figure 2d, i, 3e, and f). When mice were analyzed at a later time point (P91), Wls K14cKO hair follicles remained largely arrested at either first telogen or anagen I phase, whereas control hair follicles were in the second telogen phase (Supplementary Figure S2a online).

As the exact timing of anagen onset during the spontaneous hair cycle varies between individual mice, we determined whether the above hair cycle defects could be observed following experimentally induced anagen. Depilation of skin during second telogen phase is a potent stimulator of anagen onset (Ito *et al.*, 2002). Wls K14cKO and control mice were induced after entering second telogen phase (P50) and then depilated (Figure 2g). Histological sections from depilated areas of control mice showed a gradual progression of anagen phases, in which the earliest depilated area exhibited plucked hair follicles in anagen VI. By contrast, most plucked hair follicles of Wls K14cKO mice remained in either telogen or anagen I/II phases within all depilated areas, and only few hair follicles advanced to later stages of anagen (Figure 2h and i, Supplementary Figure S2b online). Collectively, these data suggest that epithelial Wnt ligands are essential for initiation of anagen during the hair cycle.

Wls-deficient hair follicles show decreased proliferation

During the telogen–anagen transition, sHG cells proliferate to initiate the growth phase of the hair follicle (Greco *et al.*, 2009). We analyzed proliferation in Wls K14cKO and control hair follicles. BrdU nucleotide uptake by mutant hair follicles was compared with control hair follicles that were either in telogen or depilated to induce early or late anagen (Figure 3a). As expected, control telogen hair follicles showed no BrdU uptake after a 2-hour pulse. By early anagen many cells within the sHG were BrdU+, and by late anagen most cells within the matrix were BrdU+. In contrast, none of the arrested hair follicles in Wls K14cKO mice showed BrdU+ cells. Even after a longer pulse of 6 hours, most Wls K14cKO hair follicles showed no BrdU+ cells (Figure 3a), and only rare hair follicles that appeared to be in anagen I showed one BrdU+ cell within the sHG (data not shown). When Ki67 immunohistochemistry was used to assess proliferation, Wls K14cKO mice showed a severe defect in proliferation compared with depilation-induced early anagen control skin (19.2% Ki67+ cells/hair germ vs. 67.8% Ki67+; Figure 3b and c). These results suggest that epidermal Wnts are required for proliferation of sHG cells at the onset of anagen.

HFSC and DP markers are maintained in Wls-deficient hair follicles

One possibility for the observed hair cycle arrest in Wls K14cKO mice could be that HFSCs, which are required for anagen (Oshima *et al.*, 2001; Blanpain *et al.*, 2004; Morris *et al.*, 2004), are lost in the absence of Wnt ligands. We analyzed hair follicles from Wls K14cKO and littermate control mice for the expression of HFSC markers. At P37, HFSC markers CD34 (Trempus *et al.*, 2003), K15 (Lyle *et al.*, 1998; Liu *et al.*, 2003), and S100A4 (Ito and Kizawa, 2001) were expressed in the bulge region of Wls K14cKO and control hair follicles (Supplementary Figure S3a and b online). Moreover, expression levels of K15, CD34 (Figure 3d, Supplementary Figure S3c online), and TCF3 (Nguyen *et al.*, 2006) (Supplementary Figure S4 online) were maintained in Wls K14cKO hair follicles similar to that of littermate controls by P91, 9 weeks after induction. Quantitative analysis of K15+ and CD34+ cells on histological sections did not show a significant difference between control and Wls K14cKO telogen hair follicles at P91 (Supplementary Figure S3c online). In addition, interfollicular epidermal markers of differentiation were appropriately expressed within the interfollicular epidermis of mutant mice and not within mutant hair follicles (Figure 3e and f). The DP markers alkaline phosphatase (AP) and LEF-1 were also expressed in Wls K14cKO hair follicles similar to control hair follicles (Figure 3g and h). These data show that although epidermal Wnts are required for hair growth they are not required for preservation of HFSCs or for maintenance of other constituent hair follicle populations.

HFSCs require *Wls* for hair growth

To determine whether *Wls* is required specifically in HFSCs to mediate anagen, we used the *K15-CrePR1* mouse model that expresses Cre exclusively in K15+ HFSCs following topical RU486 administration (Morris *et al.*, 2004). Dorsal skin of *K15-CrePR1*, $Wls^{fl/fl}$ (Wls K15cKO) and littermate control mice were induced during telogen and then analyzed at P33–P40 when control hair follicles were in spontaneous anagen phase (Figure 4a). Although control mice grew a new hair coat following induction, Wls K15cKO mice failed to regrow hair in the clipped area of skin (Figure 4b). Histologically, Wls expression was significantly decreased in Wls K15cKO hair follicles compared with controls (Supplementary Figure S6c online). In contrast to control mice that showed progression of all hair follicles to anagen VI, 92.4% of mutant hair follicles were arrested in telogen and only 6.7% reached anagen I (Figure 4c and d). In contrast to the Wls K14cKO mice, Wls K15cKO skin did not show thickening or enlargement of the epidermis and sebaceous glands, suggesting a cell-autonomous effect of *Wls* depletion in epithelial cells outside of the K15+ HFSC population. Ki67 immunohistochemistry revealed a marked reduction in proliferating cells within hair follicles of mutant mice (13.5% ± SD15.0 Ki67+ cells/hair germ) when compared with control early anagen hair follicles (69.3% ± SD16.9 Ki67+ cells/hair germ; Figure 4e, Supplementary Figure S6a online). Consistent with results obtained from Wls K14cKO

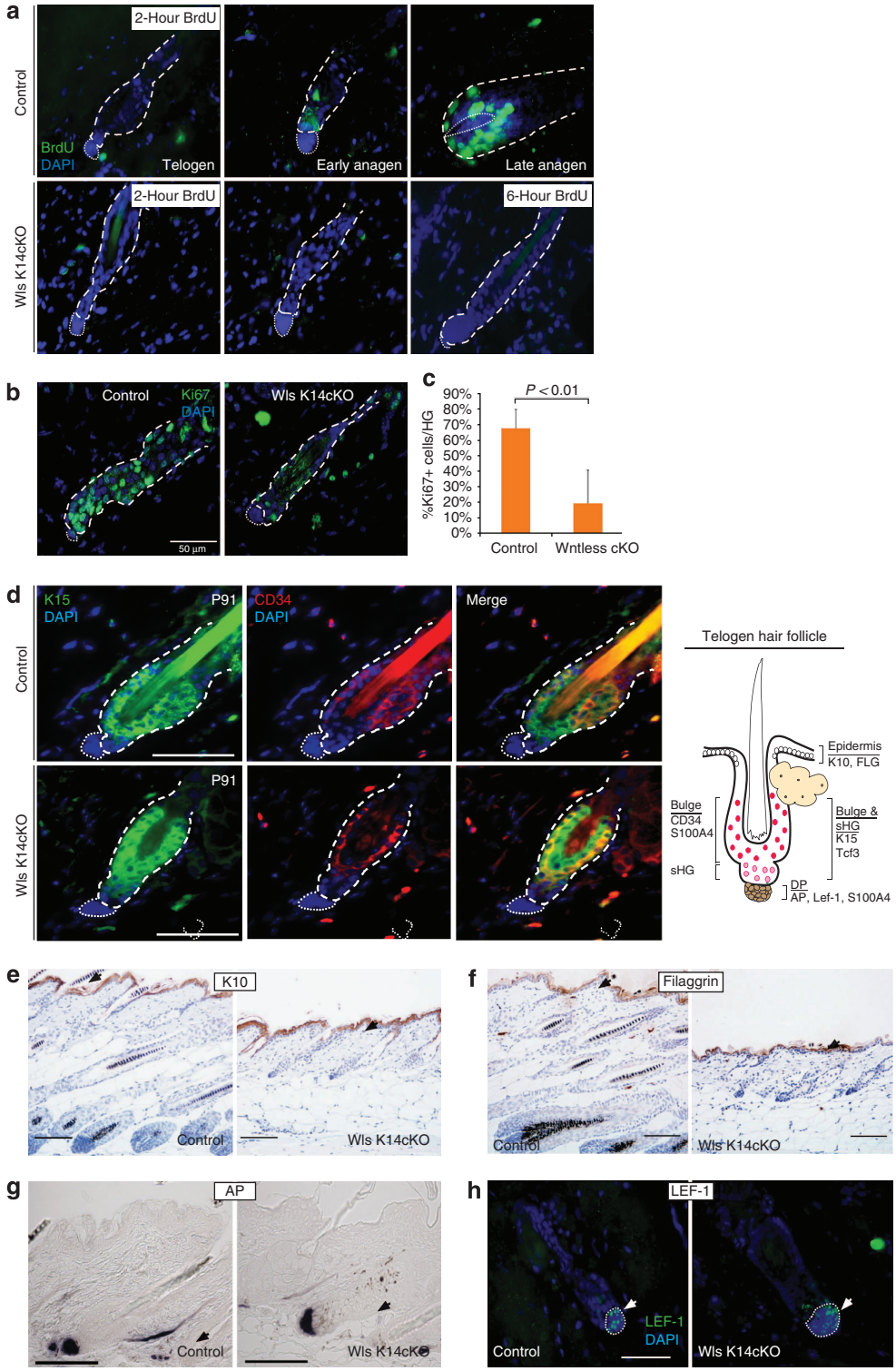


Figure 3. Epidermal Wnts are required for hair follicle stem cell (HFSC) proliferation but not HFSC maintenance. (**a**) P37 Wls K14cKO and depilated control skin were harvested 2 or 6 hours after BrdU administration. Virtually no BrdU+ cells were detected immunohistochemically in the bulge/secondary hair germ (sHG) of Wntless (Wls) K14 cKO mice even after a 6-hour pulse. (**b**, **c**) Ki67 immunohistochemistry of depilation-induced stage-matched control follicles compared with Wls K14cKO follicles. Graph represents results from three mice per group. (**d**) Double immunofluorescent detection of HFSC markers, CD34 and K15, in control (top panels) and Wls K14cKO (bottom) mice at P91. Illustration (right) of marker expression in normal telogen skin. (**e**) Immunohistochemistry of interfollicular epidermal differentiation markers K10 and (**f**) filaggrin (black arrows; bar = 100 μm). (**g, h**) AP and (**h**) LEF-1 expression are maintained in the dermal papilla (DP) of mutant hair follicles (arrows). AP, alkaline phosphatase; DAPI, 4′,6-diamidino-2-phenylindole. Black bars = 100 μm, white bars = 50 μm.

mice, expression of the HFSC markers K15, S100A4, and CD34 and the DP marker AP was maintained in Wls K15cKO hair follicles (Figure 4f, Supplementary Figure S6b online).

Wnt signaling is activated in the sHG and the DP during early phases of anagen (Figure 1b). To address whether the expression of Wls in HFSCs is required for Wnt signaling in the sHG and/or DP, we examined mutant and control hair follicles for evidence of Wnt activation as indicated by nuclear β-catenin expression (Figure 4g). Strikingly, nuclear β-catenin was not upregulated in either the sHG or DP of Wls K15cKO hair follicles. Hair follicles from Wls K14cKO mice also showed decreased number of sHG cells with nuclear β-catenin; however, the number was greater and more variable than in Wls K15cKO mice (Supplementary Figure S5 online). This suggests that Wnt ligand secretion by HFSCs is required for anagen onset and, directly or indirectly, for Wnt signal activation in both the sHG and DP.

Epidermal *Wls* is required for adult WIHN

During WIHN, hair follicles regenerate *de novo* within the center of reepithelialized wounds (Ito *et al.*, 2007). A previous study showed that nuclear β-catenin can be detected in both the hair follicle epithelium and dermal condensate/DP during WIHN. Consistent with this study, another transducer of Wnt signaling, LEF-1, is also the first to be seen in the epithelial germ and dermal condensate during the initial stages of hair neogenesis (Figure 5a). However, unlike embryonic development in which uniform upper dermal Wnt signaling precedes hair morphogenesis (DasGupta and Fuchs, 1999; Zhang *et al.*, 2009; Chen *et al.*, 2012), we could not detect Wnt activity in the upper dermis before hair germ formation by either β-catenin or LEF-1 immunohistochemistry or with a Wnt-responsive reporter (Figure 5b, data not shown).

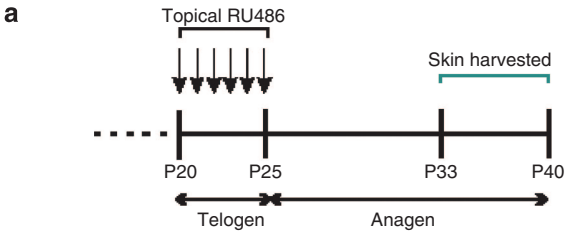
As most neogenic hair follicles are not derived from K15+ HFSCs (Ito *et al.*, 2007), we used Wls K14cKO mice to examine the requirement for epidermal Wnt ligands in promoting WIHN (Figure 5c). Re-epithelialization occurred similarly over 12–14 days in both control and mutant mice (data not shown). The DP of neogenic hair follicles was evident by AP whole-mount staining of dermis samples in healed wounds of control mice but not Wls K14cKO mice (Figure 5d, top). Anti-K17 whole-mount stains of separated epidermal sheets (middle) and hematoxylin and eosin sections (bottom) also confirmed a lack of hair follicles in the wound epithelium of mutant mice. Although control mice showed variable numbers of neogenic AP+ DP within the wound center, only one sample from mutant mice showed one AP+ DP, whereas the remainder showed no neogenic hair follicles (Figure 5e). These experiments demonstrate that epidermal Wnts are required for both the hair cycle and adult *de novo* hair follicle regeneration.

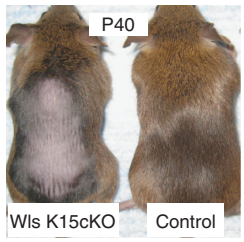
DISCUSSION

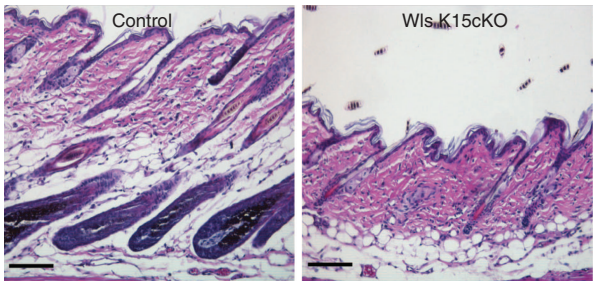
Coordinated activation of Wnt/β-catenin signaling in distinct cell types of the hair follicle is required for their synchronized growth and differentiation during anagen. Both melanocyte stem cells and HFSCs in the sHG activate Wnt signaling at anagen onset, and a previous study suggested that Wnt ligands secreted by HFSCs within the sHG are a source for the dual activation of Wnt signaling in melanocytes and HFSCs at anagen onset (Rabbani *et al.*, 2011). Wnt signaling is also activated in DP cells to support the growth of the hair follicle during anagen (Kishimoto *et al.*, 2000; Enshell-Seijffers *et al.*, 2010; Soma *et al.*, 2012). In this study, we sought to address the cellular source of Wnt ligands that activate Wnt signaling in the hair follicle during anagen onset. To this end, we deleted *Wls* specifically in the hair follicle epithelium to show that Wnt ligand secretion by HFSCs is required for activation of Wnt signaling in the sHG and DP. Furthermore, Wls-deficient hair follicles show a hair cycle arrest in telogen or early anagen phase.

Although the observed hair cycle defect is consistent with previous studies that deleted β-catenin in the basal layer of the epidermis resulting in failure to enter anagen, there are the following important differences (Huelsken *et al.*, 2001; Lowry *et al.*, 2005). First, HFSCs of Wls-deficient hair follicles have a severe proliferation defect. By contrast, loss of β-catenin in the epidermis during telogen resulted in HFSC hyperproliferation, leading to the study's conclusion that Wnt/β-catenin signaling is required for their quiescence (Lowry *et al.*, 2005). Second, expression of HFSC markers is maintained for at least 2 months following induction of Wls K14cKO mice, whereas HFSCs were undetectable within 2 weeks of Cre-mediated β-catenin deletion. Third, the expression levels of interfollicular epidermal and DP markers are maintained faithfully in the absence of epithelial Wls, in contrast to results obtained from the progressive loss of epidermal β-catenin postnatally (Huelsken *et al.*, 2001). We conclude that loss of β-catenin expression resulted in a phenotype that may be partially attributed to its function as a component of adherens junctions. It is plausible that β-catenin's function in intercellular adhesion affects the integrity of the hair follicle and its interactions with the DP, which provides signals to regulate the quiescence and activation of HFSCs. Loss-of-function mutations in specific domains of β-catenin required for its function in adhesion and Wnt signaling in the hair follicle have not been tested.

Although our study cannot differentiate the effect of Wls in canonical or non-canonical (β-catenin-independent) Wnt signaling, the previously established role of canonical Wnt signaling in hair growth and differentiation suggests that Wls is indispensible in HFSCs largely because of its function in canonical Wnt ligand secretion. Irrespective of Wnt ligand class, our data demonstrate that loss of canonical Wnt/β-catenin signaling in HFSCs is not sufficient to result in the extinction of HFSCs. In addition, a previous study showed that excessive Wnt ligand activity in the hair follicle resulted in loss of HFSC marker expression, suggesting that suppression of Wnt signaling in HFSCs is essential for their maintenance (Liu *et al.*, 2007). Taken together, we hypothesize that inhibition of Wnt ligand activity may be important for maintaining HFSCs and that the compartmentalized secretion of Wnt ligands within the sHG is one mechanism to ensure the maintenance of some HFSCs (e.g. bulge cells) with each growth phase. Our observation that a few nuclear







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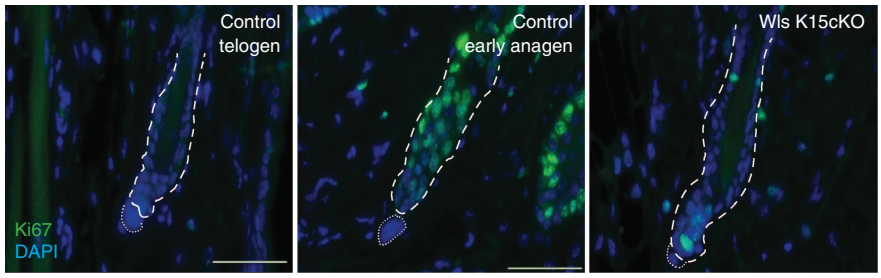
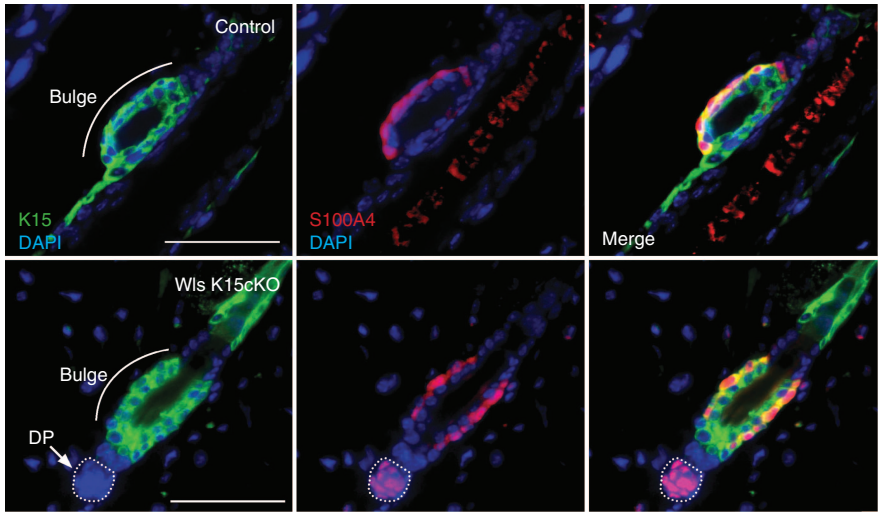
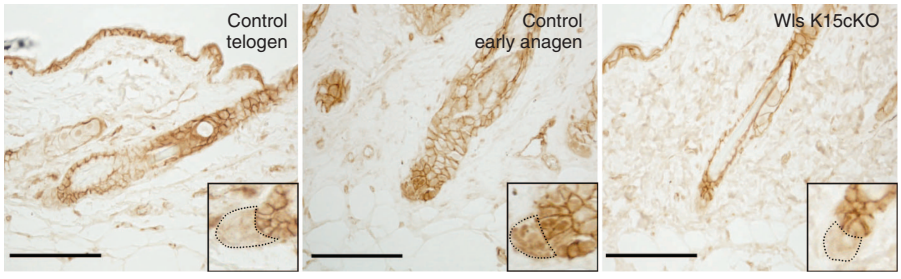
Hair cycle stage	No. HF control	No. HF Wls K15cKO
Telogen	0	110
Anagen I	0	8
Anagen II	0	1
Anagen III	0	0
Anagen IV	0	0
Anagen V	0	0
Anagen VI	90	0
Total HF	90	119
No. mice	4	5
P-value	** 0.016	







β-catenin+ and Ki67+ cells are present in the sHG of arrested mutant hair follicles may also suggest that a minimum number of cells must be activated to initiate anagen phase.

We observed that Wls is specifically upregulated in the sHG during anagen I, which correlates with Wnt ligand transcription during anagen onset (Reddy *et al.*, 2001; Greco *et al.*, 2009; Rabbani *et al.*, 2011). This partly explains the preferential upregulation of Wnt signaling and proliferation of sHG cells during anagen onset. Previous studies proposed that the DP provides inductive signals to the follicular epithelium, which leads to HFSC activation during anagen

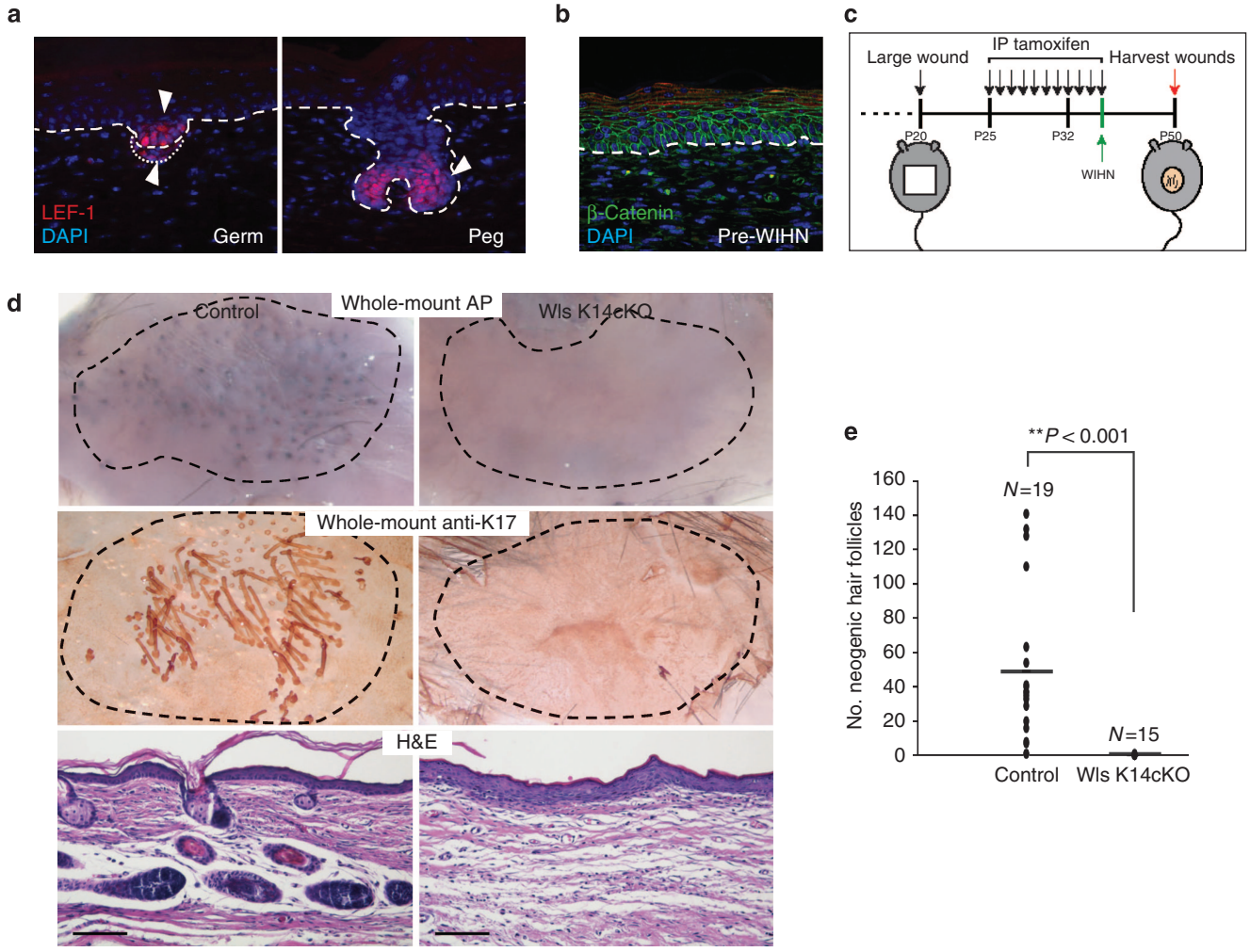


Figure 5. Epidermal Wnt ligand secretion is required for wound-induced hair neogenesis (WIHN). (**a**) LEF-1, a marker of Wnt/β-catenin signaling, is expressed in the epithelium and mesenchyme of germ- and peg-stage neogenic hair follicles from wild-type mice. (**b**) β-Catenin immunohistochemistry of WIHN in wild-type mice taken just after reepithelialization, before hair neogenesis. Nuclear β-catenin was not detected in either the epidermis or the dermis. (**c**) Schematic of tamoxifen induction and wounding. (**d**) Whole-mount alkaline phosphatase (AP) staining of dermal wound preps from control and Wntless (Wls) K14cKO mice (top), whole-mount K17 staining of underside of epidermal sheets (middle), and hematoxylin and eosin (H&E) histology of wound sections (bottom; bar = 100 μm). Control and mutant whole mounts were taken at the same magnification. (**e**) Graphical distribution of the number of neogenic hair follicles per wound in control and mutant mice determined by counting AP+ spots from dermis AP whole mounts (bar represents mean). DAPI, 4′,6-diamidino-2-phenylindole.

Figure 4. Hair follicle stem cells (HFSCs) require Wntless (Wls) to promote Wnt/β-catenin signaling and anagen phase. (**a**) Mutant and control mice were shaved at P20, and RU486 was applied topically for 6 days. Skin was analyzed during anagen (P33–P40). (**b**) Control mice grew hair over the shaved area, whereas mutant skin remained bare by P40. (**c**) Hematoxylin and eosin (H&E) histology of control and mutant skin at P40. (**d**) Corresponding morphological hair cycle stage analysis of control and mutant hair follicles. (**e**) Ki67 immunohistochemistry of Wls K15cKO hair follicles compared with control depilation-induced early anagen and telogen follicles (P33). (**f**) HFSC markers K15 and S100A4 are expressed normally within the bulge/secondary hair germ (sHG) of Wls K15cKO follicles (P40). (**g**) β-Catenin immunohistochemistry shows a lack of nuclear β-catenin in the sHG and dermal papilla (DP) of arrested mutant hair follicles, similar to control telogen follicles. White bars = 50 μm, black bars = 100 μm.

onset (Sun *et al.*, 1991). On the basis of our study and that of others, it is unlikely that the DP activates HFSCs solely by providing Wnt ligands, as Wnt signaling in HFSCs is inhibited in the absence of epithelial Wnt ligand secretion. In addition, upregulation of Wls expression in DP cells lags behind that seen within the sHG during early anagen. Nevertheless, the possibility that DP cells secrete Wnt ligands that are required for Wnt activation in HFSCs during anagen onset cannot be excluded. Reagents that specifically target the DP will be important to address this question.

We found that epithelial Wnt ligands are directly or indirectly required for Wnt signaling in the DP. Given that most canonical Wnt ligands are expressed by the follicular epithelium (Reddy *et al.*, 2001), the DP may respond to Wnt ligands secreted by adjacent sHG and matrix cells during anagen. As DP-specific depletion of β-catenin results in an early anagen arrest (Enshell-Seijffers *et al.*, 2010), the hair cycle arrest observed in the absence of epithelial Wls may be at least partly due to defective Wnt/β-catenin signaling in the DP. Downstream factors secreted by the DP such as fibroblast growth factor 7 (FGF7) and FGF10 likely have important roles in promoting anagen (Greco *et al.*, 2009; Enshell-Seijffers *et al.*, 2010). Although signals upstream of HFSC Wnt activation are still not fully understood, our data are consistent with a model in which HFSCs in the sHG respond to initiating signals from the DP (e.g. BMP inhibitors and others) to secrete Wnt ligands at anagen onset. This results in the dual activation of Wnt signaling in HFSCs themselves, as well as DP cells, and promotes their coordinated and reciprocal interactions throughout anagen (Supplementary Figure S6e online).

Finally, we demonstrate an essential role for epidermal Wnt ligands in promoting adult wound-induced *de novo* hair follicle regeneration. Similar to embryonic hair development, Wnt/β-catenin activation is essential to promote WIHN (Ito *et al.*, 2007). A recent study revealed that epidermal Wls is necessary for uniform upper dermal Wnt activation that is seen before embryonic hair follicle development (Chen *et al.*, 2012). Here, loss of *Wls* expression in the embryonic epidermis resulted in an absence of hair development, suggesting that early dermal Wnt signaling is required for hair follicle initiation. We and others found that Wnt activation is first detected in hair follicle germs and associated dermal condensates but not at an earlier stage of WIHN. Although this may reflect a limitation of detection, similar methods utilized to detect uniform upper dermal Wnt signaling before and during embryonic hair follicle development failed to demonstrate a similar finding during WIHN, suggesting that some differences in signaling requirements between WIHN and embryonic hair development may exist (DasGupta and Fuchs, 1999; Zhang *et al.*, 2009; Chen *et al.*, 2012). Regardless, these data and those of others suggest that Wnt signaling is required for WIHN, independent of uniform dermal Wnt activation.

Collectively, these data establish important concepts in our understanding of the regulation and maintenance of HFSCs by Wnt/β-catenin signaling and highlight the importance of Wnt ligand secretion by hair follicle epithelial cells in directing anagen onset and early events in hair regeneration. In addition, this study sheds light on the potential non-cell-autonomous and cell-autonomous interactions that promote hair growth and holds implications for future strategies to treat hair disorders such as alopecia or hirsutism.

MATERIALS AND METHODS

Mice

$Wls^{flox/flox}$ (Carpenter *et al.*, 2010) were bred to either *Keratin14Cre-ER* (Jackson Labs, Bar Harbor, ME) (Vasioukhin *et al.*, 1999) or *Keratin15Cre-PR1* (Jackson Labs) (Morris *et al.*, 2004) mice. All animal procedures were performed under the approval of Case Western Reserve University IACUC committee. For induction protocols see Supplementary Text online.

In situ hybridization, quantitative PCR, immunohistochemistry, proliferation assays

All assays were performed on dorsal skin as described in Supplementary Text online.

Depilation and WIHN

Depilation and WIHN were performed as detailed in Supplementary Text online.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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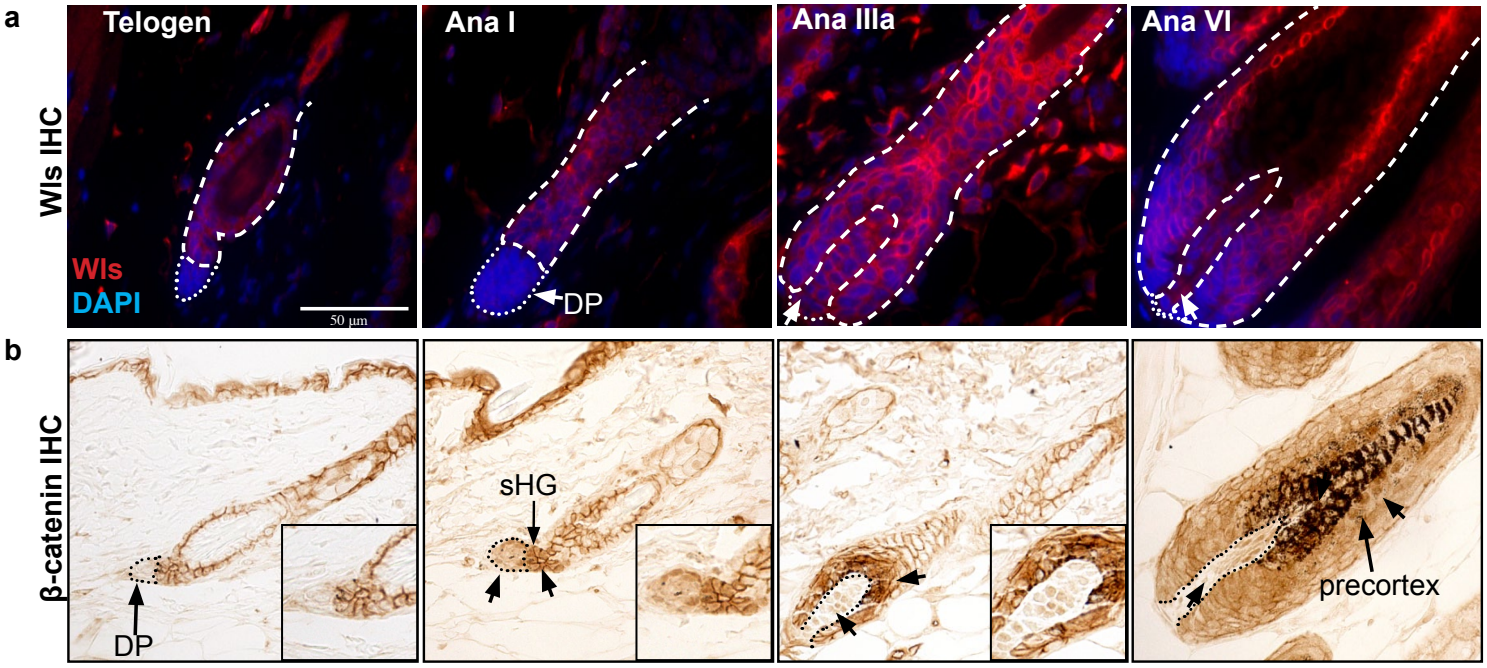

Figure S1. Wls and nuclear β-catenin expression during spontaneous anagen correlates with depilation-induced anagen. (a) Wls is upregulated in the follicular epithelium and DP during spontaneous anagen (bar = 50 um). Dorsal skin was analyzed at ages P21-P30 to obtain various stages of spontaneous anagen (b) Brightfield immunohistochemistry of β-catenin at various stages of spontaneous anagen. Short arrows indicate nuclear β-catenin-positive cells.

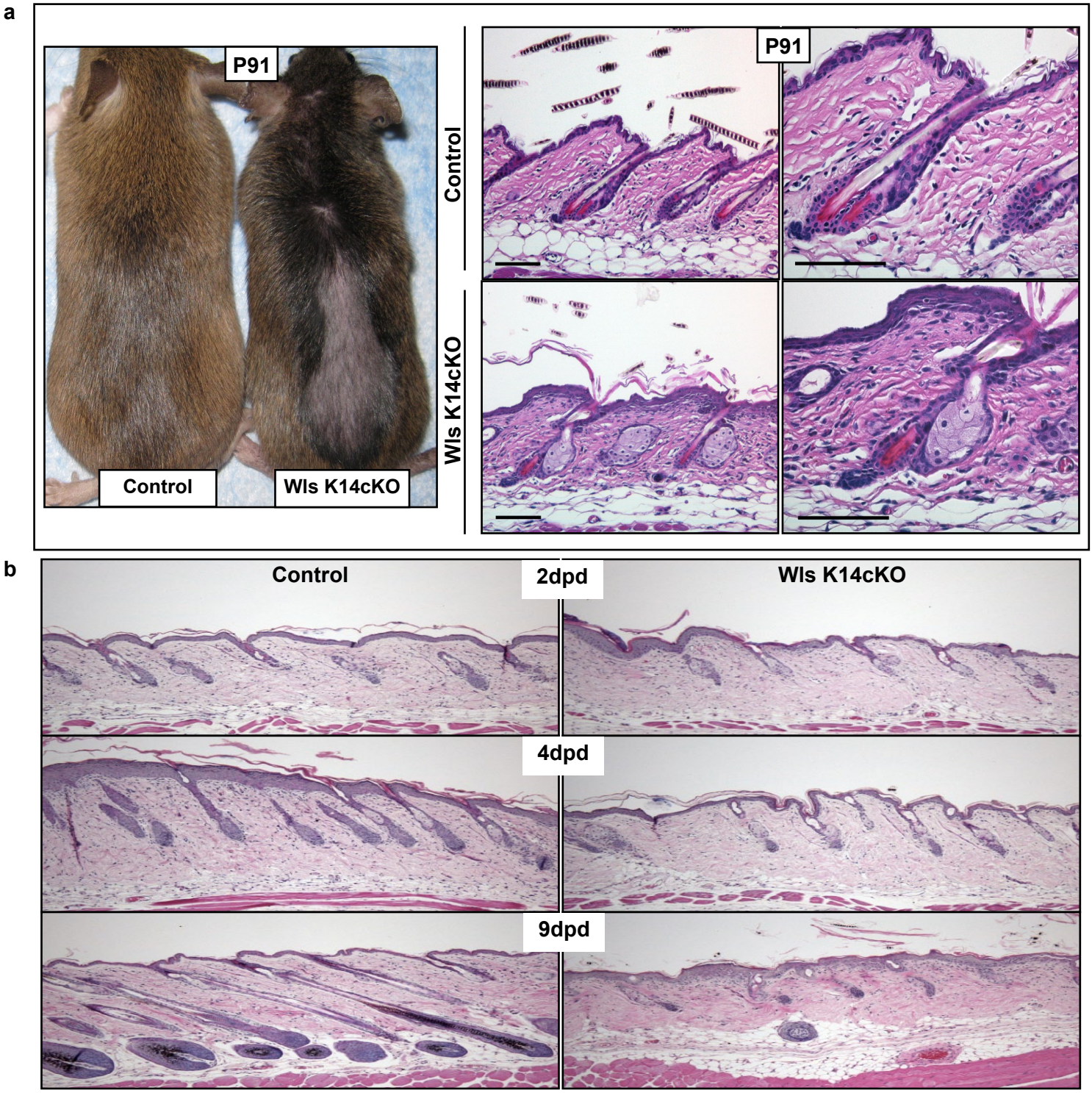

Figure S2. Hair follicles of Wls K14cKO mice show a hair cycle arrest long-term and after plucking. (a) Gross pictures of P91 mice induced with tam at P20 for 8 days. Posterior back skins were clipped at P20. H&E sections of P91 mice reveal that control hair follicles are in second telogen, whereas Wls K14cKO follicles remain in first telogen. The interfollicular epidermis of mutant mice remains thickened similar to younger mutant mice (bar=100μm). (b)H&E sections of control (left) and Wls K14cKO (right) depilated areas harvested either 2 days (top), 4 days (middle) or 9 days (bottom) following plucking during second telogen. Control plucked skin shows a gradual progression in anagen, whereas mutant hair follicles are arrested in telogen or early anagen phases with the exception of sporadic follicles that are able
to progress further in anagen.

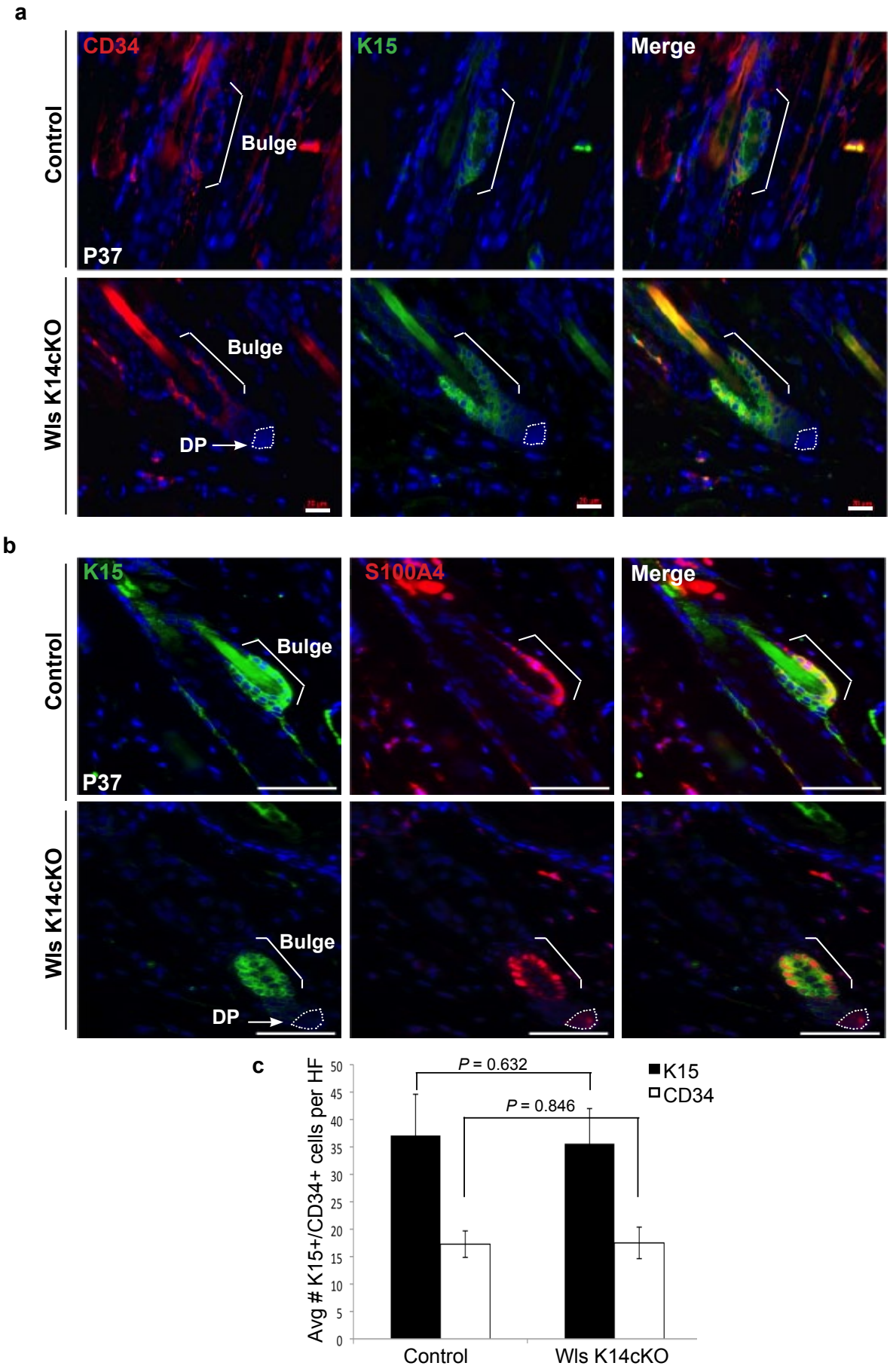

Figure S3. HFSC markers are maintained in Wls-deficient hair follicles. (a) Double immunofluorescent detection of HFSC markers, CD34 and K15 and (b) S100A4 and K15 are expressed similarly in both control and Wls K14cKO hair follicles at P37. S100A4 is expressed by both bulge cells and DP cells in telogen follicles. Bulge regions of control anagen follicles and Wls K14cKO telogen follicles are indicated. 40x (a) Bar=20 um, (b) Bar=50 um. (c) Average number of K15+ (black) and CD34+ cells counted per hair follicle (HF) section from 11 controls, 10 Wls K14cKO HFs (P91).

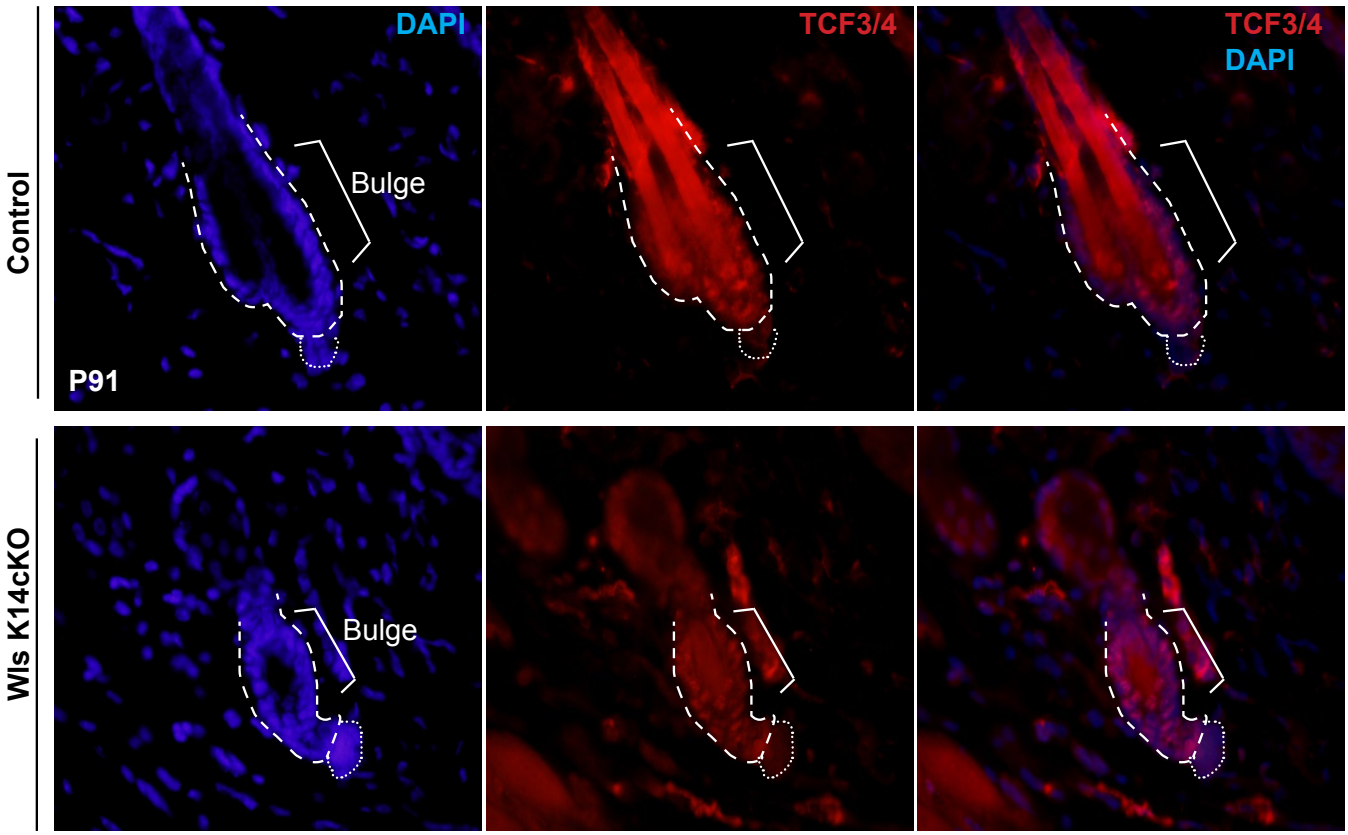

Figure S4. HFSC marker TCF3/4 is maintained in the absence of Wls expression. Immunohistochemical detection of TCF3/4 in the bulge and sHG regions of telogen hair follicles of control (top) and Wls K14cKO (bottom) mice at P91. Bulge region is indicated by brackets (controls and mutant photographs were taken at the same magnification).

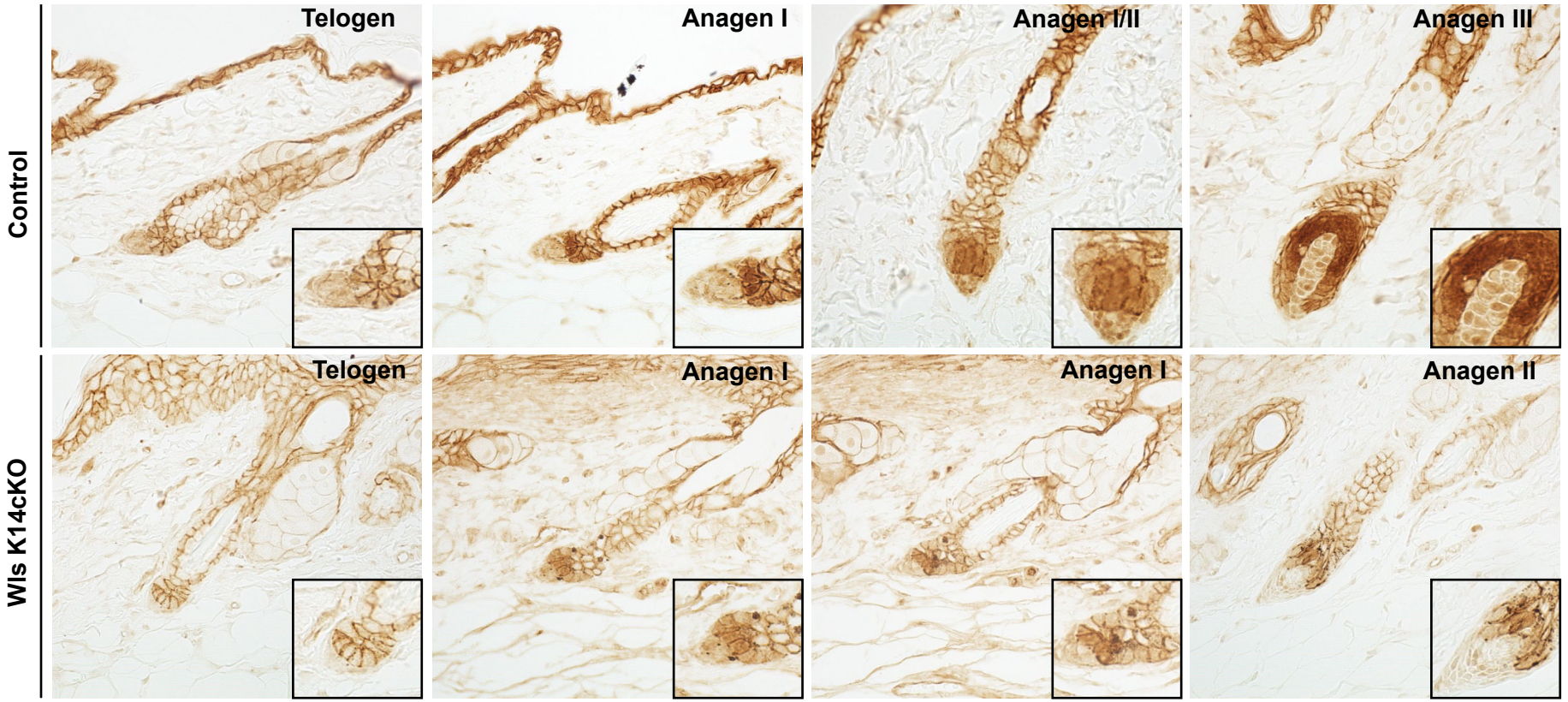

Figure S5. Depletion of epidermal Wnt ligands leads to decreased Wnt/β-catenin signaling in the sHG and DP of arrested mutant hair follicles. Brightfield immunohistochemical detection of nuclear β-catenin in telogen and early anagen control hair follicles (top panels). Note nuclear β-catenin can be detected in the sHG during anagen onset and then in both the sHG and DP of early anagen follicles. By anagen III, nuclear β-catenin is most prominent in cells adjacent to the DP that correspond to the precortex and appears more heterogenously within DP cells. In Wls K14cKO hair follicles (bottom panels), variable nuclear β-catenin staining was observed in which those that appeared to be in telogen by morphology showed a lack of nuclear β-catenin in the sHG and DP, similar to wildtype telogen follicles. In those that appear to have progressed to anagen I-II, some nuclear β-catenin can be seen in some sHG cells, but appears diminished and sometimes undetectable in associated DP cells when compared to stage-matched controls. All control and mutant photographs were taken at the same magnification. Insets show higher magnification of the sHG/DP regions of hair follicles.

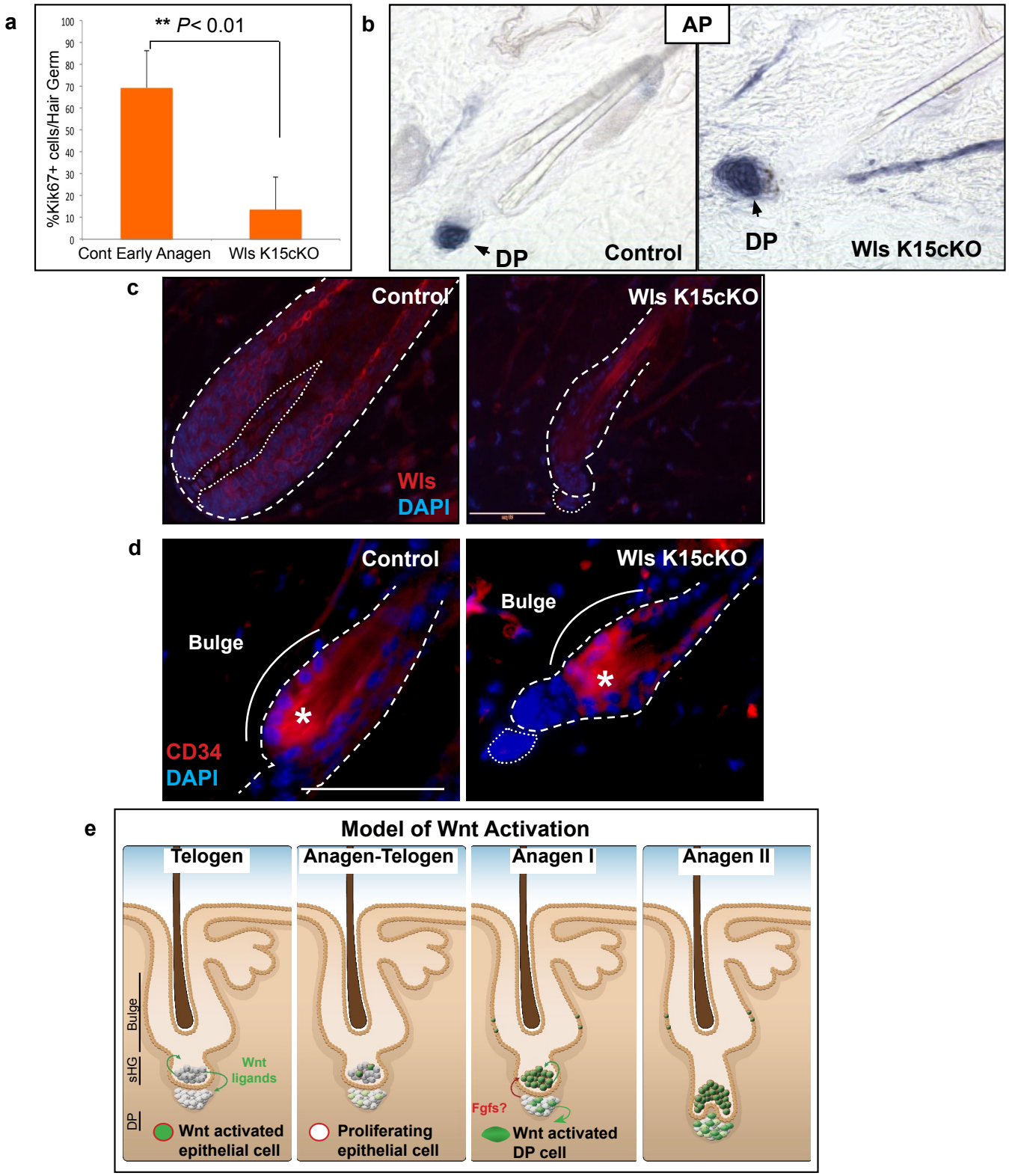

Figure S6. Wnt ligand secretion by HFSCs is required for anagen. (a) P37 mice from Wls K15cKO mice induced with topical RU486 for 6 days from P20-P25. Dorsal skin was harvested at P37 and sections were stained for Ki67. The proportion of Ki67+ cells per hair germ were significantly reduced compared to early anagen control hair follicles but more than P65 control telogen hair follicles (3.7% +/- SD6.41 Ki67+ in control telogen sHGs vs 13.5% +/- SD14.97 in Wls K15cKO; ** = stastically significant difference by Student t-test. (b) The DP marker, AP is maintained in Wls K15cKO hair follicles similar to control telogen follicles. (c) Wls immunohistochemistry of control and mutant hair follicles at P40. (d) CD34 staining of bulge cells in control and mutant hair follicles at P40 (* indicates autofluorescence; bar=50μm) (e) Model illustration of Wnt activation during anagen onset. Telogen hair follicles lack Wnt activated cells and are not proliferating (white cells). Wnt ligands secreted by the sHG activate sHG cells and possibly adjacent DP cells (green arrows). During anagen onset, sHG cells activate Wnt signaling (green) in response to Wnt ligands and begin to proliferate (red rim), while DP cells also begin to respond to Wnt ligands (light green). As anagen progresses, Wnt activated DP cells secrete other factors such as Fgf7/10 (red arrow) that support the continued growth and differentiation of epithelial cells. Wnt activated sHG cells continue to secrete Wnt ligands (green arrows).

Supplementary Materials and Methods:

Generation and induction of mouse lines

Recombination was induced by delivering 1 mg tamoxifen dissolved in corn oil (Sigma-Aldrich, St. Louis, MO) intraperitoneally daily for 7-8 days to *K14Cre-ER* transgenic mice and 1% Mifepristone (Sigma-Aldrich) in ethanol was applied topically to clipped dorsal skin of *K15Cre-PR1* mice daily for 6 days starting at P20. For each experiment, at least three to five mutants with littermate controls from one to three litters were analyzed. All animal procedures were done under the approval of Case Western Reserve University IACUC committee.

Quantitative PCR, In situ hybridization, immunohistochemistry, proliferation assays, and statistical analysis

We followed a previously described method to prepare epidermal sheets from dorsal skin (Morris *et al.*, 2004; Rabbani *et al.*, 2011). To prepare RNA from epidermal preparations, the RNeasy Micro kit (Qiagen, Hilden, Germany) was used. cDNA from total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (ABI, Carlsbad, CA) as instructed. For quantitative PCR, TaqMan primers were used according to manufacturer's instructions (ABI, Mm00509695_m1). Relative mRNA quantities were calculated based on the $\Delta\Delta$Ct method. Mid-dorsal skins were harvested, fixed in 4% paraformaldehyde and processed for paraffin embedding or dehydrated with sucrose and embedded in OCT for cryosectioning. To show skin morphology, paraffin sections were stained with hematoxylin and eosin using standard protocols. Hair cycle stage was determined morphologically, using published guidelines (Muller-Rover *et al.*, 2001). mRNA detection by *in situ* hybridization on cryosections for *Axin2* (Addgene, Cambridge, MA) was performed as previously described (Tran *et al.*, 2010). For BrdU analysis, mice were injected intraperitoneally with BrdU (50 mg/gm body weight; Roche, Basel, Switzerland). Two or six hours later, mid-dorsal skins were harvested and

cryosections were stained for BrdU. For immunohistochemistry, sections were incubated at 4 deg overnight with primary antibodies against the following: β-catenin (BD Biosciences, San Jose, CA, 1:100), Wls (courtesy of Richard Lang, 1:1000), Filaggrin (Covance, Princeton, NJ, 1:500), Keratin10 (Covance, 1:1000), LEF-1 (Cell Signaling, Danvers, MA, 1:100), CD34 (BD Biosciences,1:50), Keratin15 (NeoMarkers, Fremont, CA, 1:100), BrdU (Roche, 1:17), Ki67 (Abcam, Cambridge, UK, 1:200), TCF3/4 (Abcam, 1:50), S100a4 (NeoMarkers, 1:100). For CD34 detection, biotinylated goat anti-rat IgG secondary antibody (Vector Labs, Burlingame, CA, 1:100) followed by TRITC-conjugated streptavidin (Vector Labs, 1:200) was used. For the remaining immunofluorescence, species-specific secondary antibodies conjugated to either Alexa488 or 594 were used (Invitrogen, Carlsbad, CA, 1:200) with DAPI nuclear counterstain (Vector Labs). For brightfield immunohistochemistry, biotinylated species-specific secondary antibodies (1:200, Jackson Labs) followed by detection using the ABC kit (Vector Labs) and DAB (Amresco, Solon, OH) were used according to manufacturer instructions. Proliferation indices were calculated by counting the percentage of Ki67+ cells within the sHG; 60 control and mutant hair follicles were analyzed at 40X magnification. Statistical significance for proliferation and WIHN follicle counts was calculated using Student's *t*-test. Statistical significance of results obtained from hair follicle stage analyses was evaluated by the Mann-Whitney test. Quantification of HFSC number was performed by counting either K15+ or CD34+ cells per hair follicle (P91) from 5 um paraffin sections. Counts were taken from hair follicles in which the club hair, sHG and DP could be all visualized at 40x. For detection of AP, cryosections were fixed in acetone for 10 min and then incubated with the 0.02% NBT/BCIP (Roche) substrate. Fluorescent images were captured with Olympus BX60 microscope and DP72 camera (Olympus, Hamburg, Germany). Brightfield images were obtained with Leica DM2000 microscope and DFC490 camera (Leica, Wetzlar, Germany). Whole-mount images were obtained with Leica MZ16F dissecting microscope. Control images were all taken at the

same magnification as mutant images for each figure. Images were processed in Adobe Photoshop and InDesign software. All schematics were created with Adobe Illustrator.

Depilation and WIHN

For depilation analyses, tamoxifen was administered for 8 days and then club hairs were depilated at various times as indicated to induce anagen using previously described protocols (Ito *et al.*, 2002). 1-cm^2 full-thickness skin wounds were made as previously described (Ito *et al.*, 2007). Tamoxifen was administered daily as described and de novo hair follicles were identified by either whole-mount AP staining of dermis preparations or whole-mount anti-K17 epidermal staining (Abcam, 1:1000) as previously described (Ito *et al.*, 2007).

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