How stem cells maintain their identity and potency as tissues change during growth is not well understood. In mammalian hair, it is unclear how hair follicle stem cells can enter an extended period of quiescence during the resting phase but retain stem cell potential and be subsequently activated for growth. Here, we use lineage tracing and gene expression mapping to show that the Wnt target gene Axin2 is constantly expressed throughout the hair cycle quiescent phase in outer bulge stem cells that produce their own Wnt signals. Ablating Wnt signaling in the bulge cells causes them to lose their stem cell potency to contribute to hair growth and undergo premature differentiation instead. Bulge cells express secreted Wnt inhibitors, including Dickkopf (Dkk) and secreted frizzled-related protein 1 (Sfrp). However, the Dickkopf 3 (Dkk3) protein becomes localized to the Wnt-inactive inner bulge that contains differentiated cells. We find that Axin2 expression remains confined to the outer bulge, whereas Dkk3 continues to be localized to the inner bulge during the hair cycle growth phase. Our data suggest that autocrine Wnt signaling in the outer bulge maintains stem cell potency throughout hair cycle quiescence and growth, whereas paracrine Wnt inhibition of inner bulge cells reinforces differentiation.

Wnt signaling | hair follicle | stem cells | homeostasis | regeneration

The hair follicle is a complex miniorgan that repeatedly cycles through stages of rest (telogen), growth (anagen), and destruction (catagen) throughout life (1). During anagen, growing hair follicles emerge adjacent to the old telogen hair follicles that remain there throughout the cycle and create an epithelial protrusion known as the “bulge.” At the end of the hair cycle, in catagen, cells from the follicle migrate along the retracting epithelial strand and join the two epithelial layers of the telogen bulge—the inner and outer bulge layers—surrounding the club hair shaft (2).

Several studies have established that stem cells residing in the outer bulge are the source of the regenerative capacity of the cycling hair follicle (3–5). During telogen, these stem cells are thought to be generally quiescent (6). In response to signals from their microenvironment during anagen, the stem cells divide and produce proliferative progeny that participate in the growth of the new follicle (7). Some of these activated stem cells and their progeny are believed to migrate away from the bulge, but are subsequently able to rejoin it after anagen is complete (2, 5). Cells that return to the outer bulge take on a follicular stem cell identity, ready to divide and participate in the next hair cycle (2, 8). Conversely, cells returning to the inner bulge do not divide and, instead, form an inner bulge niche of differentiated cells for the outer bulge cells (2). Stem cells remain quiescent during telogen for an extended period, and the identity of signals that maintain stem cell identity during this time are poorly understood.

In the hair, Wnt/β-catenin signaling is required right from the earliest stages of development, for the initiation of hair placode formation (9). Wnt signals are needed later during postnatal homeostasis as well, for the initiation of anagen in postnatal hair (10). Therefore, in view of their well-established importance for stem cell maintenance in multiple adult tissues, including the skin (11), Wnts are candidate hair follicle stem cell (HFSC)-maintaining signals. However, Wnt signaling is generally believed to be inactive in the telogen bulge (8, 10, 12), which is thought to be quiescent. Wnt signaling becomes strongly elevated when bulge cells are “activated” to undergo the transition from telogen to anagen (13, 14). During anagen, Wnt signaling has been described to primarily specify differentiated cell fates in the anagen follicle (12, 15). As anagen proceeds and the follicle enters catagen and telogen again, the bulge is thought to revert to a Wnt-inhibited state (12, 13, 16, 17).

Conversely, there is evidence for a functional requirement of Wnt/β-catenin signaling in the bulge other than initiating anagen and specifying differentiation during anagen. For instance, postnatal deletion of β-catenin in outer bulge cells results in the loss of label-retention and HFSC markers, suggesting that β-catenin is required for maintenance of HFSC identity (10).

Here, beyond its role in hair differentiation and anagen initiation, we sought to determine whether Wnt/β-catenin signaling is also involved in HFSC maintenance during telogen. We found that Axin2 expression persists in HFSCs in the outer bulge throughout telogen and anagen, suggesting that active Wnt signaling is maintained in the bulge, whereas Wnt/β-catenin signaling is required for maintenance of HFSC identity during postnatal growth and catagen.


Significance

Hair follicle stem cells (HFSCs) remain quiescent for long periods of time during the resting phase of the hair cycle. How they maintain their stemness and identity during quiescence while being responsive to growth-inducing cues remains poorly understood. Here, we identify Axin2 as a previously unidentified marker of HFSCs and use it to show that quiescent HFSCs undergo and require active Wnt/β-catenin signaling. By mapping Wnt and its inhibitors with high sensitivity, we show that HFSCs secrete their own self-renewing Wnt signals and inhibitors that promote differentiation outside of the stem cell compartment.

Our findings suggest that careful modulation of Wnt signaling may be important for the derivation and maintenance of HFSCs for alopecia treatment and drug screens.
signaling is a consistent feature of bulge stem cells. Furthermore, these hair outer bulge stem cells produce autocrine Wnts and paracrine-acting Wnt inhibitors that may specify the positional identity of cells residing within the bulge niche.

**Results**

To determine whether Wnt/β-catenin signaling is active during the telogen stage, we examined telogen follicles for the expression of *Axin2*, a well-established Wnt/β-catenin target gene (18, 19), using RNA in situ hybridization. We found that *Axin2* was expressed mostly in telogen outer bulge cells (Fig. 1A). Similarly, when we examined telogen hair from *Axin2-lacZ* Wnt reporter mice, we observed clear reporter activity, specifically in the outer bulge (Fig. 1B). To determine whether Wnt signaling activity varies during telogen, we looked at *Axin2* mRNA expression during early [postnatal day 43 (P43)], mid (P56), and late (P69) telogen using RNA in situ hybridization. We found that *Axin2* mRNA is expressed in the bulge throughout telogen (Fig. S1A), suggesting that Wnt signaling persists through all of the telogen phases.

Because HFSCs are known to reside in the outer bulge (3, 5, 20), we asked whether *Axin2*-expressing telogen bulge cells have stem cell properties. We permanently labeled *Axin2*-expressing cells in the telogen bulge by exposing *Axin2*-CreERT2 Rosa26–mTmG<sup>flox</sup> mice (21, 22) to tamoxifen (Tam) during early second telogen (P49) and then tracked the fate of these cells and their progeny for various lengths of time (Fig. 1C). Consistent with our observations described above (Fig. 1A and B), *Axin2*-CreERT2-labeled cells in the bulge persisted from early to mid-telogen (Fig. 1D). As the hair follicles entered anagen, the labeled cells appeared to accumulate in the growing secondary hair germ and migrate along the elongating outer root sheath and into the developing matrix, where they lay adjacent to the dermal papilla and generated progeny spanning all of the layers of the anagen hair follicle (Fig. 1E). These cells were able to participate in successive cycles of hair follicle growth up to a year after the initial labeling (Fig. 1F). We observed the same patterns of *Axin2* expression and long-term, self-renewing potential of outer bulge cells labeled during the first telogen (P21) occurring immediately after morphogenesis (Fig. S1 B–K), suggesting that *Axin2*-expressing outer bulge cells are present during successive telogen cycles of hair growth. These data suggest that *Axin2*-CreERT2 labels long-lived HFSCs in the outer bulge.

Hair bulge cells are thought to be quiescent during early second telogen (23). To determine whether *Axin2*-expressing bulge cells were indeed quiescent cells, we performed cell-cycle analysis on *Axin2*-expressing bulge cells harvested by flow cytometry from *Axin2-lacZ* reporter mice. Almost all of these cells exhibited a G0/G1 nuclear profile (>90%; Fig. 1G), suggesting that *Axin2*-expressing telogen bulge cells are quiescent.

![Fig. 1.](https://www.pnas.org/cgi/doi/10.1073/pnas.1601599113)
To test whether Axin2 is indeed a Wnt/β-catenin signaling target gene in the hair bulge, we conditionally inactivated the β-catenin gene in Axin2-expressing cells during telogen by crossing Axin2-CreERT2 mice with β-cateninΔex2-6 floxed mice. Axin2 mRNA expression that was present in heterozygous Axin2-CreERT2/β-cateninΔex2-6/+ control hair bulge cells was abrogated in Axin2-CreERT2/β-cateninΔex2-6/- mutant bulge cells (Fig. 2A and B). This result was consistent with the observation that Axin2 expression is also greatly decreased when β-catenin is deleted in Keratin-15-expressing bulge cells (Fig. S2B) (14). Thus, Axin2 expression in the outer bulge cells is dependent on the presence of β-catenin. Furthermore, other Wnt target genes like Lgr5 are also expressed in the outer bulge (Fig. S2A) (5) and similarly require β-catenin for their expression (Fig. S2B) (14). The expression of multiple Wnt target genes suggests that Wnt/β-catenin signaling is active in the telogen bulge and that Axin2 expression is a reliable indicator of Wnt signaling activity in hair follicles.

The ability to produce differentiated cells is a defining property of stem cells. We next asked whether Axin2-expressing HFSCs require Wnt/β-catenin signaling to function as stem cells by producing differentiated hair lineages during the anagen stage of hair growth. In heterozygous Axin2-CreERT2/β-cateninΔex2-6/+ control littersmates, hair follicles entered anagen normally (Fig. 2C). In contrast, Axin2-CreERT2/β-cateninΔex2-6/- mutant hair follicles did not grow, and most exhibited an abnormal telogen-like morphology (Fig. 2D). This result resembles the arrested hair growth seen in animals where β-catenin is mutated in all outer bulge cells (10, 24). The loss of hair follicle growth suggests that Axin2-expressing bulge HFSCs require β-catenin to maintain their stem cell potency/state.

To determine the source of Wnt signals for these Wnt-dependent HFSCs, we screened for the expression of Wnt genes at various stages of the hair cycle using RNA in situ hybridization (Fig. 3A–C). We found that Wnt1, Wnt4, and Wnt7b were expressed in the bulge throughout telogen. The highest levels of expression appeared to be in outer bulge cells. Consistent with our Axin2 mRNA expression data, these Wnts remain expressed throughout telogen. In comparison, we did not detect any Wnt5a expression in the bulge throughout telogen, whereas other Wnts, including Wnt3, Wnt6, Wnt10a, and Wnt10b, are expressed, but at much lower levels (Fig. S3). These data suggest that the outer bulge HFSCs themselves are a source of Wnt signals in the telogen bulge.

How is Wnt gene expression in the bulge regulated? When we looked at Wnt gene expression using RNA in situ hybridization in Axin2-CreERT2/β-cateninΔex2-6/- mutant bulges, we found a loss of Wnt4 mRNA expression compared with Axin2-CreERT2/β-cateninΔex2-6/+ control bulges (Fig. 3D). This is similar to other reports where β-catenin deletion in keratin-15-expressing bulge cells also resulted in a great reduction in Wnt7b expression (Fig. S2B) (14). Together, these data suggest that production of at least some Wnts is regulated by and requires autocrine Wnt/β-catenin signaling in the outer telogen bulge.

Is Wnt secretion required for hair growth? We observed that Wntless, a Wnt cargo-binding protein required for Wnt secretion, is expressed in bulge cells (Fig. 4A), consistent with previous reports (25, 26). Wntless is also expressed during hair development (27), and our lineage tracing experiments show that embryonic Axin2-expressing cells become HFSCs (Fig. S1L). Conditional knockout of Wntless from this stage in mutant Axin2−rtTA/tetO-Cre/Wntlessfl/+ mice resulted in much less hair and thickened epidermis in several regions compared with their heterozygous littermate controls (Fig. 4B and C), suggesting that Wntless is required by Axin2-expressing cells for hair growth. This finding is consistent with other studies where hair is lost and remaining hairs are arrested in telogen when Wntless is deleted in the entire epidermis (25–27) and also specifically in the hair bulge (24, 25). Together, these data and the literature suggest that autocrine Wnt signaling is required by HFSCs for hair growth and maintenance.

To determine whether Wnt production and β-catenin signaling change as the hair enters the anagen growth phase, we examined follicles at the telogen-to-anagen transition and found that Axin2, Wnt1, Wnt4, and Wnt7b continued to be expressed in the bulge (Fig. 3C). However, the expression of several Wnts, including Wnt6, Wnt7b, Wnt10a, and Wnt10b, became up-regulated in cells at the boundary of the secondary hair germ and the dermal papilla (Fig. S3C), consistent with the strong expression of Axin2 in the secondary hair germ and previous reports (7, 28). This result suggests that specific Wnts may be involved in the epithelial–mesenchymal cross-talk in secondary hair germ cells needed to initiate anagen, as has previously been proposed (28).
We next asked whether the bulge becomes Wnt-inhibited during anagen, as is widely believed (12, 13, 16, 17). Contrary to expectations, we observed that \textit{Axin2} mRNA and the \textit{Axin2}–\textit{lacZ} reporter gene continued to be expressed in the anagen bulge (Fig. 5 A–C and F). At the same time, we found the persistent expression of \textit{Wnt4} and \textit{Wnt7b} in the anagen bulge (Fig. 5 D and F). This finding is in contrast to other Wnt genes like \textit{Wnt5a}, \textit{Wnt6}, \textit{Wnt10a}, and \textit{Wnt10b} that are expressed elsewhere in the anagen follicle (Fig. S4C), but not in the anagen bulge (Fig. 5E). By double-labeling RNA in situ hybridization, we found that
Axin2-expressing bulge cells also expressed Wnt4 and Wnt7b (Fig. 5F). These data suggest that the anagen bulge is not Wnt-inhibited and that Wnt production and β-catenin signaling persist in the anagen bulge. In addition, we found Axin2 (Fig. S4A) and distinct Wnt gene expression patterns (Fig. S4 B and C) in multiple differentiated lineages of the anagen hair follicle, consistent with previous reports (28). Other Wnts like Wnt7a and Wnt16 were present either at very low levels or were not expressed (Fig. S4D). Specific Wnts may thus play distinct functional roles in the different compartments of the anagen hair follicle.

Our data show that Wnt signaling, as reported by Axin2 expression, is restricted to the outer bulge (Fig. 1A and B). If Wnt signals are available in the bulge, then why do inner bulge cells not also respond to Wnt and proliferate? In seeking to address this question, we found evidence for active Wnt inhibition of the inner bulge. Using RNA in situ hybridization, we saw that several secreted Wnt inhibitors, including Dkk3 and Sfrp1, are expressed in the outer bulge during telogen (Fig. 6A). Expression of some of these Wnt inhibitors persists into the anagen phase (Fig. 6B). This finding is consistent with other studies of gene expression in isolated mouse bulge cells (3, 16) and is also similar to human hair follicle bulges, where both Dkk3 and SFRP1 are expressed in the outer bulge, but are either at very low levels or not present in the inner bulge (Fig. 6C) (29).

When we performed antibody staining for the Dkk3 protein in the telogen bulge, however, we found that the Dkk3 protein is localized to the inner bulge (Fig. 6D). This distribution of Dkk3 mRNA and protein in the bulge persists even through anagen (Fig. 6E), suggesting that HFSC stemness is restricted to the outer bulge by constant inhibition of Wnt signaling in the inner bulge cells.

Discussion

Our data lead us to propose a model in which Wnt/β-catenin signaling is a constant and persistent feature of the HFSC niche (Fig. 7). HFSCs located in the outer bulge are Wnt-dependent and produce Wnt signals that act locally, while simultaneously inhibiting Wnt/β-catenin signaling in the inner follicle bulge by

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**Fig. 5.** Anagen hair bulge cells express Axin2 and Wnts. (A) Schematic depicting cellular organization of the anagen follicle bulge. (B–E) Representative images of Axin2−lacZ (B) and RNA in situ hybridization (C) for Axin2 and Wnt gene expression (D and E) as performed on anagen hair follicles. Dotted lines denote approximate outer boundary of the hair bulge. Dotted lines denote approximate outer boundary of the hair bulge. [Scale bars: 20 μm (Axin2−lacZ image); 10 μm (RNA in situ hybridization images).] (F) Representative images of double-labeling RNA in situ hybridization in anagen hair follicles for Axin2 (red spots) and Wnt4 or Wnt7b (turquoise spots). Arrows indicate bulge cells expressing both Axin2 and Wnts. Boxes show magnified view of individual bulge cells expressing both Axin2 and Wnts. Dotted lines denote approximate outer boundary of the hair bulge. (Scale bars: 20 μm.)
secreting Wnt inhibitors like Dkk3s and Sfrp1 (Fig. 7). This autocrine Wnt activation and paracrine Wnt inhibition may help to maintain the discrete identity of cells located in each bulge layer, even as cells traffic dynamically to and from the bulge niche (2) throughout multiple hair cycles. Such a model predicts that loss of Wnt/β-catenin signaling in the outer bulge would result in disrupted compartmentalization of the inner vs. outer bulge. Consistent with this prediction, we find that Keratin-6, which is typically enriched in the inner bulge and is considered to be a marker of that compartment (Fig. S2 C and D) (2), becomes more strongly expressed in outer bulge cells upon deletion of β-catenin in Axin2-expressing outer bulge cells (Fig. S2 B and E) (14).

If HFSCs generate their own source of Wnt, and Wnt production is regulated in an autocrine manner, what keeps hair follicles in telogen and prevents them from undergoing autocrine amplification of Wnt signaling to initiate anagen? A likely candidate is BMP signaling, which has been shown to inhibit Wnt signaling and hair growth (30). It is thought that the reciprocal interplay between BMP and Wnt signaling controls HFSC activity and fate decisions and that BMP signaling suppresses Wnt production (31). We found some evidence that Wnt signaling controls BMP pathway component expression. After deletion of β-catenin in Axin2-expressing bulge cells, we observed slightly increased expression of several BMP antagonists, including Noggin, Bambi, and Gremlin1 (Fig. S2B), suggesting that autocrine Wnt signaling suppresses BMP antagonist expression. This finding leads us to speculate that a low level of autocrine Wnt signaling in HFSCs reduces BMP inhibition and thus permits BMP signaling, creating a feedback inhibition loop that moderates Wnt signaling levels and prevents autocrine entry into anagen. As environmental BMP levels are reduced, autocrine amplification could then lead to elevated Wnt signaling, which in turn promotes the transition from telogen to anagen (23, 32).

Our finding of Axin2–lacZ Wnt reporter gene expression in the telogen follicle bulge contrasts with the lack of activity in other TCF-element-based Wnt reporter constructs such as TOPGAL (13). The more limited expression pattern of TOPGAL in hair follicles may result from endogenous Wnt-responsive cis-regulatory elements being absent in the TOPGAL reporter construct (33). Consistent with this hypothesis, Axin2–lacZ is expressed widely in the anagen follicle in domains of Wnt mRNA expression, whereas TOPGAL is not. That Axin2 is a more sensitive and comprehensive Wnt reporter than TOPGAL is also observed in other stem cell niches such as the intestine, where Axin2 expression marks stem cells (22), even though TOPGAL expression is absent. The expression of other Wnt target genes such as Lgr5 in the bulge further supports the notion that Wnt/β-catenin signaling is active in the bulge, and that Axin2 is a reliable, sensitive, and comprehensive reporter of Wnt/β-catenin signaling in the hair follicle.

Whether or not Wnt/β-catenin signaling is required for stem cell maintenance has been a matter of debate. Previous studies showed that deletion of β-catenin in the bulge results in loss of hair stem cell identity, as determined by loss of both proliferation and expression of outer bulge cell markers like CD34 (10). More recently, however, others have shown that CD34 and Keratin-15 continue to be expressed even after β-catenin deletion and have proposed instead that Wnt/β-catenin signaling is not required for maintenance of stem cell identity but rather to induce anagen (14, 24). However, it is unclear that the expression of these genes is a reliable indicator of stem cell identity or function. Indeed, when CD34 is knocked out, hair follicles continue to grow, suggesting

Fig. 6. Outer bulge cells express secreted Wnt inhibitors. (A and B) Representative images of RNA in situ hybridization for Dkk3, Dkk4, and Sfrp1 during telogen (A) and anagen (B). Dotted lines denote approximate outer boundary of the hair bulge. (Scale bars: 20 μm.) (C) Dkk3 and Sfrp1 expression in laser-capture microdissected primary outer vs. inner bulge cells from human hair follicles (error bars, SD). Expression values are from Gene Expression Omnibus accession no. GSE3419. (D and E) Representative images of Dkk3 immunostaining in telogen (D) and anagen (E) bulges of Axin2-CreERT2/Rosa26–mTmG reporter mice. Representative images of RNA in situ hybridization for Dkk3 in telogen and anagen bulges are shown for comparison. Dotted lines denote approximate outer boundary of the hair bulge. (Scale bars: 20 μm (telogen Dkk3 RNA in situ hybridization image); 10 μm (anagen Dkk3 RNA in situ hybridization and Dkk3 immunostaining images).)
that it is not functionally required for hair stem cell function (34). Additionally, Keratin-15 expression persists in follicles of balding patients, whereas the expression of another HFSC marker, CD200, is lost (35). As such, although CD34- and Keratin-15-expressing cells may persist in β-catenin-deleted bulges, it is unclear that they remain as stem cells.

Stem cells are functionally defined by their potency rather than by marker expression (36–38), and a more reliable indicator of HFSC identity is a test of its ability to contribute to hair follicle growth. Consistent with our own data (Fig. 2), all of the published studies we know of involving conditional ablation of β-catenin in the bulge result in the hair follicle being arrested in telogen and no longer growing. Isolated bulge cells from these β-catenin mutants cannot form hair follicles, even when transplanted (14). We interpret these observations to mean that Wnt/β-catenin signaling is required to maintain HFSC potency and function.

Our findings complement recent work which suggests that attenuated Wnt signaling is part of the process of defining embryonic progenitors that become adult HFSCs (39). This study showed that hair progenitors start off as Axin2-expressing cells in the placode, but gradually lose Axin2 expression as they commit to becoming adult HFSCs during late embryogenesis. Our work suggests that Axin2 becomes expressed again in the adult HFSC compartment, once it is established.

HFSCs have been proposed to actively organize their microenvironment (16, 17). Here, we provide evidence that HFSCs may influence the organization of their niche by acting as their own source of self-renewing Wnt signals, while simultaneously promoting the differentiation of their progeny.

Materials and Methods

Animals. Axin2-CreERT2 mice were described (22). Axin2-lacZ (40) were a gift from W. Birchmeier, Max-Delbrück-Centre for Molecular Medicine, Berlin. Rosa26–mTmG<sup>flex</sup> (21) and β-catenin<sup>ex2–6-fl/fl</sup> (41) mice were obtained from The Jackson Laboratory. All alleles were heterozygous, except where stated. All experiments were approved by the Stanford University Animal Care and Use Committee and the A*STAR Animal Care and Use Committee and were performed according to NIH and Singapore Bioethics Council guidelines.

Labeling and Tracing Experiments. All mice received a single i.p. injection of 5–20 mg/mL stock solutions of Tam (Sigma, T5648) dissolved in corn oil/10% (vol/vol) ethanol, corresponding to specific doses per gram body weight as specified below. Axin2–CreERT2/Rosa26–mTmG<sup>flex</sup> mice received a single dose of Tam at P21 or 49 (P49), totaling 1 mg per 25 g of body weight.

Conditional Knockout of β-Catenin and Wntless. Axin2–CreERT2(lacZ<sup>ex2–6-fl</sup>), and mutant Axin2–CreERT2(lacZ<sup>ex2–6-fl</sup>) animals were injected with a single dose of Tam corresponding to 4 mg per 25 g of body weight at 3 wk of age. Skins were harvested and processed for histology upon morbidity or death after 10–11 d of chase. For Wntless ablation, mothers carrying Axin2–rtTA/Cre/Wls<sup>fl/fl</sup> and mutant Axin2–rtTA/Cre/Wls<sup>cre</sup> pups were injected with a single dose of Tam corresponding to 1 mg per 25 g of body weight at embryonic day 14.5 (E14.5). Skins were harvested and processed for histology when pups were at postnatal age 1 mo.

X-Gal Staining. Tissues were harvested and fixed in 4% (vol/vol) paraformaldehyde (PFA) for 1 h at 4 °C, washed in PBS and detergent rinse (PBS with 2 mM MgCl<sub>2</sub>, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 detergent), and then stained in staining solution (PBS with 2 mM MgCl<sub>2</sub>, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg/mL X-gal) in the dark at room temperature overnight. Tissues were then washed in PBS, postfixed in 4% PFA at 4 °C, and processed for paraffin embedding and histology.

Histology and Immunostaining. Animals were killed and skulls were removed from the back (dorsal) and the tail regions. For cryosections, tissues were fixed for 1–2 h in 4% PFA at 4 °C, washed in PBS, and incubated overnight in 30% sucrose/PBS at 4 °C. Tissues were then embedded in OCT medium and stored at −80 °C before cryosectioning. Sections of 5- to 6-μm thickness were cut using a Leica CM3050S cryostat (Leica Microsystems).

For paraffin sections, tissues were fixed overnight in 4% PFA at 4 °C. Tissues were washed in PBS, then dehydrated through an ethanol series into xylene, and embedded in paraffin wax. Sections of 5-μm thickness were cut by using a Leica RM2255 microtome (Leica Microsystems), and then rehydrated and counterstained with Eosin where specified.

All immunofluorescence staining was performed in the dark. Cryosections were washed in PBS and incubated in blocking buffer (2% normal donkey serum and 0.2% Triton X in PBS) for 1 h at room temperature. Primary antibodies were diluted in blocking buffer and incubated with cytosections overnight at 4 °C for 1 h at room temperature. Sections were then washed in PBS, and incubated with secondary antibody diluted in blocking solution for 1 h at room temperature. Sections were then washed in PBS and mounted in Prolong Gold with DAPI mounting medium (Life Technologies). The following primary antibodies were used: anti-GFP (Abcam), anti-Dkk3 (R&D systems), and anti-Keratin-6 (Covance). The following secondary antibodies were used: anti-chicken conjugated to Alexa Fluor 488 (Life Technologies, Jackson ImmunoResearch) and anti-goat conjugated to Alexa Fluor 647 (Jackson ImmunoResearch).

Fig. 7. Model: Dual roles for Wnt/β-catenin signaling in HFSC maintenance and lineage determination. (A) During telogen, Wnt-responding stem cells in the outer bulge produce autocrine Wnt1, Wnt4, and Wnt7b, and paracrine-acting secreted Wnt inhibitors like Dkk3, Dkk4, and Sfrp1. This expression pattern persists throughout the hair cycle, potentially to maintain the niche signaling microenvironment. (B) At the telogen–anagen transition, Wnt6, Wnt10a, and Wnt10b become strongly expressed in cells at the boundary of the hair germ and dermal papilla. (C) These and other Wnts continue to be expressed in various hair layers proximal to the dermal papilla during anagen and are likely involved in hair lineage specification.
RNA in Situ Hybridization. Tissues were harvested and fixed in 4% PFA for 24 h, and then dehydrated and embedded in paraffin. Tissue sections were cut at 5-μm thickness, air-dried at room temperature, and processed for RNA in situ detection by using the RNAscope 2.0 FFPE Reagent Kit according to the manufacturer's instructions (Advanced Cell Diagnostics; ref. 42). RNAscope probes used were as follows: Axin2 (NM_015732, region 330–1287), Wnt1 (NM_021279.4, region 1204–2325), Wnt2 (NM_009521.2, region 135–1577), Wnt6 (NM_009523, region 2147–3150), Wnt10a (NM_009524, region 200–1431), Wnt6 (NM_009526, region 780–2030), Wnt7a (NM_009527.3, region 1811–2013), Wnt7b (NM_009528, region 1579–2839), Wnt9a (NM_009518, region 479–1948), Wnt10b (NM_011178, region 989–2133), Wnt16 (NM_003116.4, region 453–1635) Dkk3 (NM_015814, region 1513–2703), Dkk4 (NM_145592.2, region 22–1140), and SFRP1 (NM_013834.3, region 1810–2779), which were detected using the Fast Red detection reagent.

Microscope Image Acquisition and Quantification. All sections were imaged by using the Axioplan 2 and Imager.Z2 Microscopes, the Axiocam MRm fluorescence and MRC5s (bright field) cameras, and using Axiavision AC software (Release 4.1, Carl Zeiss) or Zen software (Carl Zeiss). Image acquisitions were done at room temperature using ×10 NA 0.3, ×20 NA 0.5, ×20 NA 0.8, and ×40 NA 0.75 EC Plan-Neofluar objectives (Carl Zeiss). For some images, contrast, color, and dynamic range were globally adjusted in Adobe Photoshop (Adobe Systems).

Cell Labeling and Flow Cytometry. For isolation of bulge cells, 6- to 8-wk-old Axin2-LacZ animals were killed, hair was removed, and dorsal skins were harvested and washed in ice-cold calcium and magnesium-free Dulbecco’s PBS (DPBS; GE Healthcare). After removing fat and s.c. tissues using a scalpel, the dorsal skins were then trypsinized in 0.25% Trypsin–EDTA ( Gibco) at 37 °C for 1 h. Epidermal cell suspension was neutralized with ice-cold 10% chilled PBS (GE Healthcare) in DPBS and strained with 70 and 40 μm cell strainers (Fisher Scientific). Cells were centrifuged at 300 × g for 30 min at 4 °C, washed with 10% chilled PBS/DPBS, centrifuged at 300g for 5 min at 4 °C and resuspended into 1 mL of single cell suspension with ice-cold 10% chilled PBS/DPBS which was then incubated with primary antibodies either coupled directly with a fluorochrome or indirectly in chilled PBS-precoated propylene tubes on ice for 60 min in the dark, washed two times before incubation with secondary antibodies in 10% chilled PBS in DPBS for 20 min on ice, washed and analyzed with flow cytometry. The following primary antibodies were used in our FACS analysis: Alexa 700-conjugated anti-mouse CD34 (Affymetrix), APC-conjugated anti-human/mouse CD45 (α Integrin) reactivity, Pacific Blue-conjugated anti-mouse Ly-6E/E (E–Scai) (Biotrend), Polyclonal goat antibody against mouse LRIG1 (R&D systems). The following secondary antibodies, Donkey anti-goat IgG (H+L)-Phycocerythrin (R&D systems) was used. Cell viability was checked by staining with Zombie-Aqua Fixable viability kit (Biotrend). Living cells were sorted positive for CD34 and α Integrin, and negative for Lrig1 and Sca1. This CD34+ α Integrin+ bulge cell population was further sorted into Axin2+ and Axin2– populations.

For detection of intracellular β-galactosidase activity of Axin2-expressing cells, a fluorescein di-i–galactopyranoside (FDG) staining assay (Thermo Fisher) was used. Keratinocytes stained with the antibodies were loaded with FDG using hypotonic shock at 37 °C. Cell sorting was carried out using a FACSaria cell sorter using unstained control, single color compensation controls. Axin2– positive cells were defined by gating using fluorescence minus one without FDG. Axin2+ and Axin2– cell populations were fixed in ice-cold methanol for 15 min, washed and centrifuged in DPBS. Cell cycle analysis was performed by staining fixed cells with DNA-binding dyes with FITC- and PI-stained with RNase in the dark at room temperature and analyzed with a FACSaria cell sorter.

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