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β -Catenin Activation Regulates Tissue Growth Non–Cell Autonomously in the Hair Stem Cell Niche

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Wnt/ β -catenin signaling is critical for tissue regeneration. However, it is unclear how β -catenin controls stem cell behaviors to coordinate organized growth. Using live imaging, we show that activation of β -catenin specifically within mouse hair follicle stem cells generates new hair growth through oriented cell divisions and cellular displacement. β -Catenin activation is sufficient to induce hair growth independently of mesenchymal dermal papilla niche signals normally required for hair regeneration. Wild-type cells are co-opted into new hair growths by β -catenin mutant cells, which non–cell autonomously activate Wnt signaling within the neighboring wild-type cells via Wnt ligands. This study demonstrates a mechanism by which Wnt/ β -catenin signaling controls stem cell–dependent tissue growth non–cell autonomously and advances our understanding of the mechanisms that drive coordinated regeneration.

Adult tissue regeneration relies on the coordinated activation of resident stem cells within their niches to generate a functional organ (1). The Wnt/ β -catenin signaling pathway functions in the maintenance of stem

cells (SCs) of various lineages. However, it is unclear how this pathway controls specific subpopulations of cells to organize growth during tissue regeneration.

The hair follicle (HF) serves as a versatile model to address this fundamental question because it is highly accessible and its SCs and progeny, located in the bulge and hair germ, respectively, are anatomically and molecularly well-defined (fig. S1) (2–6). HF stem cell (HF-SC) progeny lie in direct contact with a specialized group of mesenchymal cells, the dermal papillae (DPs), that functions as a key signaling center required for

epithelial-mesenchymal interactions that govern HF growth (4, 7, 8).

Wnt signaling is required for HF development and regeneration (9–17) and is mediated by the stabilization and translocation of the key signal transducer, β -catenin, to the nucleus, where it binds TCF/LEF transcription factors to activate Wnt target gene transcription (18). Expression of activated β -catenin throughout the basal epidermis has been shown to induce de novo HFs within the epidermis, which establishes that Wnt signaling is required and sufficient for new hair growth (13, 15–17). Although these studies illustrate the importance of Wnt/ β -catenin signaling during HF regeneration, several outstanding questions remain, including (i) which dynamic SC behaviors does Wnt/ β -catenin signaling regulate, (ii) is Wnt/ β -catenin activation sufficient to promote growth within the SC pool independently of the mesenchymal DP cells, and (iii) what are the molecular mechanisms by which Wnt/ β -catenin coordinates collective tissue growth?

To investigate HF-SC behaviors regulated by Wnt/ β -catenin signaling, we genetically activated β -catenin specifically within the HF-SCs and their progeny using *K19Cre^{ER}; β -catn^{fllox(Ex3)/+}* mice (Fig. 1A and fig. S1). Tamoxifen induction during the HF resting phase results in β -catenin stabilization and constitutive activation of Wnt signaling with subsequent formation of new ectopic axes of hair growth that show HF differentiation (Fig. 1A and figs. S1 and S2, A to F). These data show that β -catenin activation specifically in HF-SCs and their progeny can induce new hair growths, despite previous studies

Fig. 1. Activated β -catenin–induced cellular mechanisms that promote new hair growths.

(A) Hair growths in tamoxifen-treated *K19Cre^{ER}; β -catn^{fllox(Ex3)/+}* mice. New hair growths are P-cadherin⁺ (green) and proliferative (Ki-67, red). Nuclei [4',6'-diamidino-2-phenylindole (DAPI), blue]. (B) Optical sections of z stacks of a *K19Cre^{ER}; β -catn^{fllox(Ex3)/+}; K14-H2B-GFP* HF depict upward nuclear movement (movie S2). Epithelial nuclei (K14-H2B-GFP, white). Arrowheads indicate points of reference of original nuclei positions. Colored circles represent moving nuclei; black circles represent stationary nuclei. (C) Formation (c' inset) and expansion (c'') of nuclear clusters in two separate *K19Cre^{ER}; β -catn^{fllox(Ex3)/+}; K14-H2B-GFP* mice. Nuclei are pseudocolored (movies S3 and S4). (D) *K19Cre^{ER}; β -catn^{fllox(Ex3)/+}; K14-H2B-GFP* HFs just after DP ablations (day 0, yellow arrows). DPs marked by Lef1-RFP (red), epithelial nuclei by K14-H2B-GFP (green). (E) Same HFs after 6 days of tamoxifen treatment (day 7). (F and G) Revisit of the same HFs (days 12 to 19). Asterisk denotes HF out of plane of view. Scale bars, 25 μ m.

Fig. 2. β -Catenin activation triggers new axes composed of both mutant and wild-type cells. (A and B) Optical sections of z stacks of *K19Cre^{ER}; β -catn^{flox(Ex3)/+}; K14-H2B-GFP; tdTom* mutant HF show early and late hair growths (K14-H2B-GFP, green; tdTom Cre reporter, red). (C) Genotype of FACS-isolated populations from the new growths based on P-cadherin enrichment (top gel) ($n = 2$ mice) or *TCF/Lef:H2B-GFP* Wnt reporter (bottom gel) ($n = 2$ mice). (D) Both tdTom⁺ and tdTom⁻ cells dividing within a new growth. Schematic depicts representative divisions within both tdTom⁺ and tdTom⁻ populations over a time-lapse recording (see movie S5). Scale bars, 50 μ m.

Fig. 3. β -Catenin mutant cells activate Wnt signaling in neighboring wild-type cells. (A) Immunohistochemistry of β -catenin in growing wild-type HF. Inset highlights membrane-bound staining. (B and C) Nuclear β -catenin in new hair growths in tamoxifen-induced *K19CreER; β -catn^{flox(Ex3)/+}* mice at early and late growth stages. Insets highlight nuclear staining. (D) Wnt reporter expression (*TCF/Lef:H2B-GFP*, green) in late growths (P-cadherin, red). Scale bars, 50 μ m.

that suggested that HF-SCs and progeny cells might be refractory to activated β -catenin signaling relative to other epidermal keratinocytes (16, 19). In contrast to ectopic HFs that form when β -catenin is activated throughout the basal epidermis (14, 16), new hair growths in $K19Cre^{ER};\beta\text{-catn}^{flox(Ex3)/+}$ mice did not harbor morphologically apparent DP structures but, instead, were surrounded by a layer of mesenchymal cells that expressed DPs or dermal sheath markers (fig. S2, G to J), consistent with previous findings (20).

Next, we coupled our genetic gain-of-function system with in vivo imaging of live mice. Time-lapse imaging of new hair growth in $K19Cre^{ER};\beta\text{-catn}^{flox(Ex3)/+};K14\text{-H2B-GFP}$ mice [in which green fluorescent protein (GFP) highlights all skin epithelial cells] captured cell divisions that were oriented along the new axis of growth (fig. S3, A to D) and displayed divergent upward displacement of epithelial nuclei toward newly forming hair growths (Fig. 1B and movies S1 and S2). These budlike clusters of epithelial cells organized themselves into a compact arrangement (fig. S3E and movie S2). To capture early changes induced by β -catenin stabilization, we

began recording when mutant follicles had not yet developed new hair growths and observed epithelial nuclei clustering into ringlike structures [Fig. 1C (top view), fig. S3E (side view), and movies S3 and S4], reminiscent of early-stage embryonic HF formation (fig. S4) (21). These findings show that activated β -catenin orients cell divisions and organizes cell movements within SC progeny cells to drive new axes of hair growth.

The mesenchymal DP constitutes one of the best-characterized niches for HF-SCs and their progeny and are required for their activation to initiate hair growth (8, 22, 23). Therefore, we hypothesized that native DP signals may be required parallel or downstream of activated β -catenin in HF-SC progeny cells to initiate new hair growths. To test this hypothesis in vivo, we first laser-ablated DP cells and then induced β -catenin activation in HF-SCs and progeny using $K19Cre^{ER};\beta\text{-catn}^{flox(Ex3)/+};K14\text{-H2B-GFP};Lef1\text{-RFP}$ mice (7) [in which red fluorescent protein (RFP) highlights DP cells]. We then revisited the same ablated follicles over several days (Fig. 1, D to G, and fig. S5). The majority of β -catenin mutant HFs regrew (62%) in contrast to wild-type

HFs (0%) after DP ablation (fig. S5F). Although we cannot exclude a potential requirement of a native DP for subsequent HF differentiation or that other mesenchymal cells may functionally promote hair growth in the absence of a native DP, these data demonstrate that β -catenin activation in the SC and progeny compartments is sufficient to initiate HF growth independent of mesenchymal DP niche signals.

To address the mechanism by which mutant β -catenin-activated cells contribute to hair growth expansion, we labeled mutant cells using a tdTomato Cre reporter (24). With in vivo imaging, we examined the contribution of tdTomato-positive cells (tdTom⁺) to the new hair growths in tamoxifen-induced $K19Cre^{ER};\beta\text{-catn}^{flox(Ex3)/+};K14\text{-H2B-GFP};tdTom$ mice. Although the initial small hair growths largely consisted of tdTom⁺ cells, later expanded growths were mosaic for tdTom expression, which suggests that mutant cells recruit wild-type cells to promote growth and expansion (Fig. 2, A and B, and fig. S6A) and are reminiscent of the mosaic contribution observed in tooth formation after β -catenin activation (25). To confirm this heterogeneity, we purified cells from growths by fluorescence-activated cell sorting

Fig. 4. β -Catenin acts non-cell autonomously via Wnt ligands.

(A) Representative qRT-PCR analysis of Wnt ligands and target genes comparing sorted $Pcad^{H1};tdTom^{+}$ versus $Pcad^{H1};tdTom^{-}$ populations from $K19Cre^{ER};\beta\text{-catn}^{flox(Ex3)/+};tdTom$ mice ($n = 2$; $P < 0.005$). (B) Model of non-cell autonomous β -catenin signaling. (C) Wntless staining (red) in $K19Cre^{ER};\beta\text{-catn}^{flox(Ex3)/+}$ mice. (D) After tamoxifen induction, hematoxylin and eosin (H&E) staining of $K19Cre^{ER};\beta\text{-catn}^{flox(Ex3)/+};Wls^{fl/fl}$ mice shows anagen-like stage HFs with growths of varying sizes versus (G) $K19Cre^{ER};\beta\text{-catn}^{flox(Ex3)/+};Wls^{fl/+}$ mutant controls, which show growths with a higher frequency and larger size ($n = 3$ experimental litters, $n = 7$ $K19Cre^{ER};\beta\text{-catn}^{flox(Ex3)/+};Wls^{fl/fl}$ mice). (E) Nuclear β -catenin within growths of $K19Cre^{ER};\beta\text{-catn}^{flox(Ex3)/+};Wls^{fl/fl}$ and controls (H). (F) Wntless (red) is absent in most of the growths of $K19Cre^{ER};\beta\text{-catn}^{flox(Ex3)/+};Wls^{fl/fl}$ compared to mutant controls (I). Nuclei (DAPI, blue). Scale bars, 50 μ m.

(FACS) using tdTom and P-cadherin expression, a marker enriched in these growths. FACS-purified mutant (tdTom⁺;P-cad^{hi}) cells showed the expected deleted β -catenin- Δ Exon3 allele by polymerase chain reaction (PCR) [700-base pair (bp) band], whereas wild-type (tdTom⁻;P-cad^{hi}) cells showed only the nonrecombined band (900 bp) (Fig. 2C, top gel). In the same way, FACS purification using the *TCF/Lef:H2B-GFP* Wnt reporter (fig. S6B) in the *K19Cre^{ER};β-catn^{fllox(Ex3)/+};tdTom* mice showed similar results (Fig. 2C, bottom gel).

To determine whether wild-type cells functionally contribute to the expansion of mutant hair growths, we performed time-lapse recordings and immunofluorescent analysis and demonstrated that wild-type cells within the growths proliferate like coresident β -catenin mutant cells (Fig. 2D, fig. S6C, and movie S5). These results show that β -catenin activation in a subset of HF-SCs and their progeny leads to the recruitment of wild-type epithelial that collectively promote coordinated hair growth expansion.

On the basis of observed cellular heterogeneity, we predicted heterogeneous activation of the Wnt/ β -catenin pathway throughout the new growths. At early stages, nuclear β -catenin was observed within the induced hair growths (Fig. 3, A and B). As growths expanded, nuclear β -catenin was still present globally in the new growth axes. However, clusters of cells with stronger nuclear β -catenin staining neighbored cells that exhibited weaker nuclear β -catenin staining (Fig. 3C). Additionally, TCF/Lef:H2B-GFP and Lef1 immunohistochemistry showed that Wnt active cells were observed throughout the new hair axes (Fig. 3D and fig. S6), which suggests that both activated β -catenin mutant cells and wild-type cells initiate Wnt signaling.

To address how a mosaic population of cells can activate Wnt signaling, we first assessed global transcriptional changes in Wnt pathway genes by quantitative reverse transcription PCR (qRT-PCR) from whole skin of mutant and control littermate *K19Cre^{ER};β-catn^{fllox(Ex3)/+}* mice. Wnt target genes (*Axin2*, *CyclinD*, and *Lef1*) and several Wnt ligands were up-regulated in β -catenin mutant skin compared to that of control littermates (fig. S7A), consistent with previous studies that show up-regulation of Wnt ligands after β -catenin activation (26–29). To determine whether β -catenin-activated mutant cells specifically up-regulate Wnt ligand expression, we FACS-purified mutant cells (tdTom⁺;P-cad^{hi}) and found that several Wnt ligands (*Wnt3*, *5a*, *10b*, and *16*), as well as a gene required for Wnt ligand secretion (*Wntless*), were all up-regulated compared to wild-type (tdTom⁻;P-cad^{hi}) cells (Fig. 4, A to C). Furthermore, wild-type cells within the growths (tdTom⁻/P-cad^{hi}/K14-H2B-GFP⁺) showed higher expression of the direct downstream Wnt target gene, *Axin2*, than wild-type cells outside the growths (tdTom⁻/P-cad^{hi}/K14-H2B-GFP⁺) (fig. S7B). This shows that Wnt signaling is active in the wild-type cells inside the new hair growths when compared to

remaining follicular epithelial cells. Furthermore, in situ hybridization analysis of *Wnt10b* and *Wnt16* showed expression localized to foci within the new growths (fig. S8).

Altogether, these experiments suggest a model in which mutant β -catenin cells influence wild-type cells to proliferate and contribute to the formation of new growths via Wnt ligand secretion (Fig. 4B). To test whether Wnt ligand secretion by β -catenin-activated cells influences the growth of wild-type cells in a paracrine manner, we used an in vitro culture assay. Wild-type keratinocytes showed increased proliferation when cultured in the presence of conditioned media collected from mutant β -catenin-activated cell cultures. This effect was abrogated when using conditioned media obtained from mutant β -catenin-activated cells cultured in the presence of a specific inhibitor of Wnt ligand secretion, IWP2 (fig. S9, A to C). To test in vivo whether Wnt secretion contributes to the development or expansion of growths, we conditionally deleted *Wntless* (Wls) using *K19Cre^{ER};β-catn^{fllox(Ex3)/+};Wls^{fl/fl}* mice (30). Activated β -catenin-induced hair growths were largely delayed and overall reduced in size in the absence of Wls compared to β -catenin mutant control mice (*K19Cre^{ER};β-catn^{fllox(Ex3)/+};Wls^{fl/+}*) (Fig. 4, D to I). When larger growths were observed in the *K19Cre^{ER};β-catn^{fllox(Ex3)/+};Wls^{fl/fl}* mice, they were associated with expression of Wls, which suggests that the efficiency of Wls elimination was inversely related to aberrant growth (fig. S9, D to F). These data show that Wnt/ β -catenin signaling acts through a non-cell autonomous mechanism to support collective cellular growth, a model that may explain previous findings of mosaic contribution to other epithelial organs. Furthermore, these results demonstrate that Wnt ligand secretion is an important downstream mediator of β -catenin-induced non-cell autonomous tissue growth.

This study brings a more detailed understanding of how a conserved regenerative signal, Wnt/ β -catenin, temporally and spatially regulates SCs and progeny behaviors during tissue growth. These data highlight how a mutation in a downstream target gene can modify the behavior of a genetic clone of cells while also co-opting surrounding wild-type cells to acquire similar behaviors. These findings hold important implications for understanding the cellular mechanisms that promote coordinated tissue growth and regeneration.

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Supplementary Materials

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