Defining the impact of β-catenin/Tcf transactivation on epithelial stem cells

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Wnt signaling has been implicated in stem cell (SC) biology, but little is known about how stabilized β-catenin functions within native SC niches. We address this by defining the impact of β-catenin stabilization on maintenance, proliferation, and lineage commitment of multipotent follicle SCs when in their native niche and in culture. We employ gain of function mutations and inducible loss of function mutations to demonstrate that β-catenin stabilization is essential for promoting the transition between SC quiescence and conversion to proliferating transit amplifying (TA) progeny. We transcriptionally profile purified SCs isolated directly from wild-type and elevated β-catenin follicles in both resting and activated states to uncover the discrete set of genes whose expression in native SCs is dependent upon β-catenin stabilization. Finally, we address the underlying mechanism and show that in the SC niche, Wnt signaling and β-catenin stabilization transiently activate Lef1/Tcf complexes and promote their binding to target genes that promote TA cell conversion and proliferation to form the activated cells of the newly developing hair follicle. We also show that these changes precede subsequent Wnt signals that impact on the TA progeny to specify the differentiation lineages of the follicle.

[*Keywords*: Stem cells; skin; microarray; β-catenin; proliferation; differentiation; slow-cycling]

Supplemental material is available at http://www.genesdev.org.

Received April 19, 2005; revised version accepted May 12, 2005.

Adult stem cells (SCs) replenish and maintain natural tissue homeostasis and repair injured tissues. Within a tissue, SCs often reside in niches that provide a specialized environment thought to regulate SC proliferation and differentiation into cell lineages that constitute their tissue of origin (for reviews, see Spradling et al. 2001; Christiano 2004; Fuchs et al. 2004; Moore and Lemischka 2004; Wagers and Weissman 2004).

In mice, each hair follicle has a reservoir (bulge) of quiescent multipotent SCs that are mobilized to repair the epidermis upon injury and fuel episodic hair cycling (Taylor et al. 2000; Oshima et al. 2001; Panteleyev et al. 2001; Blanpain et al. 2004; Morris et al. 2004; Tumbar et al. 2004). At the start of each hair cycle, SCs are stimulated to proliferate and exit at the base of the bulge. The niche then reenters quiescence, while transit amplifying (TA) progeny of SCs proliferate, beginning as a small hair germ that matures to form the follicle that produces hair (anagen).

Mature adult follicles go through cycles of growth, degeneration, and rest throughout the life of the animal. During the resting stage (telogen), the old hair shaft is encased by the bulge. Upon SC activation, the niche generates a rapidly proliferating hair germ that grows downward, envelopes specialized mesenchymal cells—the dermal papilla (DP)—and forms a matrix of proliferative TA cells at the base of the follicle. The matrix cells terminally differentiate to produce the inner root sheath and hair shaft. Anagen persists as long as matrix cells balance proliferation with differentiation, but when they apoptose, hair growth ceases and the lower follicle degenerates (catagen). As the defunct follicle regresses, the DP is drawn upward to rest under the SC niche (telogen). After some threshold for activation is reached, a few SCs initiate the next hair cycle anew. Although the mechanism of SC activation is unknown, the process depends upon the DP and its close proximity to SCs (Jahoda et al. 1984).

Several lines of evidence suggest that multiple steps in morphogenesis and cycling of the follicle are dependent upon a change in the transcriptional status of genes that are regulated by Wnt signaling. At high levels, sustained stabilized β-catenin in transgenic mice elicits de novo follicle morphogenesis, thereby bestowing committed interfollicular epidermal cells with a plasticity normally afforded only to embryonic or multipotent follicular SCs (Gat et al. 1998; see also Noramly et al. 1999; Widelitz et al. 2000). By contrast, conditional ablation of β-catenin

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Article published online ahead of print. Article and publication date are at http://www.genesdev.org/cgi/doi/10.1101/gad.1324905.

blocks follicle formation altogether (Huelsken et al. 2001), while administering stabilized β-catenin as a single potent dose at the resting stage of the cycle prompts hair growth (Van Mater et al. 2003; Lo Celso et al. 2004). Finally, older mice expressing high levels of β-catenin develop skin tumors (pilomatricomas) typified by masses of dead hair cells encased by rapidly proliferating matrix cells (Gat et al. 1998; Chan et al. 1999). In normal follicles, the strongest activity of a Wnt-responsive β-galactosidase reporter gene is seen as TA matrix cells withdraw from the cell cycle and differentiate to make hair (DasGupta and Fuchs 1999). A number of endogenous hair keratin and matrix cell transcription factor genes (*Lef1*, *Movo1*, *Foxn1*, and *Msx2*) as well as signaling factors (*Fgfs*, *Follistatin*, *Tgf*β*2*) have also been implicated as direct targets for β-catenin/Lef1 (Hovanes et al. 2001; Merrill et al. 2001; Mullor et al. 2001; Balciunaite et al. 2002; Kratochwil et al. 2002; Li et al. 2002; Jamora et al. 2005).

Although these studies underscored an importance of Wnt signaling in SC progeny and lineage determination in the follicle, they did not address whether the myriad of different effects occur only at high doses of β-catenin or whether β-catenin still exerts at least some of its effects at levels closer to those encountered physiologically. The studies also did not address whether the effects of β-catenin on hair growth are exclusive to one stage of the cycle or whether they depend upon a specific stage. Finally, the studies to date have not addressed whether stabilized β-catenin acts on the SCs themselves or only on their progeny.

Overall, these issues are of major importance in SC biology, where broad effects of Wnts have emerged in a variety of different systems, and particularly where roles for Wnts in proliferation, self-renewal, and differentiation of SCs are now hotly debated. In intestinal epithelium, for instance, loss of *Tcf4* results in a loss of the tissue (Korinek et al. 1998). Conversely, an expanded neural progenitor pool is seen upon constitutive stabilization of β-catenin in the cerebral cortex (Chenn and Walsh 2002), and hematopoietic progenitors or ES cells cultured in the presence of purified Wnt3A show enhanced proliferation (Reya et al. 2003; Willert et al. 2003; Sato et al. 2004). While tantalizing, these studies do not distinguish between SC self-renewal per se versus proliferation of SC progeny. And countering these results is the fact that transgenic stabilization of β-catenin in the neural crest seems to promote cell fate specification and differentiation rather than SC proliferation and self-renewal (Lee et al. 2004).

Why do some cells proliferate and others differentiate in response to Wnt signaling? Do differences in development, tissue specificity, and/or level of Wnt signaling dictate this outcome or is the action a universal one that is intrinsic to SCs? Does stabilized β-catenin act on the small pool of often quiescent SCs or alternatively on the larger pool of proliferating SC progeny (or both)? Do SCs change their response to Wnts when removed from their native niche and placed in culture? A molecular understanding of these disparate observations is predicated on being able to distinguish SCs from their progeny, knowing whether an SC lineage is sensitive to variations in the dose of Wnt signaling it receives, and being able to specifically monitor the consequences of physiological levels of β-catenin stabilization to SCs in their native niche. In this report, we tackle these issues and define the impact of β-catenin stabilization on the SC niche in vivo.

Results

Sustained β*-catenin stabilization in the bulge results in precocious SC activation*

We began by addressing whether stabilized β-catenin acts directly on SCs. For this purpose, it was necessary to express β-catenin continually during the hair cycle, to do so at sufficiently low levels to avoid distortion of follicle morphology, and to employ a strategy that would enable us to use fluorescence activated cell sorting (FACS) to obtain pure populations of the follicle SCs. We achieved these goals by using hemizygous and homozygous K14-ΔN β-catenin mice on a K14-GFPactin background. For our studies, we isolated follicles from matched areas of dorsal lateral backskin, since the hair cycle proceeds in an anterior to posterior wave down the back (Dry 1926; Muller-Rover 2001). Additionally, we focused our studies on the first 8 wk of life, since cycles become increasingly asynchronous as mice age.

Backskin follicles of K14-ΔN β-catenin hemizygous (ΔN) and homozygous (ΔNΔN) mice progressed through the first post-natal cycle normally (Fig. 1A). Similar to their wild-type counterparts, they entered anagen synchronously at approximately day 21 (d21) and by d28, follicles matured, were in full anagen, and appeared morphologically indistinguishable from wild type (Fig. 1A). Analogously, at the end of the hair cycle, transgenic and wild-type follicles entered telogen similarly and between d42 and d45.

In contrast to the first telogen, which lasts only 1–2 d, the second telogen is highly extended, lasting several weeks in wild-type follicles. Although the underlying reason for this extension is unknown, the DP remains positioned just below the bulge during this entire phase. At d55, wild-type follicles were still in the midst of this extended telogen. In striking contrast, ~90% of ΔN follicles had already entered the next anagen by d55. In ΔNΔN backskin, this transition occurred even earlier (Fig. 1A,B).

Importantly, while ΔNΔN skin displayed other abnormalities including signs of de novo hair follicle morphogenesis in the interfollicular epithelium, the effects on ΔN skin appeared to be largely restricted to precocious entry into anagen (see Fig. 1A). Anti-β-catenin immunoblot analysis confirmed that the level of ΔN β-catenin was ~50% higher in the homozygous versus the hemizygous state, yielding higher overall levels of β-catenin than wild-type animals (Fig. 1C). Based upon these data, we conclude that the additional defects in the ΔNΔN

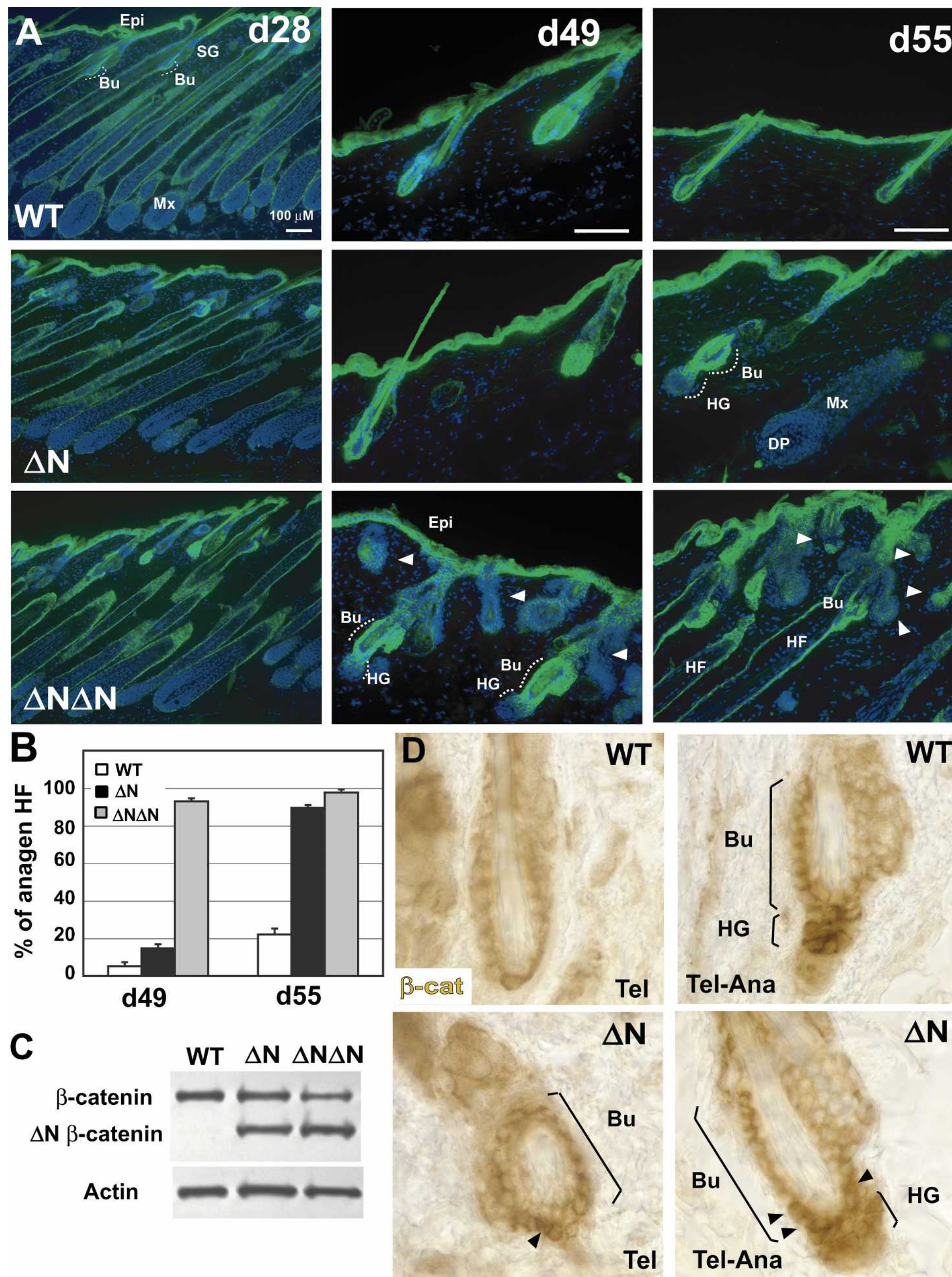

Figure 1. Synchronous, premature activation of follicle SCs expressing stabilized β-catenin throughout the hair cycle. K14-GFPactin (wild-type [WT]) and K14-ΔN β-catenin mice were mated to generate hemizygous (ΔN) or homozygous (ΔNΔN) animals displaying GFP epifluorescence in their skin epithelium. (*A*) GFP epifluorescence (green) of backskins from matched littermates at d28, when wild-type follicles are in full growth (anagen) and at d49 and d55, when wild-type follicles are in their extended resting phase (telogen) at the close of this synchronous cycle. Two dose-dependent β-catenin changes occurred: (1) precocious entry of resting (telogen) follicles into the anagen—that is, growth—phase (ΔN and ΔNΔN) and (2) aberrant activation of follicle morphogenesis in interfollicular epithelium (ΔNΔN only; arrowheads). DAPI (blue) marks nuclei. (*B*) Quantification of the data in *A*. (*C*) Immunoblot analyses with (1) a C-terminal β-catenin Ab that recognizes ΔN and endogenous β-catenin and (2) an anti-β-actin Ab (control) to show stable and dose-dependent expression of the ΔN transgene. (*D*) Anti-β-catenin immunohistochemistry of paraffin-embedded d55 skin sections treated for detection of cytoplasmic and nuclear β-catenin protein. Note prominent staining in a few cells at the base of the activated SC niche (arrowheads) and in early hair germs (additional data in Supplementary Fig. S1). Staining is elevated in ΔN and ΔNΔN follicles. (Mx) Matrix; (Bu) bulge; (Epi) epidermis; (SG) sebaceous gland; (HG) hair germ; (HF) hair follicle. Bars, 100 µm.

skin were due to the higher, less physiological levels of β-catenin. We therefore focused on ΔN as our model, and used ΔNΔN only for comparative purposes.

Immunohistochemistry of fixed follicles further revealed nuclear β-catenin in a few cells at the base of the wild-type SC compartment at the telogen-to-anagen

transition (Fig. 1D; Supplementary Fig. S1). This was even more pronounced in the ΔN follicles. Moreover, nuclear β-catenin persisted and became more prevalent in the developing hair germ—that is, the TA progeny of the activated SCs. Interestingly at other times in the hair cycle, no signs of precocious SC activation and TA progeny conversion were detected in the ΔN follicles, despite the continuously elevated levels of β-catenin in these animals. Based upon these findings, the effects of stabilized β-catenin in SC activation appeared to depend upon some additional factor(s) generated during telogen. This stimulus is likely to come from the DP–bulge interaction that happens during this phase of the cycle.

Precocious activation of SCs without gross perturbations in niche morphology

Tissue culture studies have led to the proposal that Wnts might affect SCs by promoting self renewal (for review, see Reya and Clevers 2005). To evaluate whether β-catenin stabilization might lead to self-renewal and an increase in bulge size, we conducted a three-dimensional reconstruction of wild-type, ΔN, and ΔNΔN bulges.

Remarkably, transgene expression did not seem to affect either the development or the basic biochemistry of the bulge (Fig. 2). Thus, normal bulge-preferred expression was detected with a number of markers, and inspection of XYZ planar sections revealed relatively normal bulge size and shape over the first two hair cycles. Quantification by microscopy and fluorescence activated cell sorting (FACS) documented that during this time, the numbers of follicles and CD34/α6 integrin-positive cells remained constant (Fig. 2E,F). The bulge consists of two discrete layers of cells (Blanpain et al. 2004), and both basal ($\alpha6^{high}CD34^{high}$) and suprabasal ($\alpha6^{low}CD34^{high}$) compartments maintained normal cell numbers. These findings showed clearly that sustained β-catenin stabilization caused precocious SC activation under conditions where no gross perturbations occurred in the morphology, size, or classical features of follicle SC biology.

Precocious SC activation occurs synchronously and is accompanied by transient proliferation

To understand how ΔN β-catenin causes SC activation without altering bulge equilibrium, we first tested whether transgene expression affects the quiescence of slow-cycling cells within the niche. To do so, we administered bromodeoxyuridine (BrdU) to wild-type and ΔN mice at relevant ages, and then used FACS to analyze how the cell cycle profiles of niche cells change over the hair cycle.

Even though bulge cells are quiescent relative to their TA progeny, they still undergo cycling and incorporate BrdU (Taylor et al. 2000; Blanpain et al. 2004). During anagen (d28), ΔN β-catenin expression did not affect the numbers of BrdU-labeled bulge cells, whether basal or suprabasal (Fig. 3A). In addition, anti-caspase 3 labeling was not detected in either ΔN or wild-type bulges (data not shown). Thus, the quiescent and slow-cycling characteristics of the bulge were maintained in the d28 anagen-phase bulge even in the presence of elevated stabilized β-catenin.

In wild-type mice, early anagen coincides with a transient increase in BrdU incorporation within the bulge and a constitutive burst of proliferation in the developing hair matrix. At d49, when the wild-type SC niche was still quiescent, the ΔN bulge displayed two times or more the number of BrdU-labeled cells (Fig. 3B). This difference was largely restricted to the basal population, perhaps indicating that the suprabasal bulge cells are not activated at anagen onset (Fig. 3B).

By d51–d53, as new hair germs were beginning to develop in wild-type follicles, a few BrdU-labeled cells were seen at the niche base (Fig. 3C). The proliferative hair germs of ΔN follicles were more advanced at this age, and this was reflected by the increase in BrdU-labeled cells within the bulge (lower set of panels in Fig. 3C). Quantitative analyses again revealed a more than two times increase in BrdU-labeled cells in ΔN versus wild-type bulges. Interestingly, however, by d60, when ΔNΔN follicles had progressed to full anagen, the bulges behaved as in the previous anagen (d28), displaying markedly reduced proliferation (Fig. 3D). These bulges showed no anti-caspase 3 labeling nor did they display morphological signs of apoptosis in anagen or telogen (Supplementary Fig. S2). Based upon these observations, transgenic follicle niches appeared able to return to their quiescent state following precocious activation.

β-Catenin is required for SC maintenance and niche biology

To determine whether β-catenin affects follicle SCs and their biology, we used conditional inducible gene targeting to ablate β-catenin expression when backskin follicles were in telogen (see Materials and Methods). Within 7–10 d after topical application of tamoxifen, follicles were nearing the end of telogen. By this time, β-catenin was quantitatively lost from the skin epithelium, including the SC niche, of the β-catenin fl/fl, K14-Cre^{ER} animals (Fig. 4A). Since β-catenin loss is known to impair embryonic hair follicle development (Huelsken et al. 2001), we first established that when shaved, conditional β-catenin-null skin did not grow back a normal hair coat (Fig. 4B).

Interestingly, the deleterious effects on hair follicle formation appeared to initiate in the SC niche. In the absence of β-catenin, SC niches failed to label with Abs against classical SC markers within 2 wk of treatment (Fig. 4C). This was best exemplified at the boundaries of treatment, where β-catenin(+), CD34(+) bulges were often adjacent to β-catenin(−), CD34(−) bulges. Within this first week, the overall morphology and architecture of the β-catenin-null niche was still intact (example shown).

We next determined the consequences of β-catenin loss to niche quiescence. The bulge was first described as

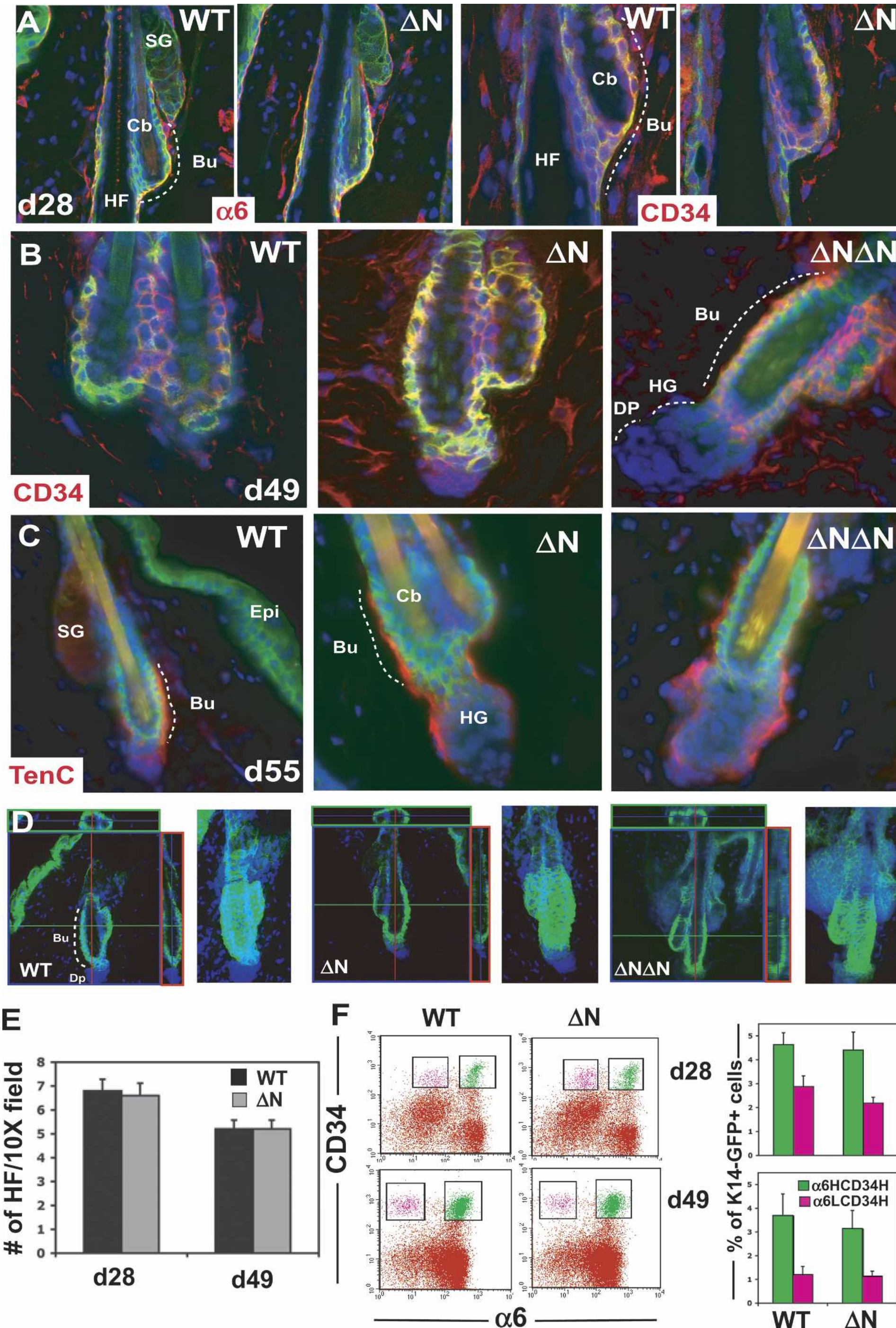

Figure 2. SC biochemistry and homeostasis is maintained even in the presence of elevated stabilized β-catenin. Mouse backskin follicles from K14-GFPactin (green) mice were stained with the indicated primary antibodies, followed by Texas red-conjugated secondary antibodies. (All images were taken at 40× magnification.) (*A–C*) Confocal immunofluorescence detects established bulge markers at different stages of the hair cycle irrespective of the level of ΔN β-catenin expression. Note: As previously reported (Tumbar et al. 2004), labeling for the basement membrane protein tenascin C (TenC) extends to the early hair germ. (*D*) Three-dimensional confocal analysis of K14GFPactin skin shows that bulge morphology and size are unaffected by the level of ΔN β-catenin expression. (*E*) Quantification shows that the number of d28 and d49 follicles per field (at 10× magnification) are analogous in wild-type (WT) and ΔN skin. (*F*) FACS analysis of the cells from d28 and d49 backskins of wild-type and ΔN mice shows that the proportion of basal ($\alpha6^{high}CD34^{high}$) and suprabasal ($\alpha6^{low}CD34^{high}$) bulge cells does not change with ΔN expression. Graphs at the *right* compile data from three experiments encompassing triplicate samples each from three different litters. Abbreviations are as in the legend for Figure 1. (Cb) Club hair; (DP) dermal papilla.

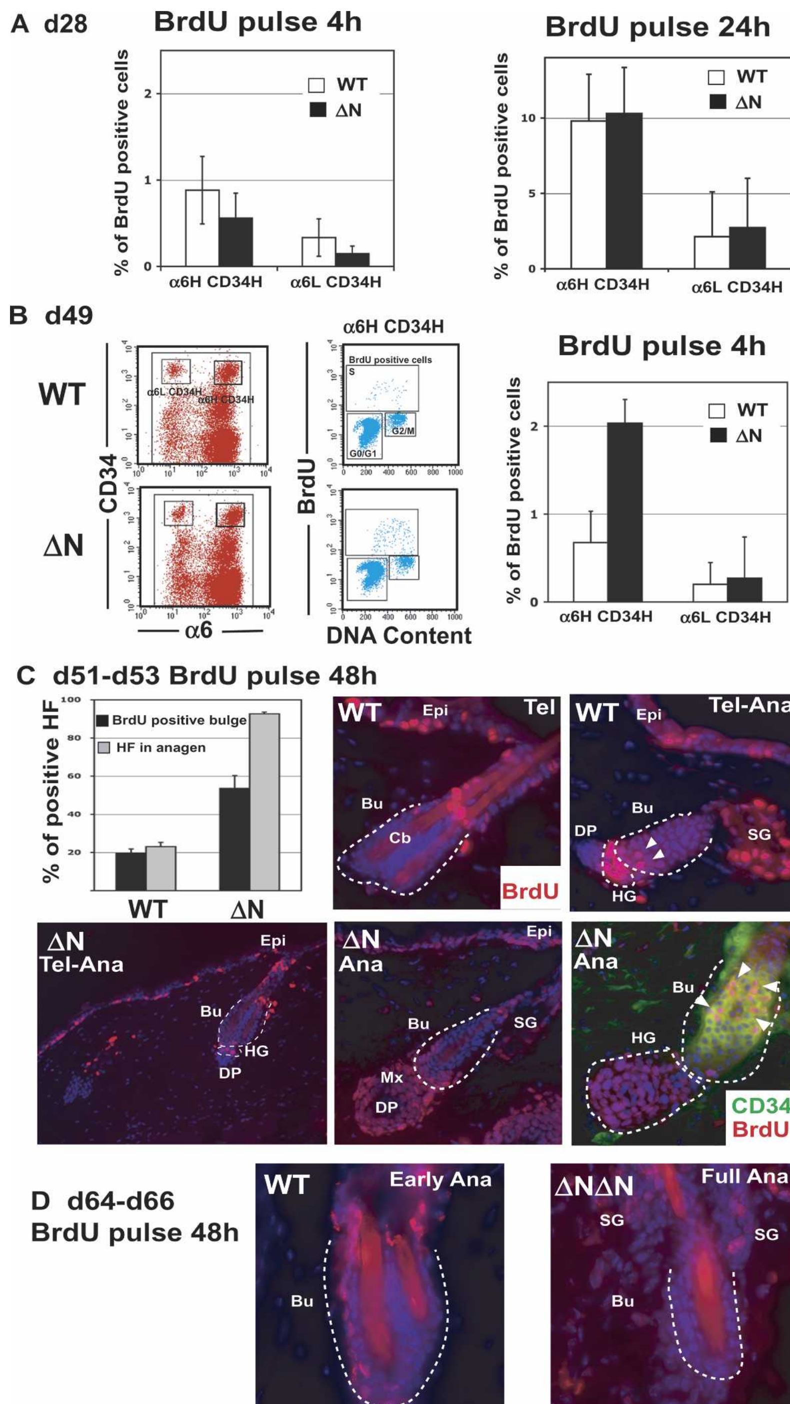

Figure 3. Premature activation of the ΔN hair cycle is accompanied by a precocious activation of proliferation in the normally quiescent SC niche. (*A*,*B*) d28 (*A*) or d48 (*B*) wild-type (WT) and ΔN mice were given 4- or 24-h pulses of BrdU prior to FACS analyses of backskin bulge cells for label incorporation. Note that at d28, the numbers of S-phase (BrdU-labeled) bulge cells are similar for wild-type and ΔN follicles. By d49, however, many more S-phase cells are present in the ΔN bulge. (*C*) At d51–d53, matched ΔN and wild-type littermates were given a 48-h pulse of BrdU, followed by immunofluorescence analyses to compare the numbers of S-phase bulge cells to that of HFs that had precociously entered anagen. Quantification is at the *left*. Note that all images are taken at 40× except the ΔN Telo-Ana which is at 20×. Note that the number of BrdU-labeled bulge cells is higher in ΔN follicles that have precociously entered anagen, and in this case, labeling is not exclusive to the bulge base. (*D*) By d65, ΔNΔN follicles have proceeded to full anagen and have regained quiescence as judged by BrdU staining. Wild-type follicles at this time are only just beginning the transition from telogen to anagen and are thus proliferative as measured by BrdU incorporation.

a group of slow-cycling SCs based on their characteristic retention of BrdU incorporation following a chase period (Cotsarelis et al. 1990). A 72-h pulse of BrdU was administered to d28 anagen phase fl/fl control and fl/fl Cre+ mice, and then chased for 4 wk. At d44, the animals were given tamoxifen, and at d57, the skins were analyzed. As expected, there were many label-retaining cells (LRCs) within the bulges of untreated or β-catenin expressing d57 follicles (Fig. 4D). In stark contrast, LRCs were not found in β-catenin-null SC niches. The loss of label retention appeared to be directly attributable to enhanced proliferation, as judged by immunolabeling with Abs against the proliferating nuclear antigen Ki67 (Fig. 4E). Again, this difference was best visualized in skin sections bordering treatment, where adjacent β-catenin-positive and -negative bulges were sometimes found (example shown).

The activation of proliferation and loss of SC markers within the bulge preceded the major morphological and biochemical aberrations described by Huelsken et al.

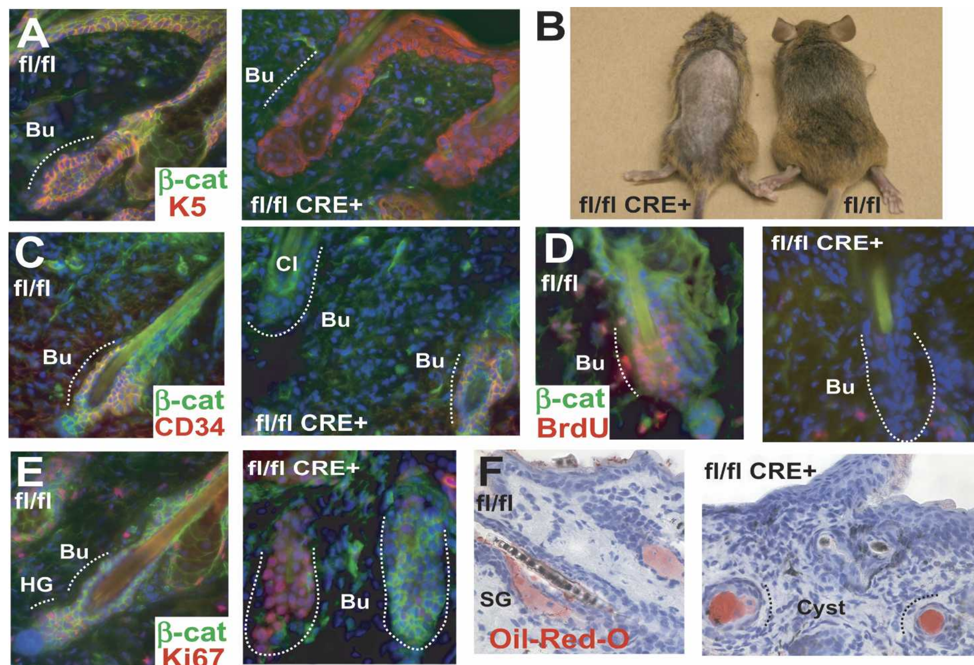

Figure 4. Loss of β-catenin leads to a loss of SC niche cell identity. The posterior backs of d49 mice homozygous floxed β-*catenin* (fl/fl) ± a Cre-ER^{tm} transgene (Cre) were shaved and topically treated with tamoxifen to activate Cre. Seven to 10 d later, Tm-treated skins were frozen and sectioned for either (1) immunofluorescence with the Abs color-coded on each frame (*A*,*C*–*E*) or (2) hematoxylin and Oil-red-O staining (positive for sebaceous gland [SG] secretions) (*F*). DAPI was used in some cases to stain nuclei (blue). (*B*) Mice at 4 wk after Tm treatment to show that only wild-type (WT) mice regrew their hair coat. (Bu) Bulge; (HG) hair germ; (Cl) companion layer (K14-promoter silent). Dotted white lines denote bulge and HG boundaries. Note: fl/fl, Cre skin regions in *C* and *E* are near boundaries of Tm treatment, and hence provide a convenient internal control to illustrate correlation between complete loss of β-catenin expression and corresponding loss of bulge markers.

(2001; see also Fig. 4F), and therefore represent the earliest and most direct effects of loss of β-catenin in SCs. Taken together, our study revealed an early impact of β-catenin ablation on the telogen-phase follicle SC niche that resulted in a failure of SCs to undergo proper activation at the onset of anagen. In addition, however, these studies suggested a more profound role for β-catenin, namely, on SC maintenance, as judged by the inability of β-*catenin*-null skin to preserve the quiescence and normal biochemistry of the bulge niche.

Interestingly, both gain and loss of function of β-catenin promoted increased proliferation, but for very different reasons leading to very different outcomes. Elevated β-catenin appeared to lower the threshold for activation by a putative DP signal to commit SCs to a hair follicle fate. By contrast, loss of β-catenin resulted in a loss of follicle SC character and a conversion to an epidermal fate.

β-Catenin stabilization in the SC niche preferentially activates TA genes associated with proliferation but not TA genes associated with differentiation

The established role of β-catenin as a transcriptional coactivator for Lef1/Tcf enhancer elements suggested that β-catenin stabilization might act directly to alter the gene expression in the SC niche. To avoid the well-established late effects of potent Wnt signaling on the hair lineage (DasGupta and Fuchs 1999; Merrill et al. 2001) and to capture the effects that occur when SCs are first activated, it was essential to first purify SCs from wild-type and from ΔN β-catenin skin. Moreover, since we observed a hair cycle-dependent difference in SC behavior, it was important to transcriptionally profile SCs from both growing (d28) and resting (d49) follicles, two time points where the follicles are morphologically indistinguishable in wild-type and ΔN β-catenin animals.

After verifying the purity of our FACS isolated populations (for details, see Blanpain et al. 2004), we performed microarray analyses in duplicate. Overall expression profiles of SC populations were remarkably similar, and the majority of genes scored as unchanged. This said, comparative analyses uncovered a number of distinguishing features of SCs at two different stages of the hair cycle and under conditions where levels of stabilized β-catenin were either wild-type or elevated to a level that specifically elicited precocious SC activation in a synchronous fashion (Table 1). The short lists of genes that scored as up- or down-regulated by ≥1.8× in the duplicates of one SC population relative to others provided "molecular signatures" for each population and revealed new insights into how SCs change behavior in response to changes in the microenvironment. The

Table 1. *Correlation between β-catenin stabilization, SC activation, and SC proliferation as revealed by transcriptional profiling*

Genes		Bulge cells	ΔN vs WT d49	ΔN vs WT d28	d28 vs d49	Bu vs TA d49	Bu vs TA d28
antigen identified by monoclonal antibody Ki 67	Mki67	a6H CD34H	2.7	3.1	NF	-10	-10
		a6L CD34H	5.2	76	2.81	-26	-200
CDC28 protein kinase regulatory subunit 2	Cks2	a6H CD34H	2.5 (2)	2.7 (2)	NF	-5.8	-5.3
		a6L CD34H	2.5 (2)	4.7 (2)	NF	-12	-11.5
cyclin A2	Ccna2	a6H CD34H	2.2 (2)	NF	NF	-4	-4
		a6L CD34H	2.6	3.4	NF	-5	-9
cyclin B1	Ccnb1	a6H CD34H	3.6	2.6	NF	-8	-10
		a6L CD34H	NF	4.7 (2)	NF	-14	-30
cyclin D1	Ccnd1	a6H CD34H	NF	1.8	NF	NF	-2
		a6L CD34H	2.6 (2)	NF	NF	-7	NF
cyclin D2	Ccnd2	a6H CD34H	NF	2.3	NF	-4	-3
		a6L CD34H	NF	3.5 (5)	3.1 (5)	-18	-12
cell division cycle 2 homolog A (S. pombe)	Cdc2a	a6H CD34H	7.8	2.7	NF	-8	-4
		a6L CD34H	NF	NF	NF	-13	-27
tissue inhibitor of metalloproteinase 3	Timp3	a6H CD34H	2.1 (2)	2.3 (2)	NF	-2.5	-2.7
		a6L CD34H	NF	14	2.5	-3.7	-3.8
biglycan	Bgn	a6H CD34H	3.4 (3)	NF	13.5 (3)	4.2	4.3
		a6L CD34H	2.4	6.8 (3)	4.3 (3)	NF	NF
transcription factor 4	Tcf4	a6H CD34H	NF	1.8	NF	2.6	2.8
		a6L CD34H	NF	NF	NF	2.6	2.2
high mobility group box 3	Hmgb3	a6H CD34H	NF	1.9	NF	-2.1	-3.9
		a6L CD34H	NF	2.4	2.4	-1.8	-1.7
high mobility group nucleosomal binding domain 3	Hmgn3	a6H CD34H	NF	2.2 (2)	2.5	NF	2
		a6L CD34H	NF	1.9	3.8	NF	-2.6
chloride channel calcium activated 1	Clca1	a6H CD34H	NF	4.2	NF	-12	-6
		a6L CD34H	1.8	NF	NF	-7.6	-3.7
chloride channel calcium activated 2	Clca2	a6H CD34H	NF	2.7	NF	-12.4	-24
		a6L CD34H	2.2	NF	NF	-5.4	NF
polycystic kidney disease 1 homolog	Pkd1	a6H CD34H	-2.1	NF	-3.8	5.7	3.9
		a6L CD34H	NF	NF	NF	4.6	3.2
growth arrest specific 1	Gas1	a6H CD34H	-1.6	NF	NF	2.80	2.00
		a6L CD34H	NF	NF	NF	2.3	2.4
growth differentiation factor 10	Gdf10	a6H CD34H	-2.4	NF	10	32	33
		a6L CD34H	NF	NF	4.6	8.5	15.2
latent transforming growth factor beta binding pro	Ltbp2	a6H CD34H	-2.5	NF	-4.5	19	12.2
		a6L CD34H	-1.5	NF	-3.6	8.3	5.6
basonuclin 1	Bnc1	a6H CD34H	NF	NF	2.5	2.9	5.2
		a6L CD34H	NF	NF	3.9	NF	2.5
early growth response 2	Egr2	a6H CD34H	NF	NF	3.2 (2)	-7.9	-7.8
		a6L CD34H	NF	NF	NF	-4.9	-12.9
inhibin beta-B	Inhbb	a6H CD34H	NF	NF	2.9	NF	2.6
		a6L CD34H	NF	3	3.7	-2.2	NF
nephronectin	Npnt	a6H CD34H	NF	NF	2.6	4	2.2
		a6L CD34H	1.9	NF	5.5	-1.9	NF
secreted frizzled-related sequence protein 1	Sfrp1	a6H CD34H	NF	NF	3.8	4.2	4.2
		a6L CD34H	NF	NF	2.6	1.7	2.3
sema domain, seven thrombospondin repeats (typ	Sema5a	a6H CD34H	NF	NF	4.3	-2.8	NF
		a6L CD34H	NF	NF	NF	-14	-4.5
SRY-box containing gene 4	Sox4	a6H CD34H	NF	NF	9.3	-3.3	NF
		a6L CD34H	NF	NF	8.5	-2.3	NF
SRY-box containing gene 7	Sox7	a6H CD34H	NF	NF	2.6	-6.3	-2.9
		a6L CD34H	NF	NF	2.2	-6.1	-3.8
cadherin 13	Cdh13	a6H CD34H	NF	NF	-3	NF	NF
		a6L CD34H	NF	NF	-3.8	NF	NF
cholecystokinin	Cck	a6H CD34H	NF	NF	-31	12.1	NF
		a6L CD34H	NF	NF	NF	NF	NF
connective tissue growth factor	Ctgf	a6H CD34H	NF	NF	-4.5	12.1	11.5
		a6L CD34H	NF	NF	NF	NF	NF
disabled homolog 2 (Drosophila)	Dab2	a6H CD34H	NF	NF	-2.2	11.1	7.5
		a6L CD34H	NF	NF	NF	3.5	2.8
thrombospondin, type I, domain 1	Thsd1	a6H CD34H	NF	NF	-3	12.9	6.2
		a6L CD34H	NF	NF	NF	11	7.9
follistatin	Fst	a6H CD34H	NF	NF	-2.9	4.1	NF
		a6L CD34H	NF	NF	-2.5	NF	NF
synuclein, gamma	Sncg	a6H CD34H	NF	-2.38	-13	13.8	NF
		a6L CD34H	NF	NF	-5.3	81.5	35.1

Duplicate microarray data sets were obtained from purified ΔN and wild-type (WT) bulge cells ($\alpha6^{high}CD34^{high}$ and $\alpha6^{low}CD34^{high}$) at d28 and d49 and from $K14^{+}\alpha6^{high}$ cells (enriched in TA cells). Data sets were subjected to the comparisons indicated, and molecular signatures were developed for genes that were at least 1.8× up-regulated (green) or down-regulated (red) in duplicate in one bulge population versus the other (white is unchanged). The numbers correspond to the fold change (+ up or – down) in the comparison described at the top of each column. For example, a positive number in the ΔN versus wild type (WT) denotes a gene up-regulated in ΔN. These signatures were then compared across the five comparisons to reveal trends.

Supplemental Material provides the details of how the comparisons were made and quantified.

Underscoring the impact of the niche on the overall program of expression, many of the established bulge markers maintained faithful expression in the presence of ΔN β-catenin (Table 1; Fig. 5A). Some SC genes, however, were differentially expressed during the hair cycle, independently of the genetically elevated levels of stabilized β-catenin (Fig. 5B; Table 1, center column: green, up-regulated; red, down-regulated).

In situ hybridizations and immunofluorescence microscopy permitted analyses of expression patterns at other stages of the hair cycle and elsewhere in the skin. Some mRNAs/proteins specifically up-regulated in the SC niche during the follicle growth phase were also expressed in proliferating TA progeny. *Sox4* was an intrigu-

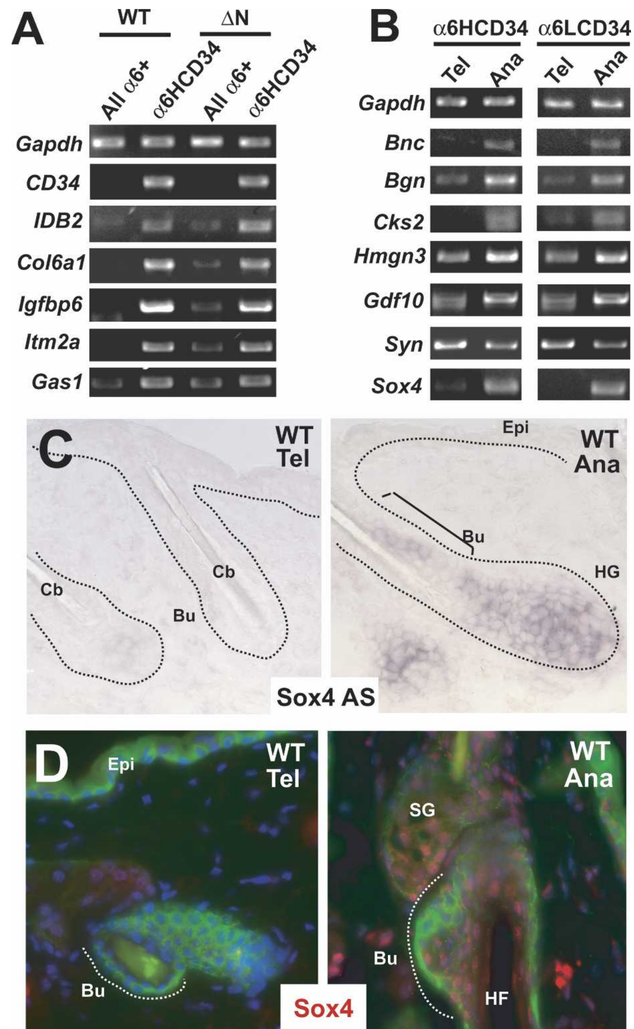

Figure 5. Two categories of SC niche genes whose expression is independent of ΔN β-catenin: Some are constitutive throughout the hair cycle and others are up-regulated at the telogen-to-anagen transition. (*A*) RT–PCR of representative bulge signature genes known to be enriched in the bulge (α6+, CD34+) relative to the TA population (>90% of the cells in the all α6+ population) (Blanpain et al. 2004). Data shown here are from bulge signature genes that scored as unchanged in our arrays from d28 versus d49 bulge cells ± ΔN. *Gapdh* was used to control for equal amounts of input RNAs. (*B*) RT–PCR of representative genes that scored in our arrays as being up-regulated in the wild-type (WT) niche during SC activation (telogen-to-anagen transition). (*C*,*D*) In situ hybridization and immunofluorescence to illustrate expression of a gene from *B*, *Sox4*, which was not only up-regulated in the niche upon SC activation, but maintained expression in the proliferating TA progeny of the hair germ (HG) and matrix (Mx).

ing example of this, as it is also a member of the HMG box superfamily of transcription factors implicated in Wnt signaling and development (Fig. 5C,D; Hurlstone and Clevers 2002; Busslinger 2004). Such findings further suggested that the transition from quiescent SCs to proliferating TA cells has its origins within the SC niche.

Of particular interest was the short list of SC genes whose expression was specifically impacted by ΔN β-catenin (Table 1, left columns). Some of these genes were up-regulated in both basal and suprabasal ΔN bulge cells, reflecting an overall impact of ΔN β-catenin on the niche (Fig. 6A,B). Intriguingly, while the ΔN bulge featured an overall transcriptional profile consistent with a quiescent state, mRNAs encoding cell cycle-promoting factors were slightly but consistently up-regulated (Table 1, left columns). Notably, this included *Cyclin D1*, an established Wnt target and cell cycle gene (Tetsu and McCormick 1999; Chamorro et al. 2005). Conversely, the growth inhibitory genes that were typically up-regulated in quiescent wild-type SCs were slightly but again significantly down-regulated by β-catenin stabilization in SCs (see Table 1). The proportion of proliferation-associated genes displaying this collective up- and down-regulation was impressive, and illuminated a subtle but striking trend in the shift of ΔN SCs to a status that resembled the early vestiges of the TA state (Table 1, cf. left and right columns). Importantly, however, since array comparisons between wild-type and ΔN β-catenin were clearly distinct from those between telogen and anagen, small differences in d49 wild-type and ΔN hair cycles did not appear to interfere substantially with these results.

As revealing as the genes that were present in the ΔN signature were those that were absent. Like many of the cell proliferation-associated genes, *Sonic hedgehog* (*Shh*), *Bone morphogenetic proteins* (*Bmp2/4*), *Lef1*, *Patched*, *Foxn1*, *Msx2*, and *mOvo1* have also been found to be markers of TA matrix cells (for reviews, see Millar 2002; Schmidt-Ullrich and Paus 2005). However, these transcription and growth factor genes did not score as up-regulated in our bulge microarrays, nor did quantitative RT–PCR reveal appreciable expression in ΔN bulge cells whereas expression was readily detected in hair follicle and even whole skin mRNA (Fig. 6A). Thus, whereas proliferation-associated TA genes were activated in the ΔN bulge, induction of the differentiation-associated TA genes appeared to be a later event in the lineage.

Mechanistic insights into Wnt signaling in the bulge

If the ΔN bulge signature is reflective of the normal process of SC activation, then expression of these genes might be expected to be even higher in the rapidly proliferating hair germs that form at the onset of anagen. To test this hypothesis, we employed in situ hybridization and immunofluorescence microscopy. Figure 6C provides representative data of some of these results.

In wild-type resting phase follicles, *Cyclin D1* (*Ccnd1*) cRNAs displayed little or no hybridization. However, as follicles transitioned into anagen, hybridization appeared at the bulge base and in the developing hair germ. In ΔN and/or ΔNΔN follicles, *Ccnd1* hybridizations were more intense and extended throughout the bulge (see Fig. 6C). In the de novo follicles arising in ΔNΔN mice, strong hybridization was also seen in the downgrowths (Fig. 6C). Consistent with the array analyses, mRNA expression was always lower in the bulge than in

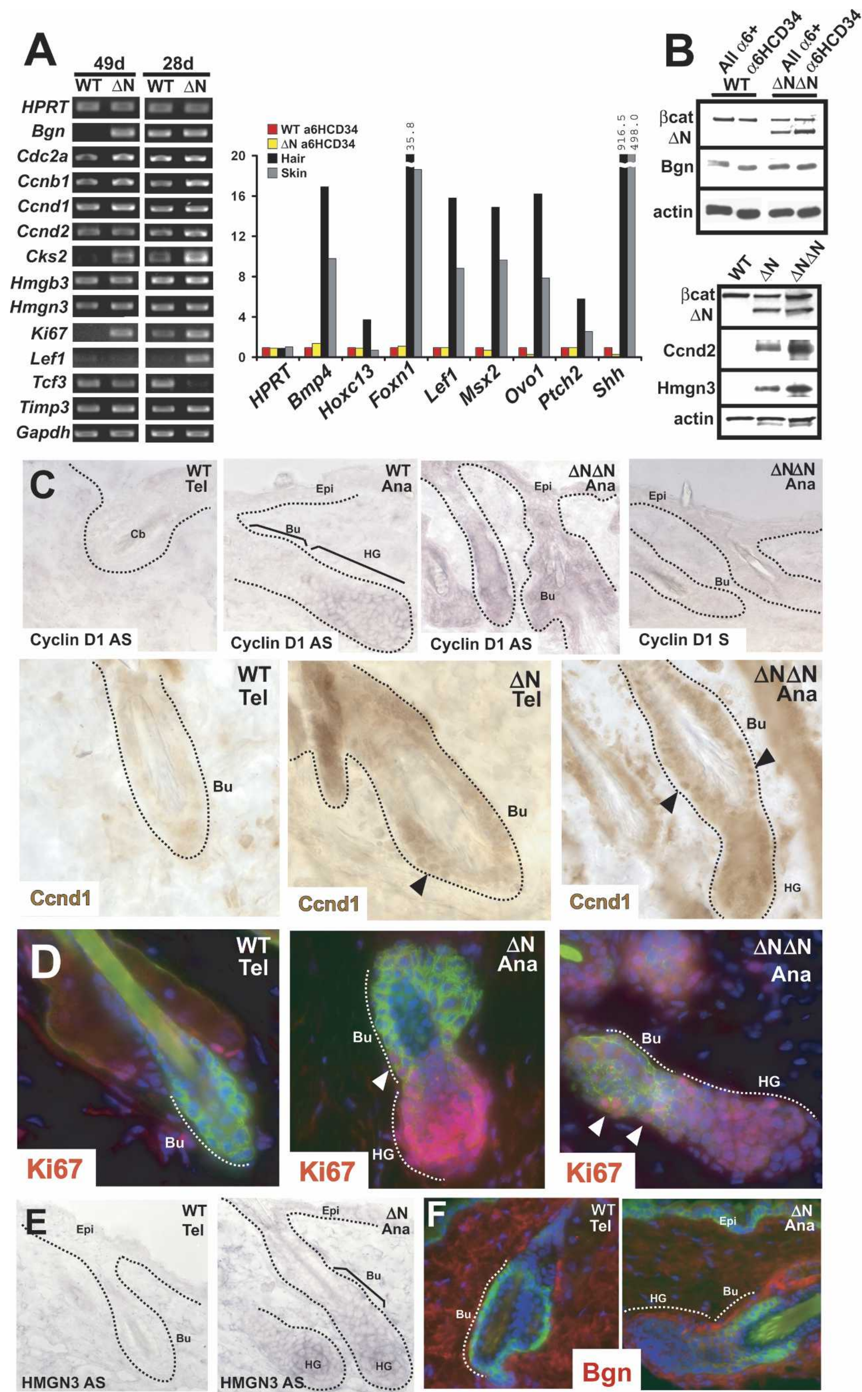

Figure 6. Some SC genes are up-regulated in the ΔN β-catenin niche and are also associated with the telogen-to-anagen transition. (*A*) Semiquantitative (*left*) and real-time (*right*) RT–PCRs of FACS purified d49 (telogen) and d28 (anagen) bulge cell populations from wild-type and ΔN skins and from dissected hair follicles and total skin from d49 wild-type (WT) mice. RT–PCRs were performed on representative genes scoring as up-regulated in a ΔN-dependent fashion. Note: Tcf3 levels are lower and Lef1, Cks2, and Ki67 levels are all higher in the anagen (d28) versus telogen (d49) SCs, reflective of early signs of SC activation and commitment. Quantitative RT–PCR (*right*) reveals that expression of Lef1 and other key genes encoding TA matrix cell-signaling molecules and transcription factors is low relative to the SC compartment. With the exception of *Lef1*, none of these matrix genes were found in our ΔN bulge signature. This contrasts with the proliferation-associated hair germ and matrix genes, present in the signature. (*B*) Immunoblots of sorted wild-type and ΔN bulge cells (*top*) and total skin epithelium (*bottom*) reveal representative mRNA changes that are also reflected at the protein level. (*C–F*) In situ hybridizations (AS, antisense; S, sense) and immunostaining of representative markers (Cyclin D1, Ki67, HMGN3, Biglycan) that scored as up-regulated in a ΔN-dependent fashion. Wild-type, ΔN, and ΔNΔN follicles are from either telogen or early anagen to highlight that these bulge genes were not only expressed in a dose-dependent manner based upon stabilized β-catenin, but in addition, they were expressed more strongly in proliferating TA progeny cells than in the more quiescent SC niche. Arrowheads denote Ki67-labeled bulge nuclei. Green is K14-GFPactin.

bulge progeny (see Table 1). This was also reflected at the protein level, where most Cyclin D1- and Ki67-positive nuclei were in the hair germ. This said, cells expressing proliferative markers were frequently at the base of the newly activated SC niche, and this was particularly notable in ΔN and ΔNΔN bulges (Fig. 6D). Notably, the proliferation-associated genes that were up-regulated in a ΔN-dependent fashion were expressed at significantly higher levels in early committed follicle TA cells than in the infundibulum or epidermis. This further underscored the distinction between proliferation per se and SC activation in hair follicle fate commitment.

Similar findings were seen with the ΔN signature genes *Hmgn3* and *Biglycan* (*Bgn*) that did not encode cell cycle proteins (Fig. 6E,F). Biglycan's unusual pattern deserves special note as it is expressed by both dermal fibroblasts and keratinocytes (Wadhwa et al. 2004). The anti-biglycan labeling along the borders of the niche and developing hair germ suggests that this proteoglycan may be a component of the basement membrane sur-

rounding the proliferative compartments of the follicle (Fig. 6F). Again, these genes were preferentially expressed in the proliferating cells of the follicle versus epidermis.

If β-catenin acts directly on some signature genes to change their expression status at the SC activation stage, then conserved Lef1/Tcf consensus-binding motifs might be expected within the 5′ upstream sequences of target genes. A computer-based analysis and cross-comparisons across mammalian genomes revealed several candidates. We focused on the conserved Lef1/Tcf motifs in the *Bgn*, *Ccnd1*, *Hmgn3*, and *Timp3* genes, since these genes were up-regulated in the activated wild-type bulge progeny and in the ΔN bulge, as might be anticipated for direct β-catenin target genes (Fig. 7A).

We then conducted in vivo chromatin immunoprecipitation (ChIP) assays, using an anti-β-catenin Ab that had recently been shown to function in in vitro ChIP assays with the *Cyclin D1* promoter (Chamorro et al. 2005). Only in ΔN skin did anti-β-catenin specifically immunoprecipitate chromatin–protein complexes that contained (1) the endogenous β-catenin, (2) the truncated transgene product, and (3) sequences that encompassed one of the putative Lef1/Tcf-binding sites (Fig. 7A). No ChIP PCR bands were observed with primers corresponding to regions that did not contain such motifs (DS sites). Even though technical limitations precluded analyses on purified SCs, these data still provided the first in vivo ChIP data from skin. Overall, these data revealed a good correlation between the up-regulation of these niche genes upon elevated nuclear β-catenin and SC activation and the β-catenin-dependent binding of proteins to their conserved Lef1/Tcf consensus-binding motifs.

To further demonstrate that direct interaction of these genes with β-catenin/Lef1/Tcf complexes affects their activation, we exposed cultured keratinocytes to Wnt3a and monitored the kinetics of endogenous *Cyclin D1*, *Biglycan*, *Timp3*, and *Hmgn3* gene expression by quantitative RT–PCR. We first pinpointed the timing for Wnt-mediated activation of target genes in keratinocytes by testing a known Wnt target gene, TOPGAL, under conditions of maximum sensitivity. Within 6 h after exposure to the potent GSK3β inhibitor LiCl, the multimerized Lef1/Tcf TOP enhancer displayed appreciable activity (Fig. 7B, left). Employing the same time course but under more physiological conditions of β-catenin stabilization, Wnt3a induced TOPFLASH activity by approximately twofold. (Fig. 7B, middle). Overall, the kinetics of Wnt target gene activation in keratinocytes was similar to other cell types (Willert et al. 2002).

With these controls in hand, quantitative RT–PCR was then used to monitor expression of our four genes. With 6 h of treatment with Wnt3a, *Ccnd1*, *Timp3*, *Biglycan*, and *Hmgn3* mRNAs were elevated by an average of twofold relative to either of two housekeeping genes (Fig. 7B, right graph). As an additional control, Sox4, a gene found to be up-regulated in the telogen-to-anagen transition, but not changed between wild-type and ΔN, was not induced by Wnt3a in vitro. These findings provide additional evidence that the SC signature genes up-regulated in response to elevated stabilized β-catenin and SC activation are also direct Wnt target genes.

To bring our functional analyses full circle, we returned to the issue of the physiological relevance of activating this set of signature genes at the β-catenin-dependent SC activation step. The domination of our signature by well-known cell cycle regulatory genes, including the Wnt target gene *Cyclin D1*, already implicated these genes in the conversion of quiescent SCs to proliferating TA progeny. To further investigate the role of Wnt signaling and stabilized β-catenin in this conversion, we first tested whether cultured FACS isolated bulge cells from ΔN mice display a proliferative advantage relative to their wild-type counterparts. Despite similar plating and holoclone forming efficiencies, after 10 d in culture there were consistently approximately three times the numbers of ΔN versus wild-type cells (Fig. 7C). Wnt3a treatment stimulated proliferation of wild-type cells to a level comparable to the ΔN state. Conversely, treatment with the Wnt inhibitor secreted frizzled protein (Sfrp) impaired keratinocyte growth. Thus, whether β-catenin was stabilized through genetic engineering or through Wnt signaling, it conferred a growth advantage to keratinocytes.

We next tested whether other signature genes; for example, *Timp3* and *Biglycan*, are also integrated in this process. Interestingly, proliferation was markedly enhanced in keratinocytes treated with purified Biglycan (Fig. 7C). In addition, enhanced proliferation was seen when >75% of keratinocytes were infected with lentiviral vectors expressing either *Biglycan*, *Timp3*, or *ΔN* β-catenin (Fig. 7D). Although cells expressing exogenous Hmgn3 did not show enhanced proliferation in this assay (data not shown), the fact that some of these genes could act independently was surprising, and indicates that up-regulation in expression of individual members of the signature can contribute to tipping the balance between SC quiescence and activation. Overall, our findings reveal functional links between β-catenin stabilization, direct activation of proliferation-associated Wnt target genes, and conversion of quiescent SCs to proliferating TA progeny.

Discussion

Roles for β-catenin in the activation and maintenance of slow-cycling, multipotent follicle SCs

Increasing evidence suggests that Wnt signaling may be a universal regulator of SCs or SC progeny, but how and where they exert their action(s) in SC lineages is by no means clear. We and others had previously shown that robust Wnt signaling occurs during hair differentiation. To address whether epithelial skin SCs regulate and respond to Wnt signaling, and when and how do they do it, we needed to devise strategies to dissect out these other effects from those on the SC niche. We succeeded by using the minimum level of stabilized β-catenin necessary to perturb the SC niche, but to do so in a way that

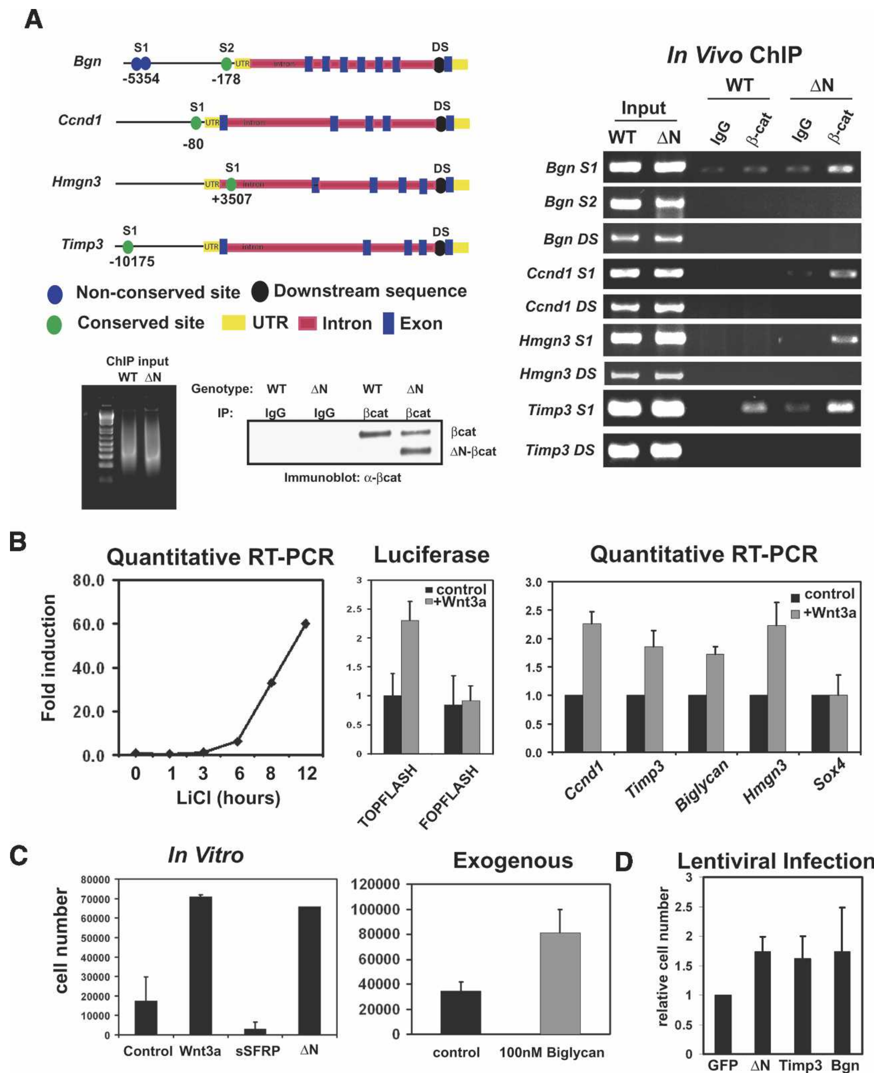

Figure 7. Functional links between Wnt signaling, β-catenin stabilization, target gene expression, and SC activation and conversion to proliferating TA progeny. (*A*) Stick diagram shows computer predictions of Lef1/Tcf sites relative to the promoters of the genes indicated (transcription initiation site = 0). The ChIP input gel shows equivalent fragmentation of total input DNA from wild-type (WT) and ΔN cells after cross-linking to protein and sonication. The ChIP immunoblot shows that β-catenin was specifically pulled down from both wild-type and ΔN chromatin samples and that the ΔN was also a part of this precipitate in the ΔN skin. Control rabbit IgG reveals no pull-down of β-catenin. ChIP PCR reveals a β-catenin dose dependency for the selective precipitation of DNA fragments that encompass Lef/1Tcf consensus-binding motifs from genes that are specifically up-regulated in the ΔN versus wild-type bulge. Primers were designed to flank conserved Lef1/Tcf-binding sites (as predicted by rVista software). Sequences not predicted to contain Lef1/Tcf-binding sites downstream of the promoter (DS) were not pulled down. (*B*, *left*) Quantitative RT–PCR to monitor kinetics of expression of the established Wnt target reporter gene TOPGAL in keratinocytes treated with 20 mM LiCl to induce stabilization of β-catenin. Amounts of β-galactosidase produced was determined relative to a standardized GAPDH control. The kinetics were similar to those reported previously for other cell types. (*Middle*) A similar experiment was conducted, this time using 40 ng purified Wnt3a and the same TOP enhancer but driving luciferase. FOPFLASH, harboring mutations in the Lef1/Tcf-binding sites, was used as a control, and the assay was made following 6 h of treatment. (*Right*) Quantitative RT–PCR for endogenous target genes in keratinocytes treated with Wnt3a for 6 h. RNAs were isolated from keratinocytes with or without 6-h exposure to Wnt3a. Shown is the average fold change calculated as a function of GAPDH for each sample from three independent experiments. Statistical analyses showed a p value < 0.05 for each of the changes shown upon Wnt3a treatment. Note that the kinetics and responses of the endogenous Wnt targets are similar to that of the TOPFLASH reporter. (*C*, *left*) A direct link between proliferation and Wnt signaling, as revealed by 10 ng/mL Wnt3a treatment of wild-type cultured bulge cells or untreated ΔN cultured bulge cells. Note that the effects of Wnt3a are obliterated by treatment with 100 ng/mL of the Wnt inhibitor, SFRP. (*Right*) A direct link between proliferation and exposure to 100 nM proteoglycan Biglycan, encoded by one of the β-catenin-dependent and SC activation-associated signature genes that we showed to be a direct target by ChIP. (*D*) Link between proliferation and lentiviral expression of individual SC activation signature genes. Keratinocytes (see *B*) were infected with lentivirus harboring transgenes encoding one of the ΔN-up-regulated genes indicated. After 10 d in culture, cells were counted. The histogram shows data averaged from five independent sets of experiments (±SEM).

permitted continuous stabilized β-catenin throughout the hair cycle. Similarly, it was essential to purify SCs under conditions where they received different levels of Wnt signaling. These considerations enabled us to discover how Wnt signaling influences the biology of the native SC niche during quiescent and activated states.

Our studies provide compelling evidence that β-catenin acts directly on follicle SCs to reduce the activation threshold imparted by the niche. Modestly elevated β-catenin levels tip the scale in favor of SC activation, as evidenced by the premature entry of follicles into anagen. Following this activation period, the niche microenvironment overrides the elevated stabilized β-catenin and returns SCs to their quiescent state.

The growth inhibitory impact of the niche was illustrated by the unrestricted proliferation observed when ΔN β-catenin follicle SCs were isolated and placed into culture. The proliferative response was also mimicked by exposing wild-type niche progeny to exogenous Wnts in vitro, similar to behavior observed for other types of

SCs in culture (Reya et al. 2003; Willert et al. 2003). By contrast, the in vivo niche appeared to maintain normal SC numbers even in the presence of stabilized β-catenin. This homeostasis could either be controlled by balancing increased proliferation with an increased activation and exit rate of SCs from the niche or by imposing a growth restraint on SCs once activation has passed. Without β-catenin, however, SCs lose quiescence and expression of conventional bulge cell markers as well as their ability to generate a new hair follicle.

Dissecting how the niche influences SC activation

The established role for β-catenin as a transcriptional cofactor for Lef1/Tcf makes it likely that enhanced β-catenin reduces the threshold for SC activation by affecting the status of genes that are regulated by Lef1/Tcf. In this regard, it is both interesting and relevant that Lef1/Tcf proteins and their Sox cousins are expressed in the bulge (van Genderen et al. 1994; Zhou et al. 1995; DasGupta and Fuchs 1999; Merrill et al. 2001), and that some of the genes up-regulated in the bulge in a β-catenin dose-dependent fashion also contained Lef1/Tcf consensus sequences that bound β-catenin in this way. However, Wnt signaling per se did not appear to be sufficient on its own to induce SC activation in vivo since even in the presence of sustained elevated levels of stabilized β-catenin, nuclear accumulation of the protein was only detected at the SC activation step, which still occurred synchronously and at one defined stage of the hair cycle.

If Wnts are not sufficient on their own, what else might be needed? In effect, the precocious activation found in β-catenin follicles resulted in a shortened exposure time of the niche to DP. Therefore, it is tempting to speculate that the DP produces some factor that, along with β-catenin transactivation, cumulatively contributes to reducing the activation threshold. Since SC contact with the DP is lost upon activation, this would explain why following activation, the niche was able to return to quiescence even though ΔN β-catenin persisted throughout the cycle.

The premature, synchronous, and transient SC activation observed in our ΔN β-catenin mice suggested that by enhancing the level of stabilized β-catenin, it may lower the threshold set by other stimuli, perhaps emanating from the DP. Precisely what these additional stimuli might be remains a topic that is beyond the scope of the present study. However, since doubling the dose of stabilized β-catenin seems to reduce the threshold even further, it seems likely that the stimuli collaborate with the Wnt pathway. Possible candidates worth exploring in the future are Wnts and Wnt inhibitors, as well as factors that alter the expression and/or DNA binding of proteins that recognize Lef1/Tcf motifs.

Wnt signaling, SC activation, and gene expression: proliferation first and then differentiation

By conducting our analyses on cells isolated directly from the niche, we were able to identify β-catenin-mediated changes in gene expression that occur at the earliest times when SCs become activated and transition from a quiescent to a proliferative state. Remarkably, among the top 30 mRNAs up-regulated in the ΔN β-catenin d49 SCs over their wild-type counterparts, 15 of them encoded proteins that are well-established players in the cell cycle. One of these, *Cyclin D1*, is a well-established Wnt target gene, and our studies extended this to keratinocytes. Conversely, *Cyclin D1* and these other genes were uniformly down-regulated in the quiescent SC niche as compared with their TA progeny. These findings unveiled a clear role for Wnt signaling in controlling genes involved in follicle SC proliferation and their activation from the resting to activated state.

The levels of β-catenin employed enabled us to identify genes involved in SC activation without perturbing the critical brake that permits SCs to return to a quiescent state following activation. Intriguingly, one of the genes that surfaced in our study, *Timp3*, has also been implicated in activation of hematopoietic stem cells (Venezia et al. 2004). It is tempting to speculate that by regulating the local concentration of critical signaling or chromatin remodeling factors (Biglycan, Timp3, and Hmgn3), the expression of such proteins may in turn affect SC activation and transit from a quiescent to a proliferative state. Importantly, our microarray, ChIP, and in vitro data were not only consistent with this notion but also suggested a direct role for Wnt signaling in enhancing the expression of these genes during SC activation.

Proliferation is one of the hallmarks of the TA population of matrix cells that are established following SC activation. However, matrix cells also express a number of signaling proteins and transcription factors that will specify these cells along one of their six programs of differentiation. Notably, although the proliferation-associated proteins figured prominently on the signature list of β-catenin activated SC genes, the signaling molecules and transcription factors of the matrix did not. Even Lef1, Movo1, and Foxn1, which are established Wnt target genes, were not among the SC signature, nor were Shh, Patched, or Glis, involved in matrix lineage determination (for reviews, see Millar 2002; Christiano 2004; Schmidt-Ullrich and Paus 2005). Thus, β-catenin appeared to selectively up-regulate genes involved in the transition from an SC to a TA cell without activating genes involved in the transition of the TA cell to a committed hair lineage.

Our findings delineate sequential roles for Wnt signaling in temporally regulating the follicle SC lineage. In the first step, β-catenin stabilization promotes SC maintenance. In the second step, it promotes activation and proliferation, as our data show here. In the third step, a more robust Wnt signal promotes the transition from proliferation to differentiation (Gat et al. 1998; DasGupta and Fuchs 1999; Huelsken et al. 2001; Merrill et al. 2001; Millar 2002; Braun et al. 2003). Figure 8 provides a model that summarizes these findings.

Whether this dual mechanism functions in other SC lineages is an intriguing question. A priori, dual action

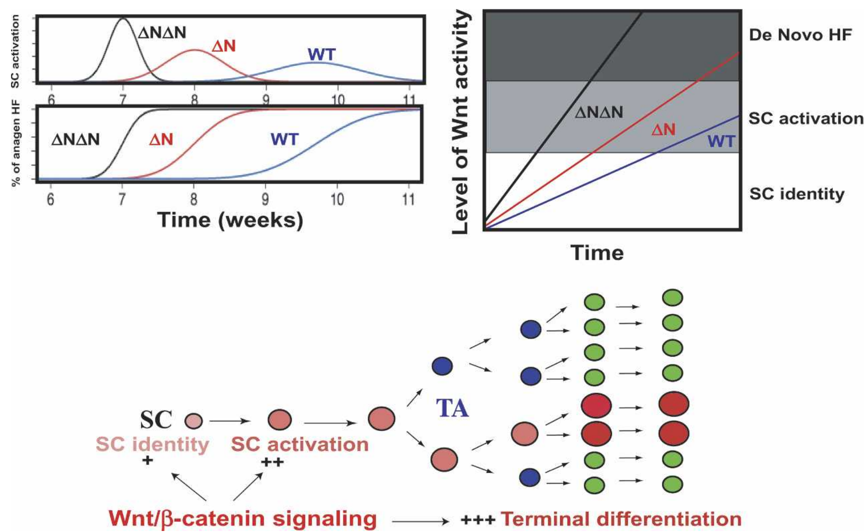

Figure 8. Model for Wnt-mediated regulation of the follicle stem cell lineage. (*Top*) The telogen (resting)-to-anagen (growth) transition is accompanied by SC activation and conversion to proliferating TA progeny. A low dose of β-catenin (ΔN) promotes this transition precociously and synchronously. A higher dose (ΔNΔN) shortens this time further and in addition endows some cells with the ability to form de novo hair follicles. (*Bottom*) The model incorporates our new findings in the context of prior models of Wnt regulation of the hair lineage (for review, see Millar 2002). Without β-catenin, SC quiescence and identity is not maintained. Stabilized β-catenin promotes SC activation and leads to a shift in the transcriptional program of SCs, prompting them to exit from the niche and convert from a quiescent SC to the rapidly proliferating TA cells of the hair germ. In the presence of ΔN, low levels of these transcriptional changes associated with SC activation occur within the SC niche, reducing the threshold necessary for the activation step and resulting in premature entry into anagen. The ΔN niche then returns to quiescence, while the TA cells of the hair germ proliferate and grow downward to form the TA matrix cells. Matrix cells proliferate, but they also receive additional Wnt signals as they activate transcription of genes encoding signaling factors and transcriptional regulators that permit these cells to differentiate along one of six different hair lineages.

might explain why some SCs seem to respond to cell fate specification while others seem to react with self-renewal and/or proliferation. In this regard, it may be relevant that disruption of β-catenin/TCF-4 activity in colorectal cancer cells in vitro blocks expression of genes that are active in the proliferative compartment of colon crypts and activates genes that are involved in intestinal differentiation (Van de Wetering et al. 2002). Additionally, perhaps analogous to the hair follicle, the strongest nuclear β-catenin in the intestinal epithelium is in the differentiated Paneth cells as opposed to the SC niche (van Es et al. 2005), and in the hematopoietic system it is in the differentiated lymphocytes (Staal and Clevers 2005). Finally, our finding that β-catenin stabilization acts on native follicle SCs to promote their proliferation provides a possible explanation as to why cancers may arise if this process goes awry in adult SCs.

Materials and methods

Mice

Homozygous K14-ΔN β-catenin mice have been described (Gat et al. 1998). For the current study, hemizygous animals were used. Inducible ablation of the β-catenin gene in adult follicles was achieved by crossing mice expressing the floxed β-catenin gene (Lee et al. 2004) to mice expressing tamoxifen-regulated Cre recombinase under control of the K14 promoter (Vasioukhin et al. 1999). Mice were anesthetized and shaved before topically applying 5 mg/mouse Tamoxifen (in ethanol) daily for 5 d. For pulse-chase experiments, 5-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich) was administered to d28 mice by injection (50 µg/g BrdU) and/or by adding 0.8 mg/mL BrdU to the drinking water for 3 d (Braun et al. 2003). Mice were then chased 28–55 d as in the text. For cell cycle analysis, d28 mice were injected once and analyzed for BrdU incorporation after 4 or 24 h.

Histochemistry and in situ hybridizations

Histology, immunofluorescence, β-galactosidase activity assays, and in situ hybridizations were performed as described (DasGupta and Fuchs 1999; Blanpain et al. 2004). Abs and dilutions employed were: α6-integrin (rat, 1:100, BD-Pharmingen), CD34 (Rat, 1:50, BD-Pharmingen), K5 (rabbit, 1:5000, Fuchs Laboratory), BrdU (rat, 1:100, Abcam), Tenascin C (1:500; IBL), Biglycan (1:500, gift of Larry Fischer, National Institutes of Health, MD), Sox4 (1:200, Chemicon), Cyclin D1 (1:100, Zymed), β-catenin (mouse, 1:50, Sigma), β-catenin (rabbit, 1: 500, Santa Cruz), Hmgn3 (1:2000, gift of Michael Bustin, University of Glasgow, Glasgow). Secondary Abs coupled to FITC, Alexa488, or Texas Red were from Jackson Laboratories. Nuclei were labeled by 4′6′-diamidino-2-phenylindole (DAPI) for immunofluoresence or TOPRO-3 (1:300, Molecular Probes) for confocal microscopy. Three-dimensional reconstructions of confocal analysis were performed using LSM510 Confocal Analyzer (Zeiss) or Imaris software (Bitplane AG).

FACS, microarray analyses, and PCR

FACS analyses and purification of bulge cells and total epithelial keratinocytes from adult mouse backskins were described (Blanpain et al. 2004). Cell isolations were performed on a FACSVantage SE system equipped with FACS DiVa software (BD Biosciences). Cells were gated for single events and viability, then sorted according their expression of K14-actin GFP, α6-integrin, and CD34. Purity of sorted cells was determined by post-sort FACS analysis and by immunofluorescence analyses of cytospun FACS populations (Blanpain et al. 2004). Purity typically exceeded 95%. FACS analyses were performed either on FACSort or BD LSR (BD Biosciences). Cell cycle analyses were described (Blanpain et al. 2004). Additional details are in the Supplemental Material.

ChIP

In vivo ChIP was performed essentially as described (Chamorro et al. 2005), but using cells directly isolated from mice (see Supplemental Material). Lef1/Tcf sites identified by rVista analysis of 5′ upstream sequences were defined by the ECR Browser and Ensemble software. Lef1/Tcf sites were chosen for ChIP analysis based on the conservation and alignment between mouse and at least one other species, including human, canine, and rat, and clustering of sites when applicable. As a control, PCR was also performed using primers that recognize other sites within the same promoter or downstream portions of these same genes to demonstrate the specificity of the pull-down.

Cell culture and Lentiviral infections

Cell cultures were described (Blanpain et al. 2004). Cells were plated in E media (15% serum, 0.3 mm CaCl) and 3 d later, treated with Wnt3a (10 ng/mL; R&D Systems), SFRP (100 ng/mL; R&D Systems), or Biglycan (100 nM; Sigma) as indicated. Following removal of feeder cells, keratinocyte numbers were determined with a Coulter Counter. For gene expression upon Wnt3a treatment, cells were starved (0.15% FBS) during Wnt or LiCl application. Quantitative RT–PCR was performed and relative RNA amounts were expressed as a function of the housekeeping genes GAPDH and HPRT. Luciferase activity was measured with the dual luciferase system (Pierce) and TOPFLASH/FOPFLASH activity was normalized to *Renilla* activity. Lentiviral stocks were produced by cloning target genes into a cloning vector supplied by Invitrogen. These plasmids were cotransfected with three vectors expressing viral coat proteins into HEK293 cells for production of virus. Keratinocytes were infected for 4 h and then overnight with a 1:10 dilution of the indicated virus 5 d after plating on a feeder layer. Cells from triplicate wells were counted every 4 d.

Acknowledgments

We thank those who provided us with special Abs, cells, and reagents, whose gifts are cited in the text. A special thank you goes to Rolf Kemler (Freiberg University) for providing the β-catenin fl/fl conditional null mice; Rockefeller University core facility staff (Svetlana Mazel and Tamara Shengelia, FCRC Facility; Fred Quimby, LARC; Alison North, Bioimaging Facility; Agnes Viale, Juan Li, and Hui Zhao, Genomics Core Facility; Memorial Sloan Kettering Cancer Research Center); Fuchs' laboratory staff for technical assistance (Maria Nikolova and Lisa Polak); and Horace Rhee, Valentina Greco, and Michael Rendl for valuable discussions and advice. C.B. is the recipient of NATO, BAEF, and HFSPO sequential post-doctoral fellowships. W.E.L. is the recipient of an NIH-NIAMS post-doctoral fellowship (AR050340-02). J.N. is an M.D./Ph.D. student supported by the NIH Medical Scientist Training Program (grant GM07739), The Keck Foundation Medical Scientist Fellowship. G.G. is a recipient of an HFSPO fellowship. E.F. is an investigator of the Howard Hughes Medical Institute. This work was supported in part by grant R01 AR31737 from the NIH (E.F.).

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