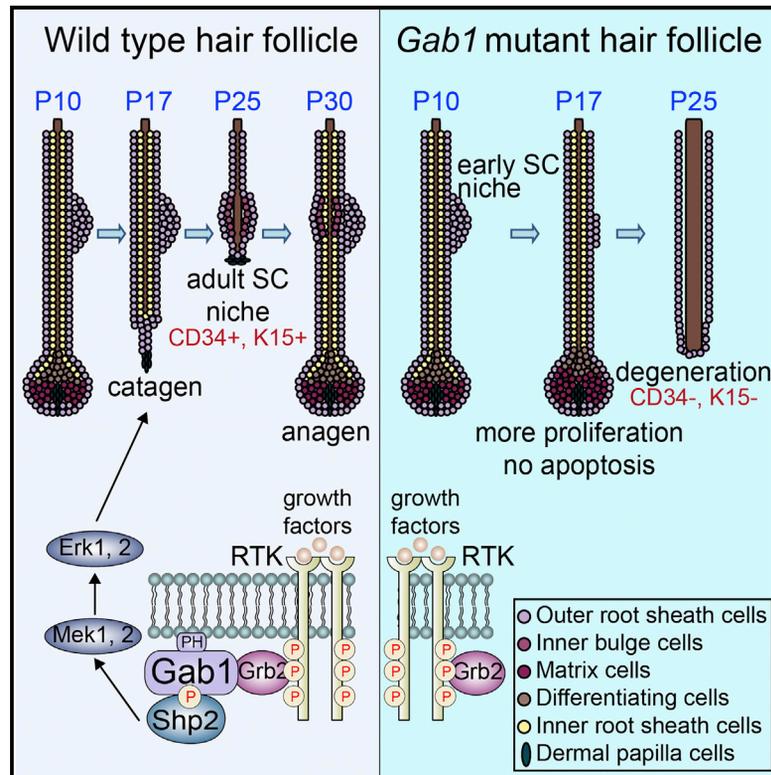


Gab1 and Mapk Signaling Are Essential in the Hair Cycle and Hair Follicle Stem Cell Quiescence

Graphical Abstract



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In Brief

Gab1 is a scaffold adaptor protein that acts in several intracellular signaling cascades. Using conditional *Gab1* mutant mice, Akilli Öztürk et al. show that Gab1 controls catagen. Deletion of Gab1 in hair follicles results in the loss of quiescence of early stem cells. Consequently, early stem cells become exhausted, leading to the absence of adult stem cell niches.

Highlights

- Scaffold adaptor Gab1 controls catagen, the destructive phase of the hair cycle
- Gab1 requires Grb2 and Shp2 binding sites and acts via Mapk signaling
- Early stem cells need Gab1 to maintain quiescence at catagen entry
- Gab1 is essential in the establishment of adult stem cells of the bulge

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Gab1 and Mapk Signaling Are Essential in the Hair Cycle and Hair Follicle Stem Cell Quiescence

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SUMMARY

Gab1 is a scaffold protein that acts downstream of receptor tyrosine kinases. Here, we produced conditional *Gab1* mutant mice (by *K14-* and *Krox20-cre*) and show that Gab1 mediates crucial signals in the control of both the hair cycle and the self-renewal of hair follicle stem cells. Remarkably, mutant hair follicles do not enter catagen, the destructive phase of the hair cycle. Instead, hair follicle stem cells lose quiescence and become exhausted, and thus no stem cell niches are established in the bulges. Moreover, conditional sustained activation of Mapk signaling by expression of a gain-of-function *Mek1^{DD}* allele (by *Krox20-cre*) rescues hair cycle deficits and restores quiescence of the stem cells. Our data thus demonstrate an essential role of Gab1 downstream of receptor tyrosine kinases and upstream of Shp2 and Mapk in the regulation of the hair cycle and the self-renewal of hair follicle stem cells.

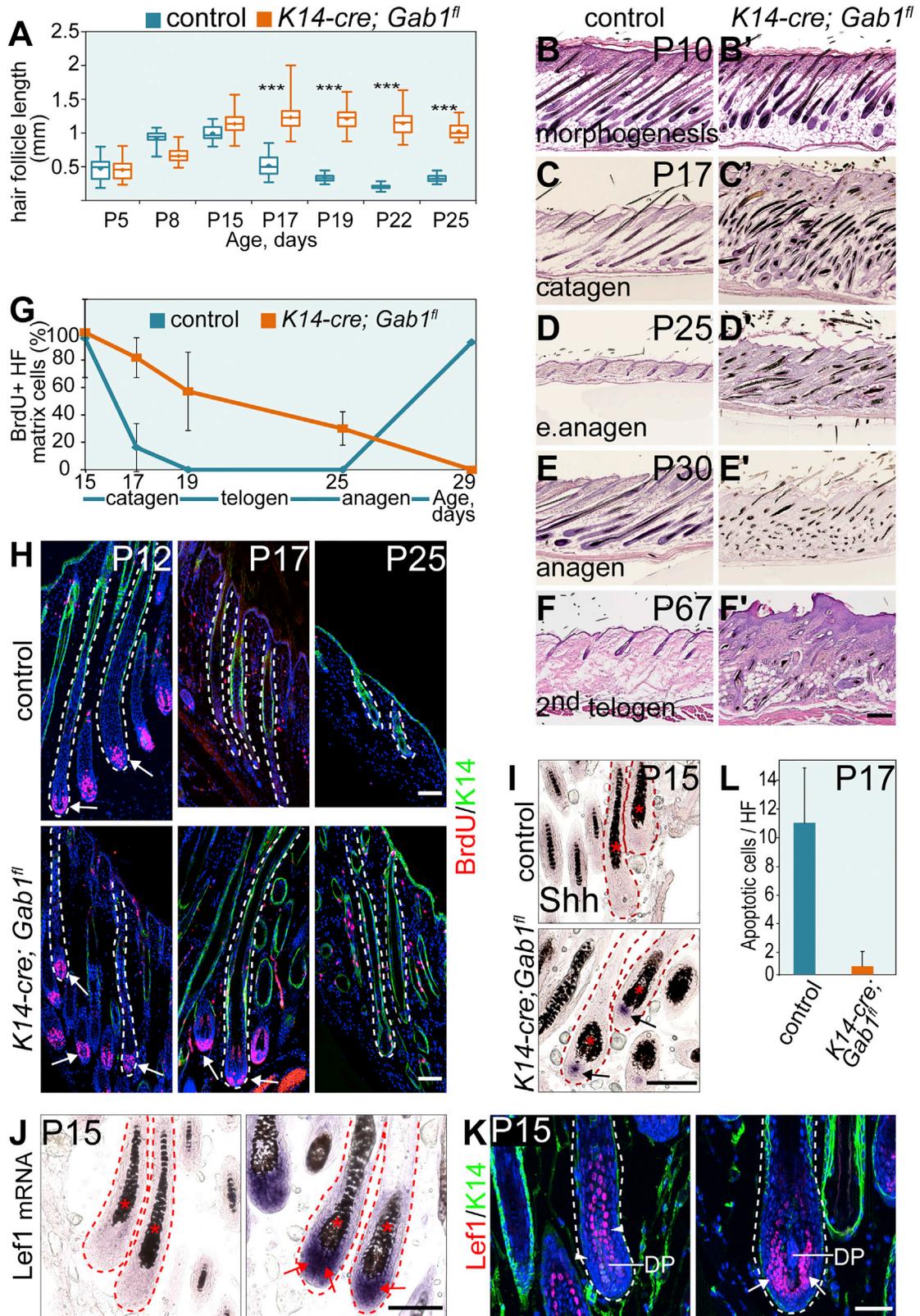
INTRODUCTION

Hair follicles are appendages of the skin, which are induced during late embryogenesis. In this process, epithelial cells of the forming hair follicles strongly proliferate and move deeply into the skin to form the hair bulbs. Matrix cells of the hair follicles then differentiate, move upward, and produce the concentric layers of the hair follicles. In mice, hair follicle morphogenesis is completed 2 weeks after birth, and then hair follicles begin to cycle (Müller-Röver et al., 2001). Catagen is the destructive phase of the hair cycle, during which the lower two-thirds of the follicles regress by apoptosis. In telogen, quiescent hair follicle stem cells accumulate in the bulges, the stem cell niches of the hair follicles. Finally, in anagen, stem cells are activated and new hair is produced (Greco et al., 2009).

Bromodeoxyuridine (BrdU) pulse-chase experiments demonstrated that quiescent stem cells, i.e., label-retaining cells, are located in the hair follicle bulges (Cotsarelis et al., 1990; Tumber

et al., 2004). Quiescence is believed to provide selective survival advantage, also under unfavorable conditions, and to protect stem cells from stress (Horsley et al., 2008; Kobiela et al., 2007). For this reason, it is important to delineate the molecular mechanisms that allow hair follicle stem cells to enter quiescence. The first hair follicle stem cells assemble during hair morphogenesis shortly after birth in so-called presumptive bulge regions and express hair follicle stem cell markers like Lhx2, Sox9, Lgr5, and Nfatc1 (Woo and Oro, 2011). Nfatc1 is required for entry of hair follicle stem cells into quiescence and for the maintenance of their undifferentiated state (Horsley et al., 2008). At the first telogen stage, the adult stem cell compartments are established in the bulges and express, in addition to the early stem cell markers, CD34 and K15 (Cotsarelis et al., 1990; Lyle et al., 1998; Morris et al., 2004; Trempus et al., 2003). Lineage-tracing experiments demonstrated that a subset of these stem cells are activated and migrate down into the hair germs to regenerate the hair follicles at anagen (Hsu et al., 2011; Morris et al., 2004; Zhang et al., 2009). It is known that in culture of normal hair follicle cells, only bulge stem cells produce so-called holoclones, which exhibit long-term self-renewal capacity over several passages (Blanpain et al., 2004).

The hair cycle represents an important biological “clock,” and its fascinating properties have been investigated for many years. Several signaling systems have been identified that participate in the control of the hair cycle. Wnt/ β -catenin and Bmp signals induce and inhibit anagen, respectively, in the hair follicle stem cells (Choi et al., 2013; Fuchs, 2007; Huelsken et al., 2001). Shh enhances proliferation of matrix cells during anagen, whereas Tgf β /Bmp signals at catagen induce apoptosis (Andl et al., 2004; Chiang et al., 1999; Ming Kwan et al., 2004; St-Jacques et al., 1998; Yang et al., 2005). Several studies report functions of receptor tyrosine kinases and their ligands in murine hair development and cycling (reviewed in Nakamura et al., 2013). Mice carrying spontaneous mutations in Tgf α and Egrf (*waved-1* and *waved-2*, respectively) and Fgf5 (*angora*) demonstrate the importance of Egf and Fgf signaling in hair formation (Hansen et al., 1997; Hébert et al., 1994; Luetke et al., 1994; Murillas et al., 1995). Different Fgfs act at different stages: Fgf7 and Fgf10 control proliferation at anagen, whereas Fgf18 prevents anagen entry and is essential to maintain quiescence of hair



(legend on next page)

follicle stem cells (Blanpain et al., 2004; Doma et al., 2013). It is thus known that receptor tyrosine kinases and their ligands play essential roles at different stages of hair morphogenesis and cycle, but the specific involvement in the catagen and the associated stem cell features could not be clearly resolved.

Grb2-associated binder1 (Gab1) is a member of an evolutionary conserved family of scaffolding proteins without enzymatic activity (reviewed by Birchmeier et al., 2003). Gab1 is recruited via Grb2 to activated receptor tyrosine kinases and directly to the receptor tyrosine kinase Met (Fixman et al., 1997; Holgado-Madruga et al., 1996; Weidner et al., 1996). Tyrosine-phosphorylation of Gab1 at various sites allows interaction to multiple downstream partners, e.g., the tyrosine phosphatase Shp2, the p85 regulatory subunit of PI3k, phospholipase C, or Crk (Fixman et al., 1997; Schaeper et al., 2000; Shi et al., 2000). Null mutations of *Gab1* in mice result in fetal lethality, and developmental changes in these mice phenocopy those observed in *Met*, *Pdgfr*, or *Egfr* mutants (Itoh et al., 2000; Sachs et al., 2000).

In the present work, we produced conditional *Gab1* mutant mice (by *K14-* and *Krox20-cre*) and show that Gab1 mediates crucial signals in the control of the hair cycle and quiescence of hair follicle stem cells. In *Gab1* mutants, hair follicles do not enter catagen, and the pool of quiescent adult hair follicle stem cells is not established. Instead, the mutant cells continue to proliferate and then become exhausted. Thus, Gab1 function is indispensable in hair follicle stem cells for self-renewal.

RESULTS

Mutation of *Gab1* in Epidermis and Hair Follicles of Mice

We examined the expression of Gab1 in mouse skin by using a *Gab1^{lacZ}* allele (Sachs et al., 2000). This is a null allele in which essential *Gab1* sequences were replaced by a *lacZ* cDNA containing a nuclear localization signal (Figure S1A); hence, the endogenous *Gab1* promoter drives *lacZ*. As judged by X-Gal staining, Gab1 was expressed in the spinous layer of interfollicular epidermis and outer root sheath cells of growing hair follicle (postnatal day 5 [P5]) (Figure S1B). No change in the Gab1 expression pattern was observed in the interfollicular epidermis throughout the stages analyzed (Figure S1B, arrows). Remarkably, expression in the hair follicles had changed during the course of the hair cycle. X-Gal staining was observed in the early hair follicle stem cells of the presumptive bulges at catagen entry (P15) (Figure S1B, p.Bu marks the presumptive bulge) and in the regressing epithelial strands at full catagen (P18). During telogen

(P20), X-Gal staining was observed in the inner bulge cells and in a subpopulation of hair germ cells (Figure S1B) (Hsu et al., 2011). In anagen (P30), Gab1 expression was observed in a subset of outer root sheath cells. Interestingly, in the second telogen, both old and new bulge cells express Gab1 (Figure S1B). Thus, Gab1 is expressed exactly in the hair follicle stem cells and progenitors, where the phenotype of conditional *Gab1* mutants occurs.

To determine Gab1 functions during hair follicle development, we introduced a conditional mutation of *Gab1* using *K14-cre* that expresses *cre* recombinase under control of the endogenous *keratin14* promoter (Huelsenken et al., 2001) and *Gab1^{loxP}* allele (Figure S1C) (*K14-cre; Gab1^{lacZ/loxP}*), hereafter called *K14-cre; Gab1^{fl}* or *coGab1* mutant mice (see Experimental Procedures). Southern blot analysis confirmed efficient recombination of *Gab1* (*Gab1^{rec}*) in the epidermis, but not in other tissues (Figure S1D). Until the end of morphogenesis (at P10, P12), skin and hair of *K14-cre; Gab1^{fl}* mice exhibited no overt phenotype (Figure S1E). BrdU labeling at P10 showed no change in proliferation in hair follicles and epidermis (Figure S1F, quantified in Figure S1G; data not shown), and immunohistological analysis of keratin10 (K10) and loricrin as well as transmission electron microscopy at P17 revealed normal epidermal differentiation (Figures S1H–S1J, quantified in Figure S1K).

Gab1 Controls Hair Cycle Entry

In normal development, hair follicles enter the first catagen, the destruction phase of the hair cycle, at P17 and then regress. Hair follicle regression was not observed in *coGab1* mutant mice (Figure 1A, orange; Figures 1C and 1C'). At early anagen (P25), hair follicles were small, and at full anagen (P30), hair follicles had regained their full size in control mice (Figures 1D and 1E). In the *coGab1* mutants, hair follicles had still not regressed but instead began to degenerate, which resulted in the appearance of naked hair shafts (Figures 1D', 1E', and S1L, arrows). Parakeratosis, characterized by the presence of nucleated cells in the keratinized outer epidermal layers, was observed at later stages, e.g., at P67 (Figures 1F, 1F', and S1L, arrowheads; data not shown; see also below). BrdU incorporation demonstrated terminated proliferation in control hair follicles between P17 and P25, but mutant hair follicles had highly proliferative matrix (Figure 1G, compare the orange and blue curves; Figure 1H, proliferating matrix cells marked by arrows). Proliferation in mutant hair follicles continued between P17 and P25, while control follicles resumed proliferation at P30, when they were in anagen (Figure 1G). Closer examination of matrix cells at P15

Figure 1. Deletion of Epithelial *Gab1* Causes Failure of First Catagen Entry

(A) Box and whisker plot quantification of hair follicle length at different ages. Maximum and minimum lengths are marked by whiskers, whereas upper and lower box boundaries mark quartile divisions with central lines indicating median. * indicates statistical significance. p values were calculated using the Student's t test. (B–F') H&E staining of control (B–F) and *K14-cre; Gab1^{fl}* (B'–F') mutant skin at the indicated time points. Scale bars, 100 μ m.

(G and H) 2hrs BrdU labeling at different stages of the hair cycle. The graph in (G) gives the percentage of hair follicles with BrdU+ hair follicle matrix at different time points. The phases of the hair cycle are indicated below. (H) BrdU staining in hair follicles at P12, P17, and P25. Arrows show BrdU+ cells. Scale bars, 50 μ m.

(I and J) In situ hybridization for *Shh* and *Lef1* at P15. Hair shafts are indicated with a red star. Arrows show positive staining. Counterstain: nuclear fast red. Scale bar, 50 μ m.

(K) *Lef1* immunostaining at P15. Arrows and arrowheads show *Lef1*+ cells in the matrix and cortex, respectively.

(L) Quantification of cleaved caspase-3+ apoptotic cells per hair follicle at P17.

Hair follicles are outlined with dotted lines. Data are the mean \pm SEM n = 3–5 mice per genotype. See also Figure S1.

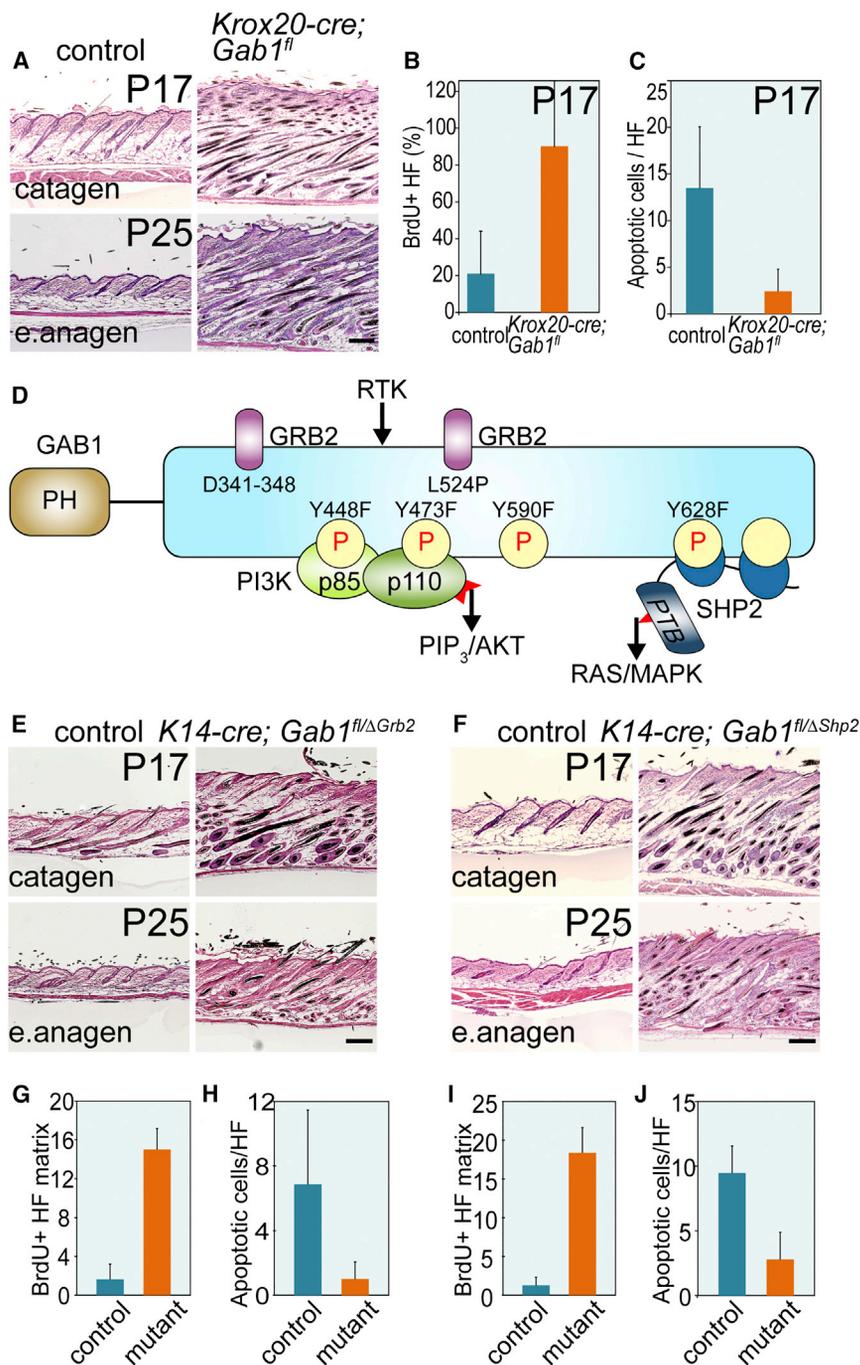


Figure 2. Gab1 Acts Specifically in the Hair Follicles and Regulates the Hair Cycle through Interaction with Grb2 and Shp2

(A) H&E staining of control and *Krox20-cre; Gab1^{fl}* mutant skin at P17 and P25.

(B and C) Quantification of BrdU+ hair follicles (B) and cleaved caspase-3+ cells (C) in control and *Krox20-cre; Gab1^{fl}* mice at P17.

(D) Schematic representation of the Gab1 protein with binding sites for Grb2 (2 sites), Pi3k (3 sites), and Shp2 (2 sites). Grb2-binding mediates the interaction to receptor tyrosine kinases. Binding of Pi3k activates Akt signaling, whereas Shp2-binding activates Ras/Mapk signaling. The Grb2-binding sites $\Delta 341-348$ and L524P sites are mutated in *Gab1^{ΔGrb2/ΔGrb2}*, the Pi3k binding sites Y448F, Y473F, and Y590F are mutated in *Gab1^{ΔPi3K/ΔPi3K}*, and Y628F of Gab1 is mutated in *Gab1^{ΔShp2/ΔShp2}*.

(E and F) H&E staining of control and *K14-cre; Gab1^{fl/ΔGrb2}* (E) and *K14-cre; Gab1^{fl/ΔShp2}* (F) mutant skin at P17 and P25.

(G–J) Quantification of BrdU+ hair follicle matrix and cleaved caspase-3+ cells of control and *K14-cre; Gab1^{fl/ΔGrb2}* (G and H) and *K14-cre; Gab1^{fl/ΔShp2}* mutant hair follicles at P17 (I and J). Scale bars, 100 μ m. Data are the mean \pm SEM; n = 3–5 mice per genotype. See also Figure S2.

showed more dispersed proliferating cells in mutants (Figure S1M, arrows). We also determined *Shh* expression, which provides proliferative signals, and expression of the Wnt effector and target gene *Lef1*, which regulates differentiation of matrix cells (DasGupta and Fuchs, 1999; St-Jacques et al., 1998; Zhou et al., 1995). *Shh* mRNAs was already downregulated at P15 in control hair follicles but persisted in the mutant (Figure 1I, arrows). Similarly, *Lef1* transcripts remained high in the mutant hair follicles (Figure 1J, arrows). *Lef1* protein was abundant in mutant matrix cells, leading to thicker hair shafts, but in the con-

trols, *Lef1* was detected only in differentiating cortex cells (Figure 1K, arrows and arrowheads, respectively). Moreover, cleaved caspase-3 staining showed that only few cells in the matrix of mutants entered apoptosis, whereas high numbers of apoptotic cells were observed in controls (Figures 1L and S1N, arrows). These data demonstrate that Gab1 is required in catagen entry and controls the block of proliferation and hence the induction of apoptosis that is associated with catagen. Gab1 function is also associated with changes in the expression of *Shh* and *Lef1* that control proliferation and differentiation signals, respectively.

We also used *Krox20 (Egr2)-cre* to conditionally delete Gab1 in hair follicles, but not in the interfollicular epidermis (*Krox20-cre; Gab1^{lacZ/loxP}*, hereafter called *Krox20-cre; Gab1^{fl}* mice). *Krox20-cre* indeed acts only in hair follicles, as shown by staining of *Krox20-cre; ROSA26R* skin sections (Figure S2A) (Gambardella et al., 2000). Catagen entry did not occur at P17 or P25 or at subsequent stages in *Krox20-cre; Gab1^{fl}* hair follicles (Figure 2A; data not shown), a phenotype indistinguishable to the one in *K14-cre; Gab1^{fl}* mice. Accordingly, hair follicles of *Krox20-cre; Gab1^{fl}* mice failed to halt proliferation and initiate apoptosis at P17 (Figures 2B, 2C, and S2B; BrdU+ and cleaved caspase-3+ cells marked by arrows). At much later stages, for instance

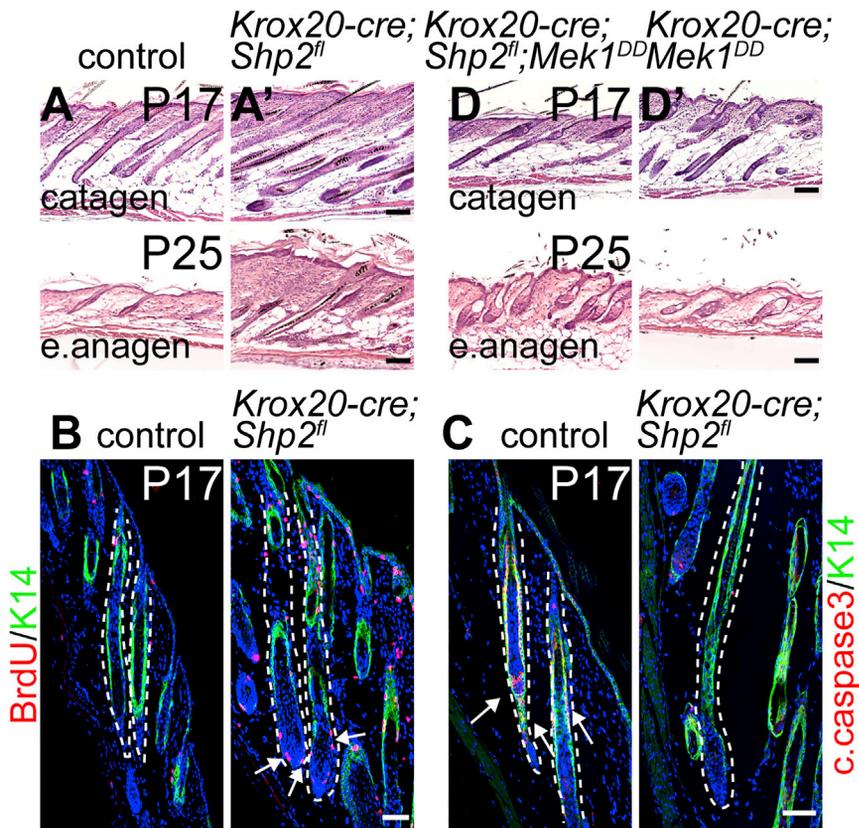


Figure 3. Rescue of Hair Cycle Alterations by Conditional Activation of Mek1/Mapk Signaling in *Krox20-cre; Shp2^{fl}* Mice

(A, A', D, and D') H&E staining of control (A), *Krox20-cre; Shp2^{fl}* (A'), *Krox20-cre; Shp2^{fl}; Mek1^{DD}* (D), and *Krox20-cre; Shp2^{fl}; Mek1^{DD}* (D') mutant skin at P17 and P25. Scale bars, 100 μ m. (B and C) BrdU after 2 hr of labeling and cleaved caspase-3 immunostaining of *Krox20-cre; Shp2^{fl}* mutant skin at P17. Arrows show BrdU+ (B) and cleaved caspase-3+ (C) cells. See also Figure S3.

at P67, we observed inflammation in the skin of both *K14-cre; Gab1^{fl}* and *Krox20-cre; Gab1^{fl}* mice, as assessed by keratin6 expression and hyperproliferation in the interfollicular epidermis (Figure S2C, compare upper and lower panels; Figure S2D, labeled by BrdU). The similarities of phenotypes of *K14-cre* and *Krox20-cre* mutations demonstrate that Gab1 functions specifically in the hair follicles to regulate catagen entry. Inflammation and hyperproliferation of the epidermis are secondary events, possibly caused by the pronounced deficits in the hair cycle.

Gab1-Dependent Recruitment of Grb2 and Shp2 Are Essential for Catagen Entry

Gab1 is recruited to receptor tyrosine kinases through upstream Grb2, and Pi3k and Shp2 are major signaling mediators that act downstream of Gab1 (Maroun et al., 2000; Schaeper et al., 2000). To address whether these interactions are essential to control the hair cycle, we analyzed three *Gab1* hypomorphic alleles, *Gab1 ^{Δ Grb2}*, *Gab1 ^{Δ PI3K}*, and *Gab1 ^{Δ Shp2}*, that encode Gab1 proteins unable to recruit Grb2, Pi3k, and Shp2, respectively (scheme in Figure 2D) (Schaeper et al., 2000). Homozygous *Gab1 ^{Δ PI3K}* mice are alive and did not show a block in the hair cycle at P17–P25 (Figure S2E; data not shown). Homozygous *Gab1 ^{Δ Grb2}* and *Gab1 ^{Δ Shp2}* mutants are embryonic lethal, and we therefore used *K14-cre; Gab1^{fl} Δ Grb2* and *K14-cre; Gab1^{fl} Δ Shp2* mice for hair cycle analysis. Remarkably, both conditional mutants showed hair cycle defects similar to *K14-cre; Gab1^{fl}* mice (Figures 2E–2J, S2F, and S2G, and data not shown).

Thus, Grb2 and Shp2 recruitments are important for Gab1 function during the hair cycle, while Pi3k binding is dispensable.

The Gain-of-Function *Mek1* Mutation Rescues the *Shp2* Mutation in the Hair Cycle

We also produced *Krox20-cre; Shp2^{fl}* mice using a *Shp2* floxed allele (Grossmann et al., 2009). Catagen entry did not occur in the *Shp2* mutant, and hair follicles remained in thick skin (Figures 3A, 3A', and S3A; data not shown) and continued to proliferate instead of entering apoptosis (Figures 3B and 3C,

arrows; quantified in Figure S3B), as had been observed in the conditional *Gab1* mutants. To further delineate the signaling cascade that acts downstream of Shp2 during catagen entry, compound mutants were produced using a conditional gain-of-function *Mek1* allele (*Mek1^{DD}*) in the *Shp2* background (Heuberger et al., 2014; Srinivasan et al., 2009) (*Krox20-cre; Shp2^{fl}; Mek1^{DD}*, also called compound mutant mice). Remarkably, the expression of activated Mek1 rescued the hair cycle deficits observed in the *Shp2* mutants, i.e., hair follicles successfully entered catagen at P17, telogen at P25, and anagen at P30 (Figures 3D and 3D'; Figure S3A, third picture). In particular, compound mutant hair follicles were no longer proliferative but contained many apoptotic cells at P17 (Figures S3B and S3C, arrowheads). In addition, compound mutants exhibited hyperplastic sebaceous glands, as did *Krox20-cre; Mek1^{DD}* mice, which also had normal hair cycle progression (Figures 3D, 3D', and S3A, right pictures). Sebaceous gland hyperplasia of *Mek1^{DD}* mice is similar to hyperplasia observed in *Kras* mutants (Lapouge et al., 2011; Page et al., 2013), further confirming elevated Ras/Mapk signaling in *Mek1^{DD}* mice. Together, these data show that Shp2 is required to function downstream of Gab1 to regulate the hair cycle and that it acts via Mapk signaling.

Gab1 Is Essential to Establish the Adult Stem Cell Compartment in Hair Follicles

Next, we assessed the cellular and molecular mechanisms of Gab1 function during the hair cycle and its effects on hair

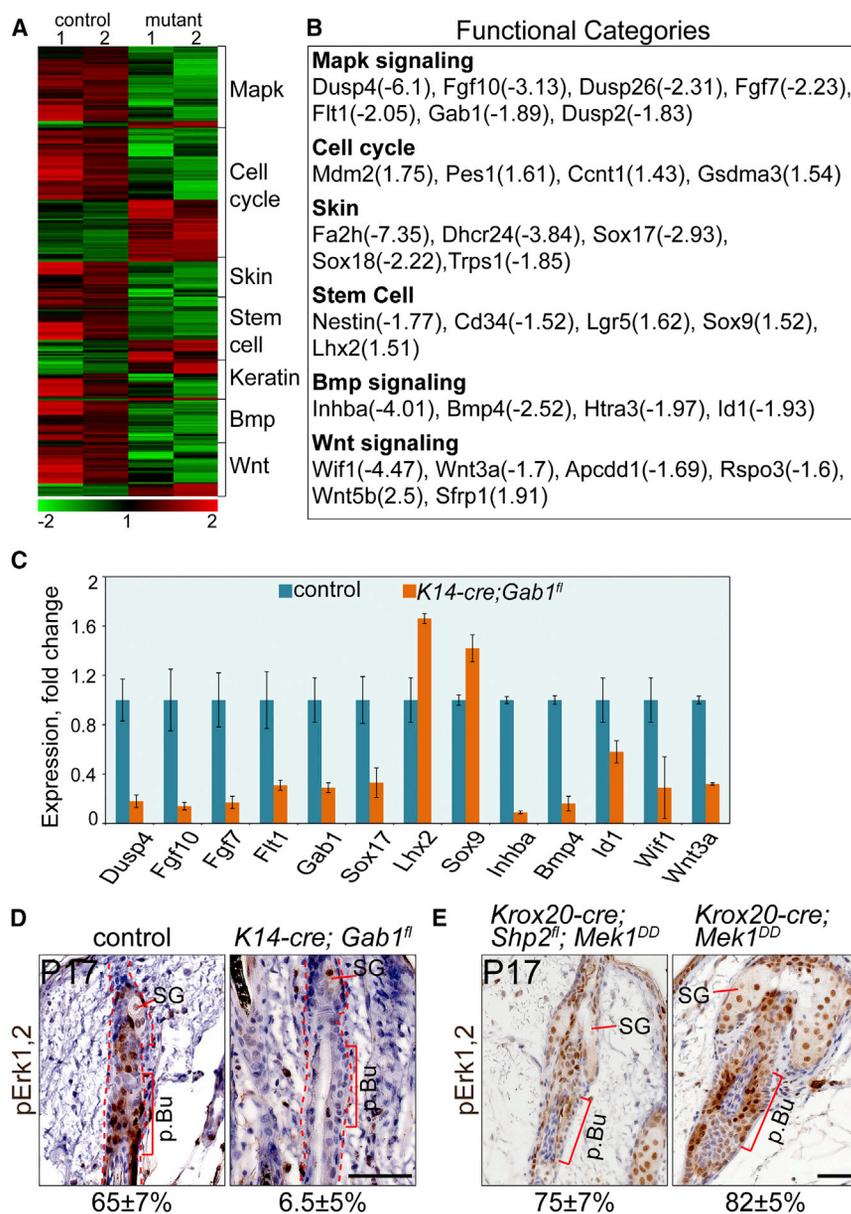


Figure 4. Gab1 Ablation in Hair Follicles Alters Gene Expression

(A) Heatmap of microarray from control and *K14-cre; Gab1^{fl/fl}* sorted P15 integrin- $\alpha 6^{\text{high}}$ hair follicle cells for selected gene categories.

(B) List of selected genes that belong to functional Gene Ontology categories.

(C) Confirmation of the expression of selected genes by qRT-PCR. The y axis represents the fold change of gene expression in mutants compared to control levels.

(D) pERK1,2 immunostaining of control and *K14-cre; Gab1^{fl/fl}* mutant skin at P17 shows absence in mutant presumptive bulges.

(E) pERK1,2 immunostaining of *Krox20-cre; Shp2^{fl/fl}; Mek1^{DD}* and *Krox20-cre; Mek1^{DD}* mutant skin at P17 shows restoration in the compound mutant.

Counterstain: hematoxylin. Hair follicles are outlined with dotted lines. p. Bu, presumptive bulge; SG, sebaceous gland. Scale bar, 50 μm . See also Figure S4 and Table S1.

follicle stem cells. Hair follicle stem cells are formed during postnatal development: they accumulate as early stem cells during hair morphogenesis in the so-called presumptive bulge region and as adult stem cells thereafter in the morphologically discernable bulges (Nowak et al., 2008; Woo and Oro, 2011). Fluorescence-activated cell sorting (FACS) of integrin- $\alpha 6^{\text{high}}$ cells (Zhang et al., 2011) was used to enrich skin epithelial cells in the morphogenesis-catagen transition period (P15), the earliest time point in which phenotypic changes in *coGab1* mutants could be visualized (Figure S4A; see also above). Microarray gene profiling was used to determine changes in gene expression of the sorted cells from control and *coGab1* mutants, which identified 900 differentially expressed genes (fold change > 1.5) that are displayed in a heatmap (Figure 4A; Table S1). Gene Ontology (GO) term analysis showed that many

genes encoding components of Mapk and canonical Wnt signaling were downregulated in the *coGab1* mutant cells (Figures 4B and 4C). We used immunohistochemistry to detect phosphoErk1,2, and we thus discovered that Mapk signaling was active at P15 to P19 in the presumptive bulge regions (located below the sebaceous glands [SGs] of control hair follicles but was conspicuously absent in the *coGab1* mutants (Figures 4D and S4B, marked p.Bu and by arrows). pErk1,2 staining was rescued in the presumptive bulges of compound *Krox20-cre; Shp2^{fl/fl}; Mek1^{DD}* and single *Krox20-cre; Mek1^{DD}* mutants (Figure 4E, marked p.Bu). Thus, our data demonstrate that a specific Erk1,2 activation occurs in the presumptive bulge regions at catagen, but this is prevented in the *coGab1* mutant hair follicles. Moreover, activation by *Mek1^{DD}* rescues the Mapk deficits of *Shp2* mutants.

Changes in the presumptive bulges of *coGab1* mutant hair follicles also affected the label-retaining stem cells that are known to require *Nfatc1* (Horsley et al., 2008). Remarkably, *Nfatc1* expression was downregulated in the *coGab1* mutant skin starting at P17, and was not restored subsequently (Figure 5A; compare orange with blue bars; Figure 5B, marked p.Bu; Figure S5A, below SG). *Lhx2* and *Sox9*, two markers of early hair follicle stem cells (Nowak et al., 2008; Rhee et al., 2006; Vidal et al., 2005), were expressed in the presumptive bulge cells in control and mutant (Figures 5C and S5B, marked p.Bu, located below the SGs [see merged fluorescence]). To ensure that *coGab1* mutants have established early stem cells, we analyzed label-retaining cells by repeated injection of BrdU between embryonic day

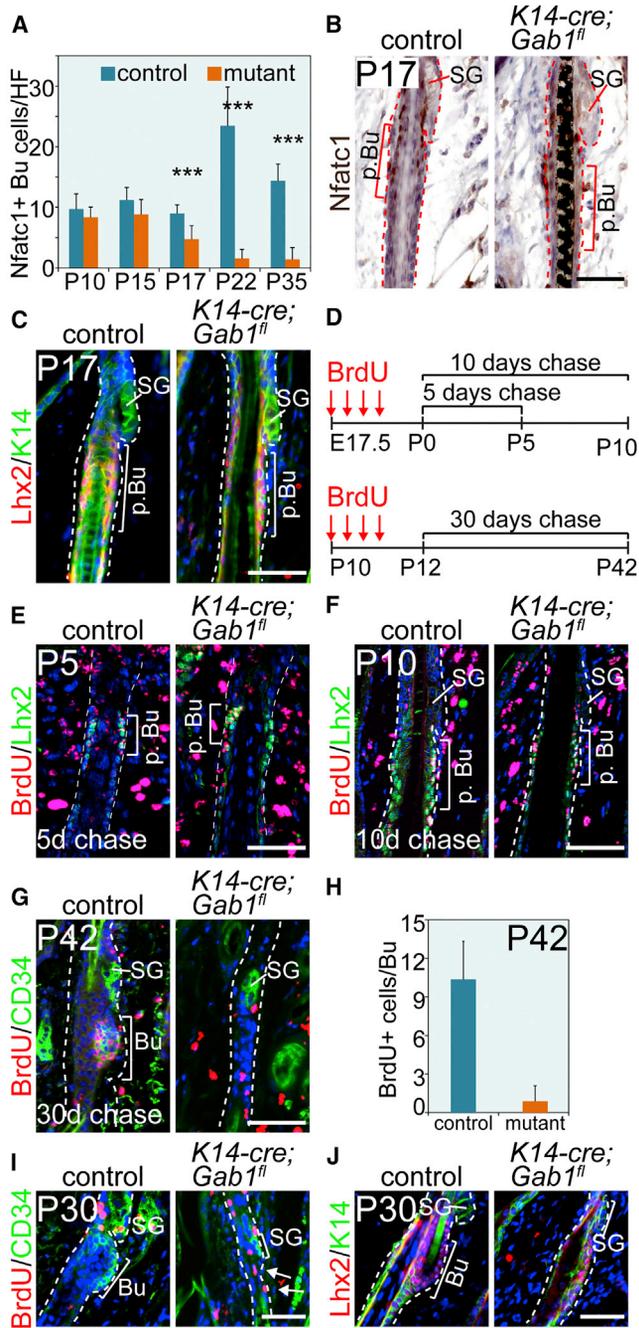


Figure 5. Maintenance of Quiescence of Hair Follicle Stem Cell Requires Gab1

(A) Quantification of Nfatc1+ cells per hair follicles at the indicated time points. Nfatc1+ cells in regressing epithelial strands are excluded from quantification. * indicates statistical significance. p values were calculated using Student's t test.

(B and C) Immunostaining for Nfatc1 (B) and Lhx2 (C) in the presumptive bulges at P17. Counterstain in (B): hematoxylin. Bu, bulge; p. Bu, presumptive bulge; SG, sebaceous gland. Scale bars, 50 μ m.

(D) Scheme of experimental design for BrdU pulse-chase experiments.

(E-G) Immunostaining after BrdU pulse-chase experiments at the indicated time points for progenitors and adult stem cell markers. Scale bars, 50 μ m.

17.5 (E17.5) and P0, followed by subsequent chases over 5 or 10 days (Figure 5D, upper scheme) (Cotsarelis et al., 1990). BrdU label-retaining cells were concentrated at the presumptive bulge regions at comparable levels both in control and mutant skin (Figures 5E and 5F, marked p.Bu, see merged fluorescence; Figure S5C). These data indicate that quiescence of early stem cells is correctly established in *coGab1* mutants during hair morphogenesis, as in controls, but that quiescence is then not maintained in mutants as reflected by the loss of Nfatc1 expression at catagen entry.

We also analyzed label-retaining adult stem cells in the bulges during the course of the hair cycle. BrdU was repeatedly injected between P10 and P12, followed by a 30-day chase (Figure 5D, lower scheme). In control hair follicles, BrdU+ label-retaining cells were detected that co-expressed CD34 (Trempus et al., 2003), a marker of adult hair follicle stem cells (Figure 5G, left, marked by Bu; quantified in Figure 5H; hair follicles were BrdU+ after 2 days of pulse, Figure S5D, quantified in Figure S5E), while mutant hair follicle cells located in the bulge area were neither label-retaining nor CD34-expressing (Figure 5G, right, below SG; quantified in Figure 5H). In order to test the proliferation status of stem cells, we used short BrdU labeling at P30 without chase. In control mice, CD34+ and K15+ bulge cells were not proliferating (Figures 5I, left, marked Bu; Figure S5F, arrows). In contrast, in the corresponding regions of the mutants, BrdU+ proliferating cells were observed that did not express the adult stem cell markers CD34 and K15 but did express the progenitor markers Lhx2 and Sox9 (Figures 5I and 5J, right; Figures S5F and S5G, arrows), indicating that mutant cells are progenitors. We conclude that in *coGab1* mutant hair follicles, label-retaining adult stem cells are not established. Instead, early stem cells in the mutants lose quiescence at catagen and become exhausted. Interestingly, the junctional zone stem cell compartment was not affected by loss of Gab1, as judged by the presence of Lrig1 expression at P30 (Figure S5H) (Page et al., 2013).

Shp2 Mutation Prevents Adult Stem Cell Formation in Hair Follicles, and This is Rescued by Activated Mapk

We asked whether *Shp2* mutants show a similar deficit of hair follicle stem cells, as observed in *coGab1* mutants. Indeed, Sox9+ hair follicle cells of *shp2* mutants proliferated aberrantly at P17, as shown by short BrdU labeling (Figure 6A, middle picture, merged fluorescence marked by arrows). Moreover, Nfatc1 expression was downregulated in the presumptive bulges of *Shp2* mutants (Figure 6B, middle picture). We then asked whether activation of Mapk by *Mek1^{DD}* could rescue the *Shp2* stem cell phenotype. Indeed, presumptive bulge stem cells in the compound mutant mice were quiescent (Figures 6A and 6B, right pictures). We also analyzed the adult stem cell compartment in *Shp2* and compound mutants. Adult stem cells were not

(H) Quantification of BrdU+ label-retaining cells per hair follicles of *K14-cre; Gab1^{fl/fl}* mice at P42.

(I and J) Immunostaining for BrdU and CD34 staining (I) and Lhx2 (J) after 2 hr of BrdU labeling. Scale bars, 50 μ m.

Hair follicles are outlined with dotted lines. Bu, bulge; p. Bu, presumptive bulge; SG, sebaceous gland. Data are the mean \pm SEM; n = 3 mice per genotype. See also Figure S5.

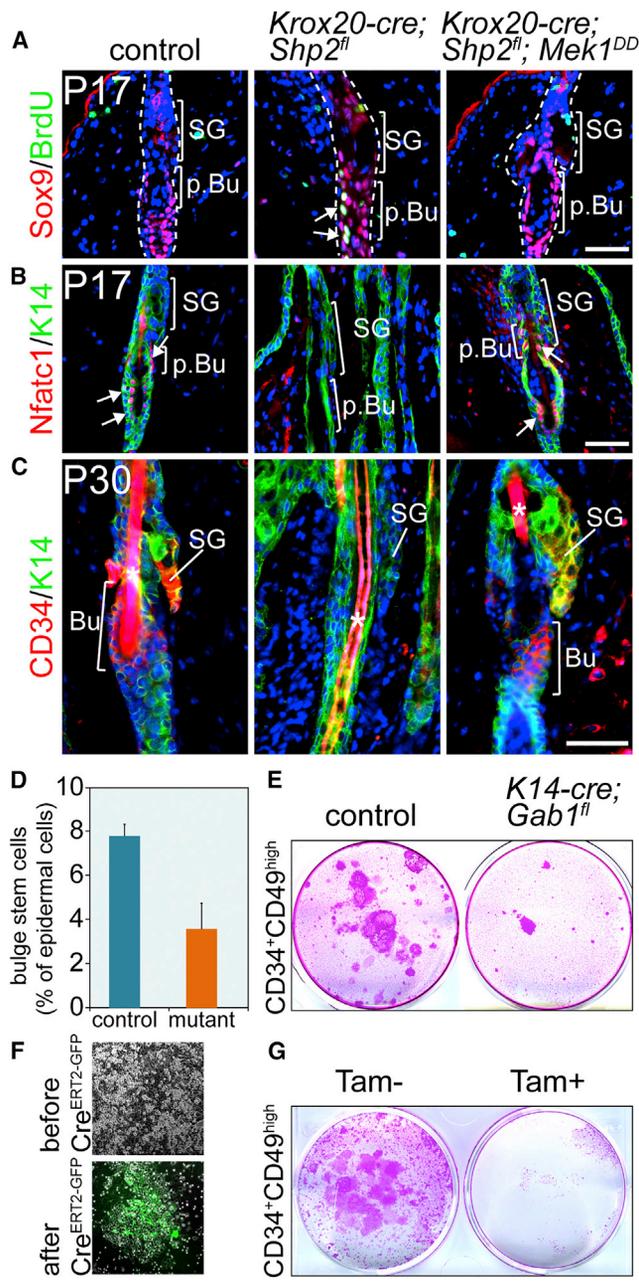


Figure 6. Stem Cell Functions of Gab1 Mutant Hair Follicle Are Diminished In Vitro
 (A–C) Sox9 and BrdU (A), Nfatc1 (B), and CD34 (C) immunostaining of *Krox20-cre; Shp2^{fl}* and *Krox20-cre; Shp2^{fl}; Mek1^{DD}* mutant skin after 2 hr of BrdU labeling. Arrows show BrdU and Sox9 double-positive cells (A) and Nfatc1+ cells (B). Hair follicles are outlined with dotted lines (A). Hair shafts are indicated with a red star (C). Bu, bulge; p.Bu, presumptive bulge; SG, sebaceous gland. Scale bars, 50 μ m.
 (D) Quantification of percentages of FACS bulge cells ($CD34^+$ integrin- $\alpha 6^{\text{high}}$) of five independent assays.
 (E) Representative photo of a colony-forming assay of primary epidermal keratinocytes isolated from control and *K14-cre; Gab1^{fl}* mice stained with Rhodamine B. n = 5 mice per genotype.
 (F) Representative images of FACS purified bulge cell colonies from *Gab1^{fl/null}* before and after retroviral transduction.
 (G) Representative photo of a colony-forming assay of primary epidermal keratinocytes isolated from *Gab1^{fl/null}* mice stained with Rhodamine B. n = 3 mice per genotype.
 See also Figure S6.

present in *Shp2* mutants, as assessed by the absence of $CD34^+$ cells in the bulges (Figure 6C, middle), but were present in the compound mutants (Figure 6C, right; data not shown). Thus, activation of Mapk signaling rescues the loss of *Shp2* and restores label-retaining properties of adult hair follicle stem cells.

Gab1 Mutant Stem Cells Cannot Form Holoclones in Culture

We used FACS to enrich for $CD34^+$ integrin- $\alpha 6^{\text{high}}$ cells (Blanpain et al., 2004), i.e., adult bulge stem cells (Figures S6A and S6B, marked by squares, quantified in Figure 6D), and cultured these cells on feeder layers of mitomycin-treated 3T3 cells. $CD34^+$ integrin- $\alpha 6^{\text{high}}$ cells from controls produced large and compact growing colonies (Figures 6E, left; quantified in Figure S6C), so-called holoclones (Barrandon and Green, 1987; Blanpain et al., 2004). Cells from *coGab1* mutant hair follicles produced typically only few and small colonies (Figures 6E, right; quantified in Figure S6C), and in some experiments, no colonies were formed. Moreover, in mutant colonies, single cells exhibited changed morphologies, i.e., failed to make close cell-cell contacts, but instead remained dispersed and were often very flat (Figure S6D, large cells are encircled). Furthermore, the few colonies formed by *coGab1* mutant cells could not be passaged (data not shown). We have also sorted $CD34^+$ integrin- $\alpha 6^{\text{high}}$ cells from *Gab1^{fl/null}* mice (Figure S6E) and then ablated *Gab1^{fl}* by retroviral Cre^{ERT2-GFP} in cell culture, as assessed by GFP+ expression (Figure 6F). After tamoxifen-induced Cre expression, cells died and no colonies were formed (Figure 6G). These data confirm that Gab1 is essential for the self-renewal of hair follicle stem cells.

DISCUSSION

We produced conditional mutant mice in which the adaptor protein *Gab1* was deleted in skin epithelia (by *K14-cre*) and specifically in hair follicles (by *Krox20-cre*). Remarkably, hair follicles in both *Gab1* mutants did not enter catagen. Loss of *Gab1* caused continuous proliferation in hair follicles, and hence catagen-associated apoptosis was not initiated. Instead, stem cells became exhausted, and thus no adult stem cell niches were established in the hair bulges. By analyzing different mouse lines, we identified the signaling cascade of *Gab1* in the hair cycle. *Gab1* recruits *Shp2*, which in turn activates *Mapk* to regulate catagen entry and quiescence of hair follicle stem cells. At much later stages in conditional *Gab1* mutants, we observed inflammation and hyperproliferation of the skin, which are secondary to the hair cycle and stem cell deficiencies. We thus discovered functions of *Gab1*, *Shp2*, and *Mapk* signaling in hair cycle control and hair follicle stem cells.

Dissecting the Gab1/Mapk Signaling Cascade during Catagen Entry

By taking advantage of our different *Gab1* knockin mouse lines, we found that *Gab1* needs to interact with upstream *Grb2* to

(G) Representative photo of a colony-forming assay of primary epidermal keratinocytes isolated from *Gab1^{fl/null}* mice stained with Rhodamine B. n = 3 mice per genotype.
 See also Figure S6.

exert its role in catagen entry. The question arises which ligands and receptors might act upstream of Gab1 and Grb2 to regulate catagen entry. *Fgf5* functions at late anagen to control catagen entry on time. *Angora* mice (a deletion mutation in the *Fgf5* gene) show excessively longer fur formation due to prolonged anagen (Hébert et al., 1994). Gab1 also acts as an adaptor downstream of Fgf receptors in other contexts (Eswarakumar et al., 2005). However, it should be noted that the phenotype of *Gab1* and *Fgf5* mutant mice are distinct: in *Gab1* mutants, the hair cycle is blocked, not only delayed, and hair shafts are not extended. Interestingly, the hair phenotypes of dominant-negative *Egf receptor* mutants in part resemble those observed in *Gab1* mutants (Murillas et al., 1995). Based on our analyses as well as others, neither the genes encoding the Egf receptor nor those encoding any of its ligands are differentially expressed during the hair cycle (Lien et al., 2011; Lin et al., 2004). Furthermore, the Egf receptor is essential at several stages of skin development (reviewed in Doma et al., 2013). Thus at catagen entry, transient changes might occur in the expression, production or compartmentalization of components of Egf signaling.

Here, we show that Gab1 needs to recruit the downstream tyrosine phosphatase Shp2 to mediate its effects in the hair cycle: a hypomorphic *Gab1* mutation that interferes with Shp2 recruitment as well as a conditional *Shp2* mutation produced similar phenotypes as *coGab1*. Direct activation of the Pi3k/Akt cascade by Gab1 was however dispensable for hair cycle control. In many cellular contexts, Shp2 is required for sustained activation of Ras-Mapk signaling (Chan et al., 2008; Grossmann et al., 2009; Heuberger et al., 2014; Sheean et al., 2014). Indeed, activating Mapk signaling by a gain-of-function mutation, *Mek1^{DD}*, sufficed to rescue the disrupted hair cycle in *Shp2* loss-of-function mutants. Our data thus demonstrate that Gab1 plays an essential role downstream of receptor tyrosine kinases and upstream of Shp2 and Mapk in its role in the hair follicles.

A previous report showed that high levels of Ras/Mapk activity can directly downregulate *Shh* in the hair follicles (Mukhopadhyay et al., 2013), and *Shh* controls proliferation of hair follicle cells during anagen (Gritti-Linde et al., 2007; St-Jacques et al., 1998). In line with these findings, our experiments showed that *Shh* continued to be expressed in the hair follicles of *coGab1* mutant mice when catagen entry occurred in normal postnatal development. It had been shown that *Shh* promoted proliferation of quiescent hair follicle stem cells by inducing *Gas1*, a co-receptor of *Ptch1* (Hsu et al., 2014). However, our expression profiling did not reveal a change in *Gas1* expression. The effect we observed in *Gab1* mutants should therefore be independent of *Gas1*. Tgf β /Bmp signaling is essential to regulate apoptosis in catagen (Andl et al., 2004; Foitzik et al., 2000; Ming Kwan et al., 2004; Yang et al., 2005). Although our array data indicate a decrease in the expression of *Inhba* and *Bmp4*, overall Tgf β /Bmp signaling was unchanged in *Gab1* mutants. We also show here that Gab1 is not required for the development of the interfollicular epidermis. It has been shown previously that in cell culture, Gab1 stimulates Ras/Mapk signaling in epidermal cells to promote cell proliferation and oppose differentiation (Cai et al., 2002). However, our in vivo BrdU-labeling and histological analyses of *coGab1* mutants showed no proliferation, differentiation,

and developmental defects in the interfollicular epidermis. Our data thus show that the role of Ras/Mapk signaling is independent of Gab1 in the interfollicular epidermis.

One might expect to see longer hair shafts in *Gab1* mutant mice, as in the case of *angora* mice. However, mutant hair shafts in *Gab1* mutants were stunted and thicker. The Lef1 expression pattern in mutant hair follicles in part explains this phenotype: Lef1 is expressed by hair shaft precursor cells to control their differentiation to hair shafts (DasGupta and Fuchs, 1999; Zhou et al., 1995). Interestingly, our data show that Lef1 expression is expanded to the matrix in mutants leading to thicker hair shafts. This altered differentiation might prevent longer hair shaft formation.

Gab1 and Mapk Signaling Controls Bulge Stem Cells

Adult hair follicle stem cells are formed first at \sim P20, express the markers CD34 and K15, and are quiescent (Cotsarelis et al., 1990; Lyle et al., 1998; Morris et al., 2004). We found that early hair follicle stem cells are correctly established in *coGab1* mutants during hair morphogenesis, as judged by the presence of label-retaining cells. However, quiescence is not maintained in mutants, as reflected by loss of *Nfatc1* expression at catagen entry. Interestingly, Gab1 is expressed in the regressing epithelial strand during catagen, where *Nfatc1* is also expressed (Horsley et al., 2008). The cells of the regressing epithelial strands are essential in the establishment of the quiescent adult stem cell niches, where Gab1 is also expressed at telogen (Hsu et al., 2011).

Remarkably, label-retaining adult stem cell compartments are not established in *coGab1* mutant skin. Instead, Sox9⁺ and Lhx2⁺ proliferating progenitors are found in the expected bulge regions. Thus, in *coGab1* mutant hair follicles, stem cells lose quiescence at catagen and finally become exhausted. It is known that in cultures of normal hair follicle cells, only bulge stem cells produce so-called holoclones, which exhibit long-term capacities for self-renewal over several passages (Blanpain and Fuchs, 2014). Cells from *coGab1* mutant hair follicles had lost their self-renewal properties and produced only a few colonies, which were small and could not be passaged. To explore the role of Gab1 after the establishment of adult stem cell compartments, we deleted Gab1 specifically in adult hair follicle stem cells in culture. We discovered that adult stem cells also lose their self-renewal properties upon inducible loss of Gab1. Thus, the stem cell phenotype we observed might be independent of the hair cycle defect. We also analyzed the adult stem cell compartments in *Shp2* and *Shp2; Mek1^{DD}* compound mutants. Indeed, adult stem cells were not present in *Shp2* mutants but were restored in the compound mutants. Overall, our data show that Gab1 and the associated Ras/Mapk signaling are essential components of bulge stem cells.

EXPERIMENTAL PROCEDURES

Preparation of Conditional Mutations in Mice

The conditional mutation of *Gab1* in the skin was generated by crossing mice carrying *K14-cre* first to *Gab1^{lacZ null/+}* mice, then these mice were crossed to *Gab1^{loxP/loxP}* mice to obtain *K14-cre; Gab1^{fl}* mice. All mice were obtained by crossbreeding of heterozygous males carrying the *cre* recombinases with the respective homozygous females. All animal experiments were conducted

according to regulations established by the Max Delbrück Center together with the Berlin authorities (Landesamt für Gesundheit und Soziales [LAGeSo]) and the European Union.

Histopathology and Immunostaining

Dorsal skin was dissected, fixed in 4% formaldehyde, and embedded in paraffin, from which 10- μ m sections were cut and stained with H&E or by immunostaining as described previously (Chmielowiec et al., 2007). For BrdU incorporation experiments, animals were injected once with 0.1 mg/g body weight BrdU (Sigma-Aldrich) in PBS, and skin samples were collected after 2 hr, fixed in 4% formaldehyde, and embedded in paraffin followed by immunofluorescence staining. To identify BrdU-label-retaining cells, pregnant mice and 10-day-old mice were injected four times over 2 days with 50 mg/kg body weight BrdU (Bickenbach et al., 1986) and sacrificed at the indicated time points.

β -Galactosidase staining was performed either on whole mounts or on cryosections. 10- μ m sections were cut, fixed in 2% glutaraldehyde, and stained in β -galactosidase solution at 37°C until suitable blue color was observed. After the staining, slides were washed in PBS, and counterstained with nuclear fast red. In situ hybridization was performed using digoxigenin-labeled RNA probes (Roche) on 10- μ m sections as described (Chmielowiec et al., 2007).

FACS and Colony-Formation Assays

Dorsal skin of mice in telogen (P21 or 8 weeks old) was used for isolation of bulge keratinocytes on a BD FACS Aria II cell sorter following established protocols (Sotiropoulou et al., 2010). For colony-formation assays, FACS bulge cells were seeded onto mitomycin-treated Swiss 3T3 cells at 1×10^4 cells per well in six-well plates, and cultures were maintained in E-media (15% fetal bovine serum, 0.3 mM CaCl₂) for 7–10 days. Cultures were gently treated with 0.05% trypsin-EDTA to remove feeders, fixed with 4% formaldehyde, and stained with 0.5% Rhodamine B (Sigma). The number and diameter of cell colonies in scanned images were measured using Adobe Photoshop. Virus infections were performed as in Lan et al. (2015). Supernatants were added in 10%–20% confluency, and GFP+ cells were resorted and cultured.

Microarray Hybridization, Data Analysis, and qRT-PCR

Total RNA was isolated from P15 dorsal skin cells after FACS using TRIzol (Invitrogen), purified by RNeasy kit (QIAGEN), biotin-labeled using Illumina Total-Prep RNA amplification Kit (Ambion), and hybridized to MouseRef-8 v2.0 Expression Beadchips (Illumina) as specified by the manufacturer.

ACCESSION NUMBERS

The accession number for the microarray data reported in this paper is GEO: GSE64402.

SUPPLEMENTAL INFORMATION

Supplemental information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.09.015>.

AUTHOR CONTRIBUTIONS

Ö.A.Ö., J.C., and W.B. designed research. Ö.A.Ö., H.P., J.C., and S.S. performed research. Y.S. and K.R. contributed mice. L.L. instructed the use of retroviral Cre^{ERT2-GFP}; Ö.A.Ö., J.Q., and W.B. analyzed data. Ö.A.Ö. and W.B. wrote the paper.

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