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The ciliopathy gene *Rpgrip1l* is essential for hair follicle development

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Abstract

The primary cilium is essential for skin morphogenesis through regulating the Notch, Wnt, and hedgehog signaling pathways. Prior studies on the functions of primary cilia in the skin were based on the investigations of genes that are essential for cilium formation. However, none of these ciliogenic genes has been linked to ciliopathy, a group of disorders caused by abnormal formation or function of cilia. To determine whether there is a genetic and molecular link between ciliopathies and skin morphogenesis, we investigated the role of *RPGRIP1L*, a gene mutated in Joubert (JBTS) and Meckel (MKS) syndromes, two severe forms of ciliopathy, in the context of skin development. We found that *RPGRIP1L* is essential for hair follicle morphogenesis. Specifically, disrupting the *Rpgril1* gene in mice resulted in reduced proliferation and differentiation of follicular keratinocytes, leading to hair follicle developmental defects. These defects were associated with significantly decreased primary cilium formation and attenuated hedgehog signaling. In contrast, we found that hair follicle induction and polarization and the

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CONFLICT OF INTEREST

None to declare.

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development of interfollicular epidermis were unaffected. This study indicates that *RPGRIP1L*, a ciliopathy gene, is essential for hair follicle morphogenesis likely through regulating primary cilia formation and the hedgehog signaling pathway.

Keywords

Rpgrip1l; ciliopathy; hair follicle; cilia; hedgehog signaling

INTRODUCTION

The primary cilium is an antenna-like subcellular structure protruding from the cell surface (Goetz and Anderson, 2010; Singla and Reiter, 2006). Primary cilia are found in keratinocytes and dermal fibroblasts (Bershteyn *et al*., 2010; Dai *et al*., 2011; Ezratty *et al*., 2011; Lehman *et al*., 2009). Blocking cilium formation through disrupting ciliogenic genes can cause epidermal and hair follicle morphogenesis defects due to attenuated Notch or Hh signaling pathways (Croyle *et al*., 2011; Dai *et al*., 2013; Dai *et al*., 2011; Ezratty *et al*., 2011; Lehman *et al*., 2009; Woo *et al*., 2012). However, prior studies in the skin were based on the investigation of genes, such *Ift88*, *Kif3a* and *Ift74*, which have not yet been linked to ciliopathy in humans. Therefore, it remains unclear whether genes causative for ciliopathies are also essential for normal skin development.

Retinitis pigmentosa GTPase regulator interacting protein-1 like (*RPGRIP1L*), also known as nephronophthisis 8 (*NPHP8*), KIAA1005, MKS5, JBTS7, and fantom (*Ftm*, in mouse), encodes a 151 kDa protein, containing five coiled-coil domains in the N-terminus, two protein kinase C conserved region 2 (C2) domains in the central region, and an RPGR-interacting domain (RID) in the C-terminus (Arts *et al*., 2007; Delous *et al*., 2007; Remans *et al*., 2014; Vierkotten *et al*., 2007). Autosomal recessive mutations in *RPGRIP1L* can cause Joubert (JBTS) and Meckel (MKS) syndromes, two of the most severe forms of ciliopathies (Arts *et al*., 2007; Delous *et al*., 2007). JBTS is characterized by cerebellar ataxia, developmental delay, hypotonia, and the “molar tooth sign” when cerebellar vermis hypoplasia and dysplasia with accompanying brainstem defects are visualized by axial magnetic resonance imaging (Doherty, 2009; Sattar and Gleeson, 2011; Valente *et al*., 2013). MKS is characterized by occipital encephalocele, polydactyly, cystic kidney, and hepatic fibrosis. Interestingly, no remarkable skin manifestations were reported in JBTS and MKS patients or fetuses.

Disrupting the mouse homolog (*Rpgrip1l* or *Ftm*) of *RPGRIP1L* results in ciliogenesis defects in a number of cell types, such as neuroepithelial cells, embryonic fibroblasts, and cardiac cells (Besse *et al*., 2011; Gerhardt *et al*., 2013; Mahuzier *et al*., 2012; Vierkotten *et al*., 2007). In accordance, mutant mice displayed polydactyly, laterality defects, kidney cysts, liver and heart defects, craniofacial malformation, and patterning defects in the brain and in the posterior neural tube (Besse *et al*., 2011; Delous *et al*., 2007; Gerhardt *et al*., 2013; Vierkotten *et al*., 2007). Some of these phenotypes were attributed to attenuated Hh signaling pathway. Since the development of hair follicles requires primary cilia and

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activation of the Hh signaling pathway, it is interesting to determine whether this ciliopathy gene is also required for hair follicle morphogenesis.

Beyond controlling ciliogenesis, *Rpgrip1l* is required for convergent extension and the establishment of planar cell polarity (PCP) in zebra fish and in the mouse cochlea (Khanna *et al*., 2009; Mahuzier *et al*., 2012). Whether *Rpgrip1l* also participates in the establishment of PCP in the skin is unknown. Prior studies demonstrated that PCP is essential for the establishment of the global patterning of the hair follicles such that disrupting core PCP genes could result in hair follicle polarization defects (Chen and Chuong, 2012). Therefore, whether *Rpgrip1l* could influence PCP in the skin, thereafter, the polarization of the hair follicles remains to be determined.

In this study, we investigated whether *Rpgrip1l* is involved in the development of the skin and hair follicle. We found that *Rpgrip1l* is essential for primary cilia formation, Hh signaling, and follicular keratinocyte differentiation during hair follicle morphogenesis. Interestingly, disrupting *Rpgrip1l* did not affect hair follicle induction and polarization or epidermal differentiation. This study establishes the role of *Rpgrip1l* in hair follicle development at the genetic and molecular levels, and suggests that other genes involved in the development of ciliopathies may also be essential for the morphogenesis of hair follicles.

RESULTS

Expression of *Rpgrip1l* in the skin and hair follicle

Expression profile of *Rpgrip1l* was examined in developing mouse skin. Quantitative RT-PCR revealed that *Rpgrip1l* mRNA is expressed throughout the embryonic development of the skin (Supplemental Fig. S1). *In situ* hybridization showed that *Rpgrip1l* is expressed ubiquitously in both epidermal and dermal compartments of the skin and immunofluorescence confirms the localization of the protein at the Ciliary base in both cell types (Supplemental Fig. S1), suggesting that this gene may be involved in the function of epidermal keratinocytes and dermal fibroblasts.

Rpgrip1l is essential for the proliferation and differentiation of follicular keratinocytes but not epidermal keratinocytes

Histologically, the dorsal epidermis of E15.5 *Rpgrip1l*$^{-/-}$ fetuses did not reveal any noticeable defect (Fig. 1a). Immunofluorescence labeling of p63 and keratin 1 (KRT1) did not reveal any remarkable difference between *Rpgrip1l*$^{-/-}$ and controls at E15.5 (Fig. 1a), suggesting that *Rpgrip1l* is not involved in the stratification and differentiation of the epidermis. At E18.5, the proliferation of interfollicular epidermal keratinocytes, as determined by bromodeoxyuridine (BrdU) and phospho-histone H3 (Supplemental Fig. S2a and b, respectively), the expression of early and late differentiation markers (KRT1 and loricrin (LOR)), and the activation of the Notch signaling pathway were also comparable between *Rpgrip1l*$^{-/-}$ and control skins (Fig. 1f and i).

The dorsal skins of E15.5 embryos of controls and *Rpgrip1l*$^{-/-}$ mutants contained comparable number of early hair follicles (stage 1 and stage 2) (3.44 ± 0.38 and 2.83 ± 0.86 per microscopic field, respectively), $p = 0.45$ (Fig. 1d), which were also positive for keratin

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17 (KRT17) (Supplemental Fig. S3). The expression of canonical Wnt target genes, such as *Lef1* and *Ctnnb1*, were also comparable between control and *Rpgrip1l*$^{-/-}$ mutants (Fig. 1a, g and h). Examination of dermal condensates of E15.5 embryos with nerve growth factor receptor (NGFR) revealed normal aggregation of hair follicle-inducing dermal mesenchymal cells (Fig. 1a). These observations suggested that the induction of hair follicle morphogenesis was unaffected in the *Rpgrip1l*$^{-/-}$ mutants.

At E18.5, a significantly reduced number of hair follicles was observed in the dorsal skin of *Rpgrip1l*$^{-/-}$ mutants. Specifically, the number of hair follicles, irrespective of developmental stages, were 15.57 ± 1.74 and 10.66 ± 0.77 per microscopic field for controls and *Rpgrip1l*$^{-/-}$ mutants, respectively, $p < 0.01$ (Fig. 1c and d). Furthermore, classification of the developmental stages of hair follicles (as described by Paus et al. (Paus *et al*., 1999)) revealed that the development of hair follicles in *Rpgrip1l*$^{-/-}$ skin was significantly delayed. Specifically, the percentage of stage 1−2, stage 3−4, and stage 5−6 hair follicles in the control skins were 15.8% ± 14.3%, 74.0% ± 10.8%, and 10.2% ± 5.9%, respectively, whereas the *Rpgrip1l*$^{-/-}$ skin contained a significantly increased proportion of stage 1−2 hair follicles (55.2% ± 17.9%, $p = 0.003$) and reduced proportions of stage 3−4 hair follicle (37.9% ± 15.8%, p=0.002) and stage 5−6 hair follicles (6.8% ± 2.5%, $p = 0.26$) (Fig. 1e). The proliferation of follicular keratinocytes contained in stage 2 and stage 3 hair follicles, as determined by BrdU and phospho-histone H3 stainings (Supplemental Fig. S2c and d, respectively), was also reduced. Interestingly, alkaline phosphatase (AP) staining of E18.5 embryonic skins demonstrated robust AP activity, albeit the number of AP-positive cell clusters was significantly reduced in *Rpgrip1l*$^{-/-}$ skin (Fig. 1b).

Disrupting *Rpgrip1l* in the skin causes hair follicle morphogenesis defects

To determine the function of *Rpgrip1l* in postnatal skin and the fate of the developmentally delayed hair follicles, we transplanted the dorsal skins of E18.5 *Rpgrip1l*$^{-/-}$ mutants onto nude mice. Four weeks after grafting, we observed that *Rpgrip1l*$^{-/-}$ skin was able to engraft as control skin, however, the *Rpgrip1l*$^{-/-}$ skin was almost completely devoid of visible hair shafts (Fig. 2a). Histological examination confirmed that the *Rpgrip1l*$^{-/-}$ skin contained almost no hair follicles (Fig. 2b). The hair follicle-like structures in the *Rpgrip1l*$^{-/-}$ transplants were highly disorganized, despite containing keratin 75 (KRT75) and AE13 (hair cortex cytokeratin) positive cells (Fig. 2c and d). TUNEL staining showed numerous apoptotic cells along the hair canal in wild-type skin transplants, whereas there were essentially no apoptotic cells in the hair follicle-like structure in *Rpgrip1l*$^{-/-}$ transplants (Supplemental Fig. S4). This result demonstrates that *Rpgrip1l* is not required for the postnatal maintenance of the epidermis. However, disrupting the *Rpgrip1l* gene during skin development could cause permanent growth arrest and, ultimately, the clearance of developing hair follicles.

To gain insight into the cause of the disappearance of developing hair follicles in *Rpgrip1l*$^{-/-}$ skin, proliferation of *ex vivo* cultured skin explants and skin transplants was examined. We found that hair follicles in *Rpgrip1l*$^{-/-}$ skins progressively lost proliferative hair follicle matrix keratinocytes (Supplemental Fig. S5). The loss of proliferating follicular

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keratinocytes might have resulted in the loss of hair follicles in post-natal skin, probably in response to attenuated mitogenic signals in the hair follicles (see below).

Formation of primary cilia was disrupted in *Rpgrip1l*$^{-/-}$ skin

Immunofluorescence labeling of primary cilia and basal body with ARL13B and γ-tubulin, respectively, revealed significantly reduced number of ciliated keratinocytes and fibroblasts in *Rpgrip1l*$^{-/-}$ skin (Fig. 3a and Supplemental Fig. S6). Cells that were able to form cilia contained severely truncated ciliary axonemes (Supplemental Fig. S6). This observation is consistent with cilia defects described in previous reports (Besse *et al*., 2011; Gerhardt *et al*., 2013; Vierkotten*et al*., 2007).

To further examine the ciliogenic potential of *Rpgrip1l*$^{-/-}$ cells, we collected dermal fibroblasts from E18.5 embryos and examined their ciliogenic capability *in vitro*. After 48-hour serum starvation, 31.80% ± 4.90% control fibroblasts were able to form cilia; whereas only 2.32% ± 1.07% *Rpgrip1l*$^{-/-}$ cells formed cilia ($p < 0.0001$) (Fig. 3b − d). These data demonstrated that *Rpgrip1l* is indispensable for primary cilia formation in dermal fibroblasts *in vitro*.

Hh signaling is attenuated in *Rpgrip1l*$^{-/-}$ skin

Because the primary cilium is essential for Hh signaling and the hair follicle phenotypes observed in *Rpgrip1l*$^{-/-}$ mutants were reminiscent of those in Hh mutants (Chiang *et al*., 1999; Gat *et al*., 1998; Mill *et al*., 2003; St-Jacques *et al*., 1998; Woo *et al*., 2012), we examined the Hh responsive genes in the *Rpgrip1l*$^{-/-}$ mutants. *In situ* hybridization demonstrated remarkably reduced levels of *Gli1* and *Ptch1* in E18.5 *Rpgrip1l*$^{-/-}$ skin (Fig. 4a and b), which was confirmed by quantitative RT-PCR ($p < 0.01$) (Fig. 4c). Furthermore, we found that, in the control skin graft, the hair follicle bulbs contained a large number of *Gli1* positive cells; whereas in the *Rpgrip1l*$^{-/-}$ skin transplants, we were only able to observed a few *Gli1*-positive cells scattered along the epidermis or clustered in disorganized epidermal invaginations under a thickened epidermis (Fig. 4d and Supplemental Fig. S7). These data demonstrated that Hh signaling was severely attenuated but not completely abolished in *Rpgrip1l*$^{-/-}$ skin and that the hair follicle phenotype of the *Rpgrip1l*$^{-/-}$ mutants was associated with attenuated Hh signaling.

To further determine the role of *Rpgrip1l* in the transduction of Hh signals, we treated primary dermal fibroblasts isolated from E18.5 *Rpgrip1l*$^{-/-}$ mutants with SAG, a potent agonist of smoothened (SMO). Quantitative RT-PCR showed that SAG was able to robustly induce the expression of Hh target genes (*Gli1* and *Gli2*) in control cells ($p < 0.0001$ and $p < 0.001$, respectively) but not in *Rpgrip1l*$^{-/-}$ cells (Fig. 4e). This experiment suggested that *Rpgrip1l* is essential for primary dermal fibroblasts to respond to Hh signals.

Polarization of hair follicles was unaffected in *Rpgrip1l*$^{-/-}$ skin

Since *Rpgrip1l* was implicated in the establishment of PCP, such as the polarization of cochlear hair cells in mice, (Mahuzier *et al*., 2012) and because PCP is essential for establishing the global polarization of hair follicles (Devenport and Fuchs, 2008; Guo *et al*., 2004; Tissir *et al*., 2010; Wang *et al*., 2010), it is of interest to determine whether *Rpgrip1l*

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is also involved in polarizing the hair follicles along major body axes of *Rpgrip1l*$^{-/-}$ mice. First, we observed that hair germs in E15.5 embryos were consistently polarized, which is especially evidenced by the asymmetric localization of dermal condensate cells (Fig. 1a). Then, we determined the acute angles formed between the hair follicle projection and the planar plane (surface) along the midline of the dorsal skin of E18.5 embryos (Fig. 5 and Supplemental Fig. S8). The angles formed in wild-type and *Rpgrip1l*$^{-/-}$ skins were essentially indistinguishable at 60.74° ± 1.42° (n = 137) and 52.10° ± 1.88° (n = 79), respectively (Fig. 5a), suggesting a normal polarization of the hair follicles in *Rpgrip1l*$^{-/-}$ mutants. To further determine whether mutant hair follicles were also molecularly compartmentalized, the distribution of a polarity marker, E-cadherin, in stage 2 hair follicles was examined in control and *Rpgrip1l*$^{-/-}$ hair follicles. We found that E-cadherin was asymmetrically distributed to cells that were localized to the posterior side of control and *Rpgrip1l*$^{-/-}$ hair follicles (Fig. 5b). Finally, immunofluorescence labeling of VANGL1 revealed normal distribution of this core PCP protein at the lateral membrane of basal keratinocytes in *Rpgrip1l*$^{-/-}$ skin (Fig. 5c).

DISCUSSION

Here, we show that *Rpgrip1l* is essential for primary cilia formation, Hh signaling, and hair follicle keratinocyte differentiation during hair follicle morphogenesis, but is dispensable for hair follicle induction and polarization and for epidermal differentiation. This study suggests that genes involved in the development of ciliopathies may also be essential for the morphogenesis of hair follicles.

Ciliopathies represent a number of clinically and genetically heterogeneous syndromes caused by dysfunctions of primary or motile cilia (Badano *et al*., 2006; Hildebrandt *et al*., 2011; Tobin and Beales, 2009). Clinical manifestations of ciliopathies are heterogeneous due to the diverse morphogenetic functions of cilia during the development of multiple tissues (Eggenschwiler and Anderson, 2007). Dermatological manifestations are generally unremarkable and only described in a subset of ciliopathies, notably skin laxity and sparse hair in cranioectodermal dysplasia (CED, also known as Sensenbrenner syndrome) (Arts and Knoers, 1993; Lin *et al*., 2013; Walczak-Sztulpa *et al*., 2010) and Ellis–van Creveld (EvC) syndrome (also known as chondroectodermal dysplasia) (Atasu and Biren, 2000; Baujat and Le Merrer, 2007). The lack of documentation of skin phenotypes in most ciliopathy patients may be due to the fact that there is no remarkable dermatological manifestation associated with ciliopathies. It is also possible that skin phenotypes are dwarfed by more severe clinical conditions, such as cystic kidney, cardiac anomalies, and developmental delay, or that skin phenotypes are frequently associated with lethal developmental defects. Since the current study provides evidence that a ciliopathy gene, *Rpgrip1l*, is essential for the normal development of hair follicles, together with the recently established role of primary cilia in skin and hair follicle development, this suggests that skin phenotypes may be more prevalent in ciliopathy patients than currently reported. However, because hair follicle morphogenesis requires complex neuroectodermal/mesodermal interactions, which is also under the influence of many temporally and spatially regulated genetic and environmental factors, it is also possible that genetic make-ups and species differences are confounding factors underlying variations in phenotypic outcome or penetrance. Genetic backgrounds of the

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Rpgrip1l$^{-/-}$ mice might affect the phenotypic presentations of this mutant allele, as previously shown for TMEM67 mutant ((Abdelhamed *et al*., 2013) and CL personal communication). Also, as human hair follicle morphogenesis is completed already *in utero*, subtle phenotypes in the skin of ciliopathy patients may get lost during the substantial post-natal remodeling that human skin undergoes.

Ciliopathies are caused by mutations in genes encoding the cilium or basal body/centrosome-related proteins. The mechanisms of genotype-phenotype correlation of ciliopathy remains perplexing, which are likely under the influence of heterogeneity of the genetic locus, multiple allelism (mutational load), modifier genes (effect), and true oligogenicity (oligo-genetic inheritance) (Hildebrandt *et al*., 2011). Ciliopathies caused by *RPGRIP1L* mutations are often associated with more severe or even lethal cases of ciliopathy, such as for JBTS and MKS, in which ciliary functions are more severely impaired. Because strong cilia and hair follicle phenotypes were observed in the *Rpgrip1l*$^{-/-}$ mice, we speculate that hair follicle or skin abnormalities may be more prominent in severe or lethal cases of ciliopathies. However, it is worth noting that phenotypes seen in JBTS and MKS patients are caused by point mutations in the *RPGRIP1L* gene; whereas the phenotypes in *Rpgrip1l*$^{-/-}$ mice resulted from targeted gene disruption. Although some patient mutations could theoretically result in truncation of most of the functional domains of the RPGRIP1L protein (Arts *et al*., 2007; Delous *et al*., 2007), variations in phenotypic severity warrant further investigation of the impact of a particular mutation on the functional domains of RPGRIP1L. A number of recent studies have shed light on protein-protein interactions mediated by specific domains of RPGRIP1L (Arts *et al*., 2007; Delous *et al*., 2007; Remans *et al*., 2014; Sang *et al*., 2011).

CED and EvC are the only two ciliopathies that demonstrate hair phenotypes, namely, fine, sparse and slow-growing hair. Mutant mouse models of the CED genes (*Ift121*, *Ift122* and *Ift144*) were unable to develop beyond midgestation for further analysis (Ashe *et al*., 2012; Cortellino *et al*., 2009; Mill *et al*., 2011); mutant mouse models of the EvC genes (*Evc1* and *Evc2*) display perinatal lethality (Caparros-Martin *et al*., 2013; Ruiz-Perez *et al*., 2007), however, the skin remained uncharacterized. Overlapping phenotypes observed in the *Rpgrip1l* mutants and CED or EvC mutants, such as bone and cardiovascular defects, and disrupted Hh signaling suggest that CED and EvC genes may control hair follicle formation in a similar fashion to *Rpgrip1l*. Whether the hair phenotypes in CED and EvC patients are caused by impaired cilia formation or function and, consequently, attenuated Hh signaling, remains to be determined.

Birt–Hogg–Dube (BHD) syndrome is a recently characterized ciliopathy, caused by autosomal dominant mutations in the folliculin (FLCN) gene (Luijten *et al*., 2013). BDH patients are frequently affected by fibrofolliculomas, benign tumors of hair follicles (Menko *et al*., 2009). The formation of fibrofolliculoma in BHD patients are associated with increased activation of the canonical Wnt signaling pathway (Luijten *et al*., 2013), whereby represents another type of aberrant signaling due to ciliary dysfunction. It will be interesting to determine whether the Hh signaling pathway is also involved in the development of the hair follicle phenotypes in BHD patients.

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PCP is essential for global polarization of hair follicles. Interruption of PCP in the skin through the disrupting core PCP genes, such as *Fzd6*, *Vangl2*, and *Celr1*, could result in disrupted polarization of the hair follicles and altered asymmetric localization of molecules within the hair follicles. In the current study, we found no morphogenetic evidence of disrupted hair follicle polarization. This observation is reminiscent of findings in the *Ift88* mutant, in which apparent PCP defects was observed in the cochlea (Jones *et al*., 2008), however without apparent hair follicle polarization defects (Croyle *et al*., 2011; Ezratty *et al*., 2011; Lehman *et al*., 2009). These findings suggest that these ciliary gene may function downstream of core PCP genes or that disrupting them in the skin is not sufficient to cause hair follicle polarization defects.

In summary, the current study establishes the genetic and molecular basis of hair phenotypes in association with a ciliopathy gene, *RPGRIP1L*. Further characterization of the ever growing list of ciliopathy genes, which are essential for the development and function of many tissues and organs, will advance our understanding of their functions in skin development and homeostasis.

MATERIALS AND METHODS

Rpgrip1l mouse model

Genetic modification and PCR genotyping of the *Rpgrip1l* locus was described previously in the *Ftm* mouse (Besse *et al*., 2011; Gerhardt *et al*., 2013; Vierkotten *et al*., 2007). *Rpgrip1l*$^{-/-}$ homozygous mutants were obtained by crossing *Rpgrip1l*$^{+/-}$ heterozygous. E15.5 and E18.5 fetuses were obtained by timed-mating. All procedures related to mice were conducted according to relevant European rules and approved by a French ethic committee and the Institutional Animal Care and Use Committee of Stony Brook University.

BrdU labeling and tissue processing

BrdU labeling was performed by injecting BrdU labeling reagent at 10 μg per gram body weight intraperitoneally two hours prior to euthanization. Skin specimens were obtained by removing the full thickness dorsal skin along the midline and processed for routine histology analysis. For all analyses, a minimum of three embryos obtained from at least two different litters were examined.

Alkaline phosphatase staining

Alkaline phosphatase (AP) staining was performed as previously described (Tsai *et al*., 2010). Specifically, fresh skin was fixed in 4% paraformaldehyde for 10 minutes, soaked in B3 buffer (0.1 M Tris, pH 9.5, 0.1 M NaCl, and 0.05 M $MgCl_2$) for 10 minutes, and NBT/BCIP solution (1:200 of Nitro Blue tetrazolium and 1:267 of 5-bromo-4-chloro-3-indolyl phosphate, Roche, Indianapolis, IN) for 20 minutes. Stained skins were imaged on a Zeiss Stemi 2000-C dissecting microscope fitted with an Infinity 2 camera.

Cell culture and *in vitro* assays

The isolation of primary skin keratinocyte and dermal fibroblast was conducted as described elsewhere (Dai *et al*., 2011). Briefly, skins of E18.5 fetuses were digested by dispase II

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(Roche) to separate epidermis and dermis. Dermis was then digested with collagenase to dissociate dermal fibroblasts. Fibroblasts were cultured in Dulbecco's modified Eagle's medium (4.5 g/l glucose) supplemented with 10% fetal bovine serum and antibiotics. To examine ciliogenesis, cells were grown to confluency and serum-starved for 48 hours before being fixed in cold methanol. To examine Hh responsiveness, fibroblasts were grown as described above, serum-starved for 24 hours, treated with 100 nM SAG (Calbiochem, Danvers, MA) for 24 hours, before lysed in RLT buffer (Qiagen, Valencia, CA) for RNA extraction.

Quantitative RT-PCR

RNA was isolated with RNeasy kit (Qiagen) and quantitative RT-PCR analyses were performed as described previously (Dai *et al*., 2013). The following probes were used for TaqMan analysis: *Lef1*, Mm00550265_m1; *Ctnnb1*, Mm00483039_m1; *Hes1*, Mm01342805_m1; *Gli1*, Mm00494645_m1; *Gli2*, Mm01293111_m1; and *β-actin*, Mm00607939_m1 (Life Technologies, Grand Island, NY). Results were analyzed using ΔΔCt method. Relative expression levels of target genes were determined by comparing with wild type or treatment controls after normalizing with β-actin.

Immunofluorescence labeling and microscopy

Immunofluorescence and TUNEL staining were performed as described previously (Dai *et al*., 2013). The following primary antibodies were used: RPGRIP1L, 1:100 (Origene, Rockville, MD); p63, 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA); KRT14, 1:1,000 (Covance, Princeton, NJ); KRT1, 1:500 (Roop *et al*., 1987); KRT17, 1:1,000 (Abcam, Cambridge, MA); LOR, 1:100 (Covance); NGFR, 1:100 (Promega); LEF1, 1:100 (Cell Signaling, Danvers, MA); BrdU, 1:50 (Life Technologies); acetylated α-tubulin, 1:1,000 (Sigma, St. Louis, MO); γ-tubulin, 1:1,000 (Abcam); ARL13B, 1:1,000 (#73-287, NeuroMab, Davis, CA); phospho-histone H3, 1:100 (Cell Signaling). AlexaFluor-conjugated secondary antibodies (1:250) were from Life Technologies. Sections were sealed in mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Images were acquired by Nikon 80*i* fitted with Nikon DS-Qi1Mc camera and processed with Photoshop 5.5 CS.

In situ hybridization

In situ hybridization was carried out on formalin-fixed paraffin embedded tissue sections using the RNAScope system (Advanced Cell Diagnostic, Hayward, CA) per manufacturer's instructions and as previously described (Wang *et al*., 2012).

Skin transplantation and explant culture

Full-thickness skin transplantation was performed as previously described (Dai *et al*., 2011). Briefly, dorsal skins of E18.5 littermates were dissected and seeded in silicon grafting chambers placed on the back of 8 – 12 week old nude mice (*Foxn1*$^{-/-}$). Graft chambers were removed 10 days later. Skin grafts were harvested two weeks thereafter. All experiments were performed at least three times. Skin explants were cultured as previously described (Zhang *et al*., 2009). Specifically, dorsal skins were harvested at E18.5 embryos and

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cultured at the air-liquid interface in Corning Transwell plates containing DMEM and 10% FBS. These *ex vivo* cultured skin explants were then fixed in formalin and processed for routine histology analysis.

Statistical analyses

All quantifications are presented as means ± S.D. Student t-test was used for statistical analysis. $P < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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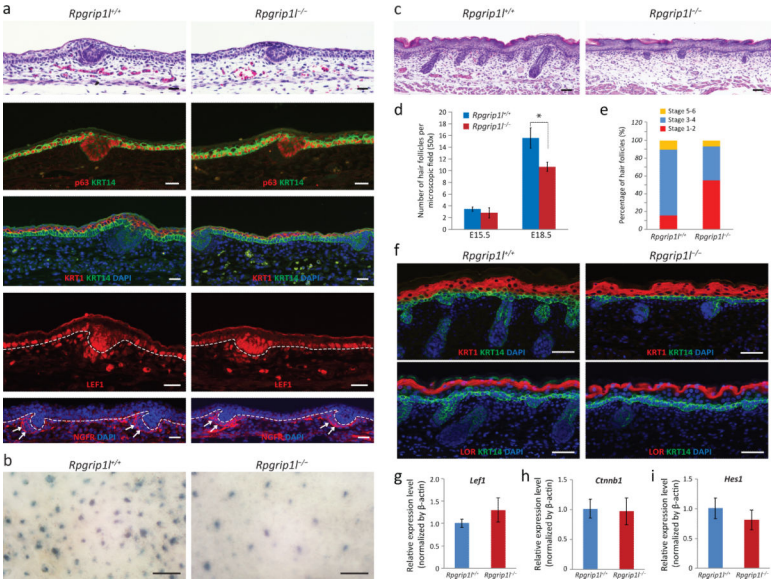

Fig. 1.

Rpgrip1l in skin morphogenesis. (a) H&E staining and immunofluorescence labeling of p63 (red), KRT1 (red), KRT14 (green), LEF1 (red) and NGFR (red) in dorsal skins of E15.5 controls (*Rpgrip1l*$^{+/+}$) and homozygous mutants (*Rpgrip1l*$^{-/-}$). (b−c) Alkaline phosphatase and H&E stainings of E18.5 skins. (d) Quantification of hair follicles in the dorsal skin of E15.5 and E18.5 fetuses, n ≥ 4. *, P < 0.01. (e) Distribution of developmental stages of hair follicles in dorsal skin of E18.5 fetuses. n ≥ 6. (f) Immunofluorescence labeling of KRT1 (red), LOR (red), and KRT14 (green) in E18.5 skins. Nuclei were stained with DAPI (blue). (g−i) QRT-PCR of *Lef1*, *Ctnnb1*, and *Hes1* in E18.5 skins. Scale bars = 15 μm in (a); 250 μm in (b); 50 μm in (c and f).

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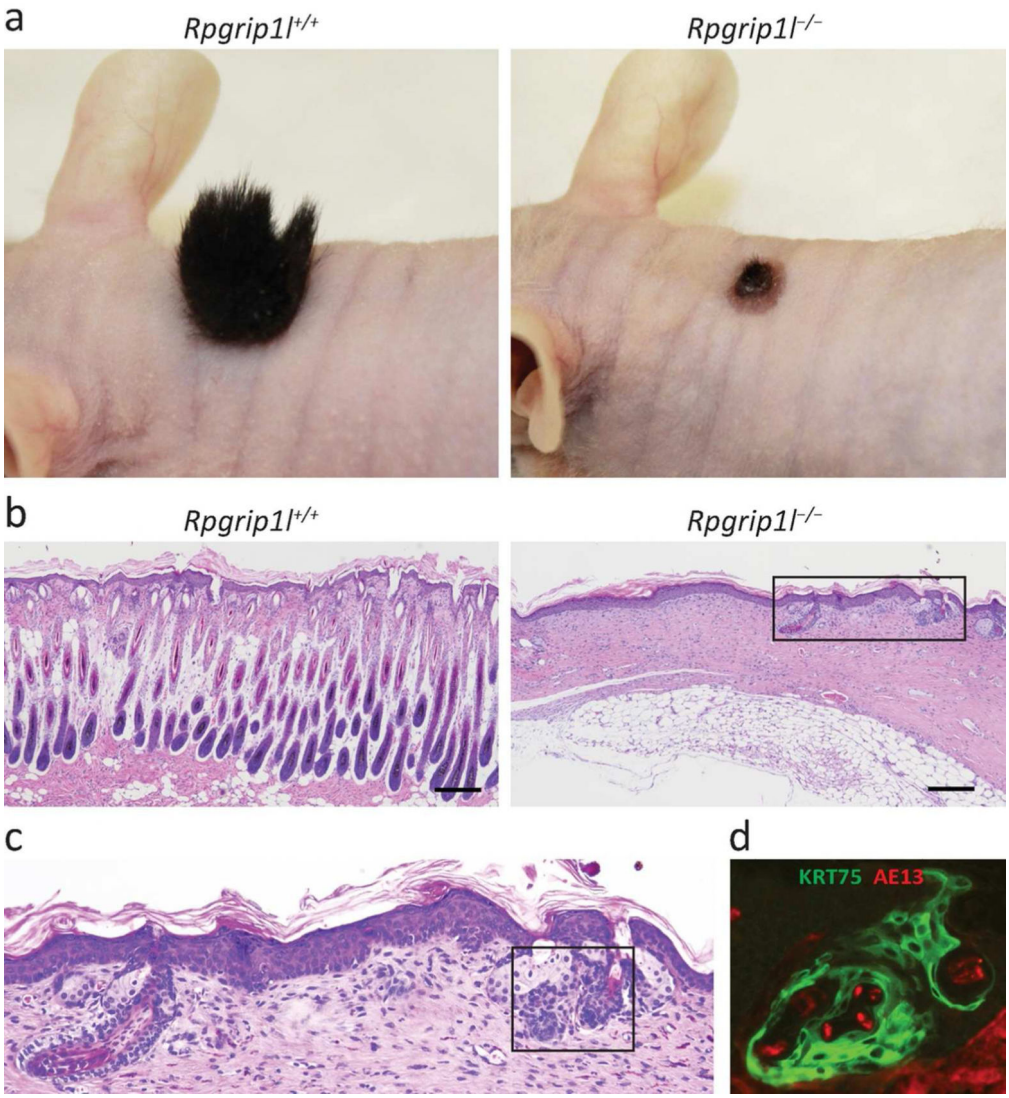

Fig. 2.

Skin transplantation of control (*Rpgrip1l*$^{+/+}$) and mutant (*Rpgrip1l*$^{-/-}$) littermates. (a) Representative gross images of skin transplants at 34 days post-grafting. (b) H&E staining of skin transplants in (a). (c) Magnified boxed area in (b). (d) Immunofluorescence labeling of AE13 (red) and KRT75 (green) of boxed area in (c). Note that the lack of hair in mutant skin transplants was caused by the lack of hair follicle and that the hair follicle-like structures in mutant skin contained disorganized but AE13 and KRT75 positive cells. $n \geq 4$. Scale bars = 200 μm.

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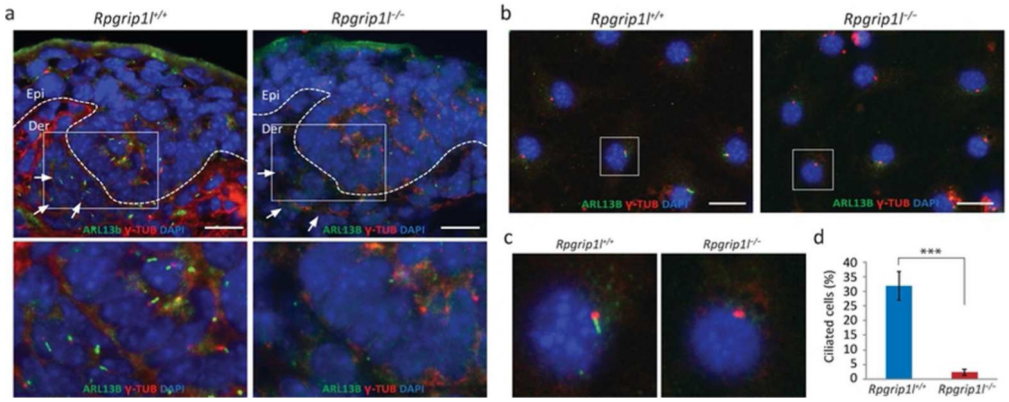

Fig. 3.

Ciliogenesis was disrupted in mutant (*Rpgrip1l*$^{-/-}$) skin. (a) Immunofluorescence labeling of primary cilia and basal bodies with ARL13B (green) and γ-tubulin (red) in dorsal skins of E15.5 control (*Rpgrip1l*$^{+/+}$) and mutant embryos. Nuclei were stained with DAPI (blue). Dotted lines illustrate the epidermal-dermal junction; arrows point to dermal papilla cells. Epi, epidermis; Der, dermis. Lower panels are enlarged boxed area in the upper panels. n ≥ 6. (b) Labeling of cilia and basal body (as described in a) in primary dermal fibroblasts. Three independent experiments were performed with similar results. (c) Enlarged boxed area in (b). (d) Statistically analyses of the percentage of ciliated cells in (b). ***, $P < 0.0001$. Scale bars = 50 μm in (a) and 25 μm in (b).

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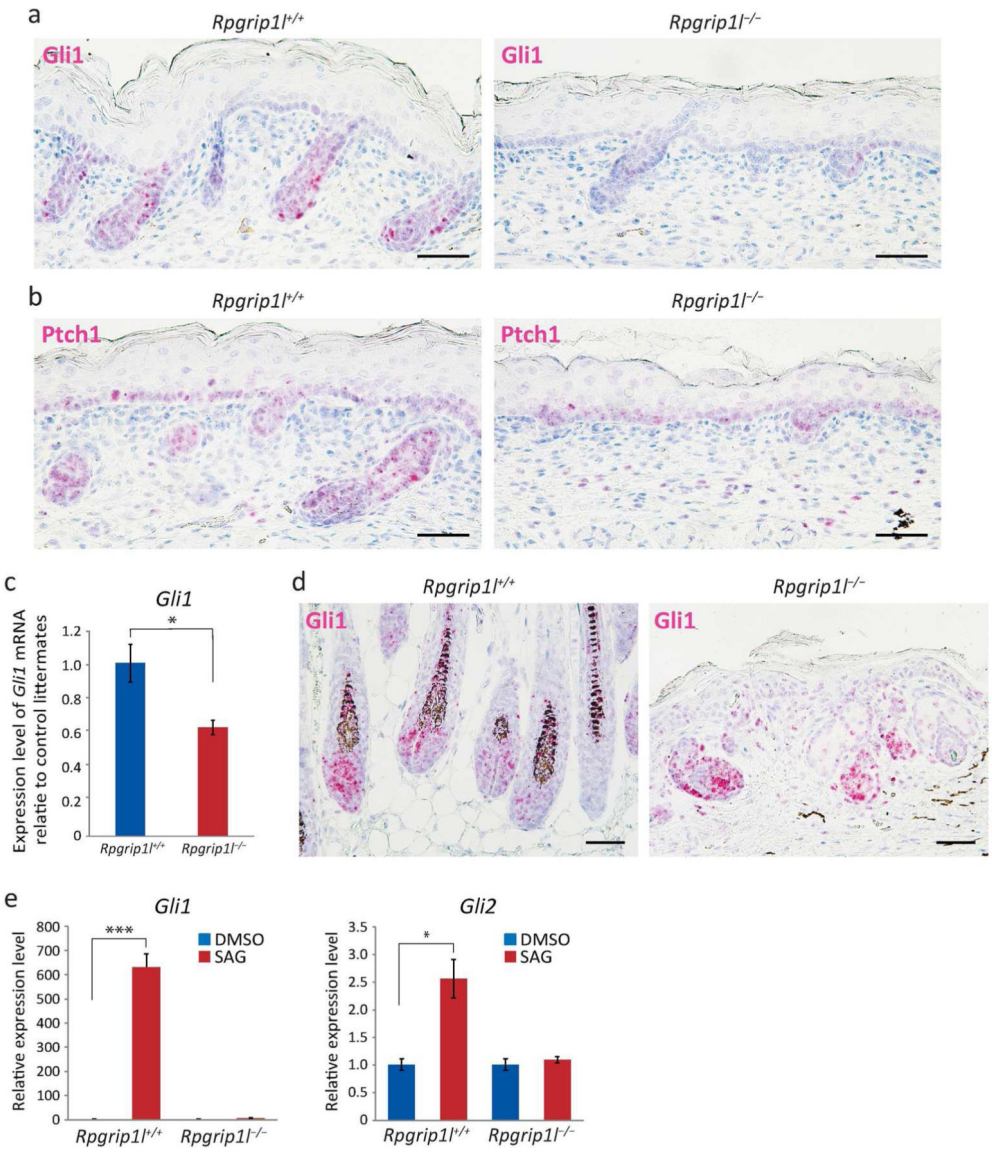

Fig. 4.

Hedgehog signaling is attenuated in mutant (*Rpgrip1l*$^{-/-}$) skin. (a − b) Representative *in situ* hybridization of *Gli1* (a) and *Ptch1* (b) in dorsal skins of E18.5 controls (*Rpgrip1l*$^{+/+}$) and *Rpgrip1l* mutants, n = 3. (c) Expression levels of *Gli1* in skins of E18.5 fetuses by quantitative RT-PCR; n = 3; *, P < 0.01. (d) *In situ* hybridization of *Gli1* in skin transplants. Some *Gli1*-positive cells scattered along the thickened epidermis and amorphic cell aggregates invaginated in the dermis. (e) Relative expression levels of *Gli1* and *Gli2* in SAG-treated control and mutant primary dermal fibroblasts as determined by qRT-PCR. DMSO treatment was used as control. *, p<0.05. ***, P < 0.0001. Experiments were conducted three times. Scale bars = 50 μm.

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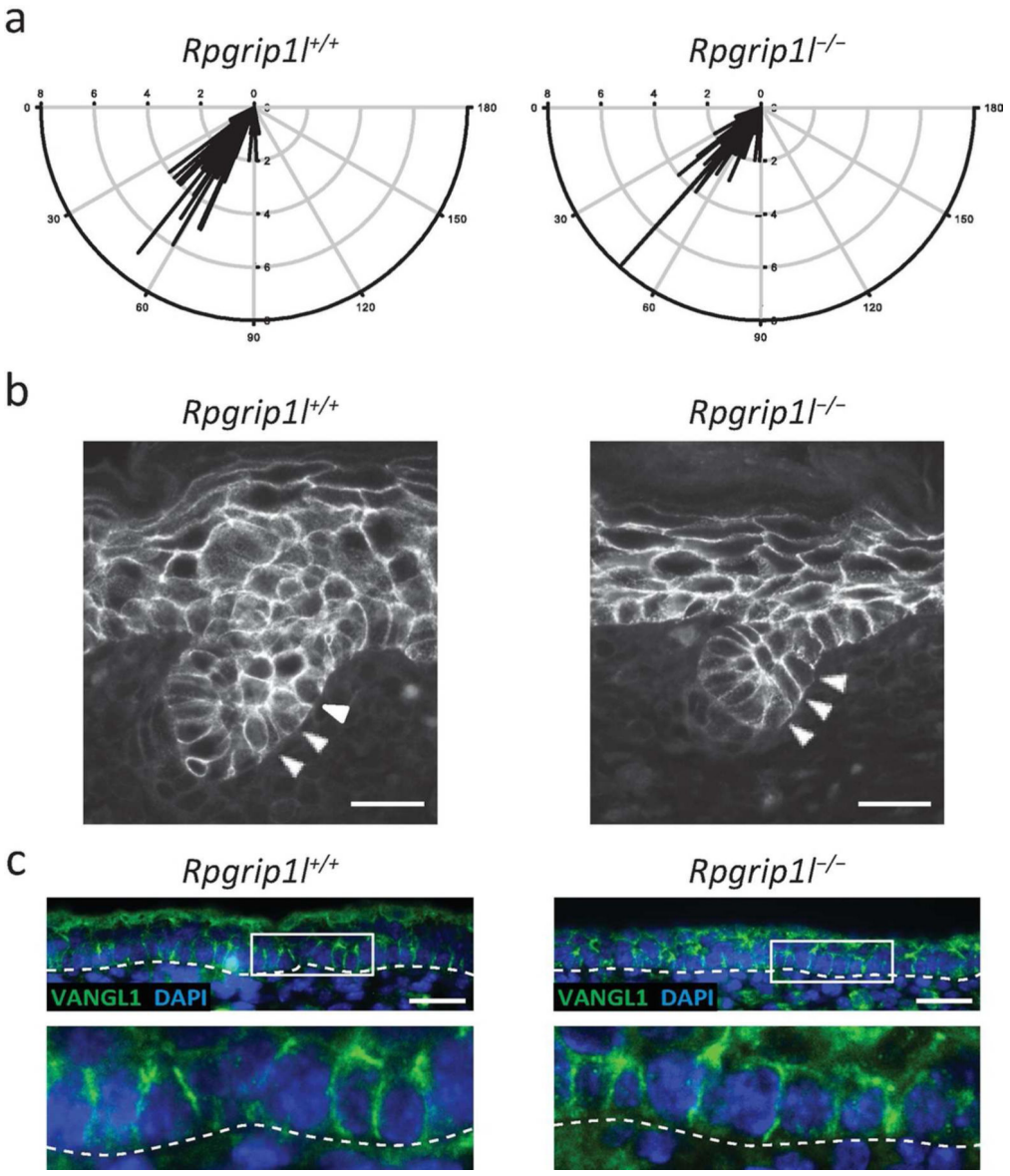

Fig. 5.

Orientation of hair follicles in sagittal sections of E18.5 dorsal skins of control (*Rpgrip1l*$^{+/+}$) and *Rpgrip1l* mutant (*Rpgrip1l*$^{-/-}$) skins. (a) Quantification of the acute angle between hair follicle and the surface of the epidermis. (b) Polarized distribution of E-cadherin in follicular keratinocytes. Triangles point to E-cadherin positive cells on the posterior side of the hair germs. (c) Immunofluorescence labeling of VANGL1 (green). Lower panels represent magnified boxed areas in upper panels. Scale bar = 25 μm.