Fuz Controls the Morphogenesis and Differentiation of Hair Follicles through the Formation of Primary Cilia

Daisy Dai[1,2], Huiping Zhu[3,6], Bogdan Wlodarczyk[3,6], Lei Zhang[1], Li Li[1,7], Allen G. Li[4,8], Richard H. Finnell[3,5,6], Dennis R. Roop[1] and Jiang Chen[1]

Planar cell polarity (PCP) signaling is essential in determining the polarity of cells within the plane of an epithelial sheet. Core PCP genes have been recently shown to control the global polarization of hair follicles in mice. *Fuz*, a homologue of the *Drosophila* PCP effector gene, *fuzzy*, is critical in ciliogenesis in vertebrates, and is required for the development of a wide range of organs in mice. Here, we report that disruption of the *Fuz* gene in mice severely blocked the development of hair follicles in the skin. In contrast to the loss of hair follicle polarization in mice deficient in core PCP genes, hair follicles in mice lacking the *Fuz* gene retained their typical anterior–posterior orientation. We show that disruption of *Fuz* impaired the formation of primary cilia and the hedgehog signaling pathway in the skin. In addition, using skin grafts and skin reconstitution assays we demonstrate that the expression of *Fuz* is required in both epidermal and dermal cells and that the formation of primary cilia is a cell-autonomous process that does not require cross talk between the epithelia and mesenchymal compartments during hair follicle formation.

Journal of Investigative Dermatology (2011) **131,** 302–310; doi:10.1038/jid.2010.306; published online 21 October 2010

INTRODUCTION

The development of multicellular structures, such as the hair follicle of mammals, requires specification of both cell fate and cell polarity in a precisely controlled temporal and spatial manner (Wang and Nathans, 2007; Strutt and Warrington, 2008; Schneider *et al.*, 2009). Planar cell polarity (PCP) proteins exert critical effects in the determination of cell polarity within an epithelial plane, acting on both subcellular and multicellular structures to determine local and global polarization of tissues and organs along body axes (Wang and Nathans, 2007). The hair follicle is an ideal multicellular appendage of the skin that can be used to study PCP functions in skin pattern formation.

PCP genes have been extensively studied in *Drosophila melanogaster*. *Drosophila* PCP genes are grouped as core PCP genes and tissue-specific PCP effector genes. Core PCP genes include *frizzled* (*fz*), *dishevelled* (*dsh*), *prickle* (*pk*), *diego* (*dgo*), *strabismus* (*stbm*, or *Van Gogh* (*Vang*)), and *flamingo* (*fmi*, or *starry night* (*stan*)). PCP effector genes include *inturned* (*in*), *fuzzy* (*fy*), *fritz* (*frtz*), *multiple wing hairs* (*mwh*), and *nemo* (*nmo*) (Wong and Adler, 1993; Klein and Mlodzik, 2005; Zallen, 2007; Strutt and Warrington, 2008). In vertebrates, PCP genes participate in convergent extension, neural tube closure, eyelid closure, inner ear sensory cell hair bundle orientation, and hair follicle orientation (Wang and Nathans, 2007). Disruption of core PCP genes usually results in extensive and severe defects in these processes (Jones and Chen, 2007; Wang and Nathans, 2007; Simons and Mlodzik, 2008). Mutations in PCP effector genes generally result in more subtle defects (Lee and Adler, 2002; Park *et al.*, 2006). Therefore, unlike core PCP genes, PCP effector genes may function more locally.

PCP genes provide one of the earliest developmental cues to govern epidermal and hair follicle morphogenesis (Fuchs, 2007). Core PCP genes have been implicated in the development of pelage hair in mice. Mutations in genes encoding these proteins resulted in disruption of hair follicle polarization in mice (Guo *et al.*, 2004; Devenport and Fuchs, 2008; Ravni *et al.*, 2009). However, to date, there are no

[1]*Dermatology and Charles C. Gates Center for Regenerative Medicine and Stem Cell Biology, University of Colorado at Denver, Aurora, Colorado, USA;* [2]*Program in Developmental Biology, Baylor College of Medicine, Houston, Texas, USA;* [3]*Institute of Biosciences and Technology, Texas A&M Health Sciences Center, Houston, Texas, USA;* [4]*Department of Dermatology, Oregon Health Science University, Portland, Oregon, USA and* [5]*Texas A&M Institute for Genomic Medicine, Houston, Texas, USA*

[6]*Current address: Dell Pediatric Research Institute, College of Natural Sciences, University of Texas at Austin, Austin, Texas, USA.*

[7]*Current address: Department of Dermatology, Peking Union Medical College Hospital, Beijing, China.*

[8]*Current address: Genomics Institute of the Novartis Research Foundation, San Diego, California, USA.*

Correspondence: Jiang Chen, Dermatology and Charles C Gates Center for Regenerative Medicine and Stem Cell Biology, University of Colorado, Denver, 12800 East 19th Avenue, RC-1 North, P18-8404D, Mail Stop 8320, PO Box 6511, Aurora, Colorado 80045, USA.
E-mail: jiang.chen@ucdenver.edu

Abbreviations: Fuz, *homologue of the* Drosophila fuzzy *gene; Hh, hedgehog; PCP, planar cell polarity; Shh, sonic hedgehog*

Received 15 December 2009; revised 8 August 2010; accepted 6 September 2010; published online 21 October 2010

reports describing the effects of PCP effector genes on hair follicle formation in mice.

Fuzzy (*fy*) is a member of the tissue-specific PCP effector gene family (Simons and Mlodzik, 2008). In *Drosophila*, *fy* specifies wing hair initiation site and orientation, and is involved in cytoskeleton maintenance to control wing hair number (Collier and Gubb, 1997). Disruption of the *fuzzy* homologues in vertebrate animals resulted in a number of development defects (Park *et al.*, 2006; Gray *et al.*, 2009; Heydeck *et al.*, 2009), such as neural tube closure defects, disruption of dorsal–ventral patterning of the spinal cord, and disruption of anterior–posterior patterning of the limb buds in mice (Gray *et al.*, 2009; Heydeck *et al.*, 2009), abnormalities often associated with defects in primary cilia formation and function.

Most cells in vertebrate animals possess a single nonmotile (primary) cilium. Genes that are involved in PCP functions are often required for cilia formation (Beales, 2005; Bisgrove and Yost, 2006; Davis *et al.*, 2006; Singla and Reiter, 2006; Jones *et al.*, 2008; Veland *et al.*, 2009). In fact, one of the best-studied PCP genes affecting cilia formation is the *fuzzy* gene. Disruption of *fuzzy* homologues in *Xenopus* (*fuz*) and mice (*Fuz*) disrupted ciliogenesis (Park *et al.*, 2006; Gray *et al.*, 2009; Heydeck *et al.*, 2009). Recently, it was shown that the disruption of another PCP effector gene, *Intu*, could also result in defective cilia formation (Zeng *et al.*, 2010).

It is well established that primary cilia are essential for hedgehog (Hh) signaling during development (Corbit *et al.*, 2005; Haycraft *et al.*, 2005; Huangfu and Anderson, 2005; Liu *et al.*, 2005; Rohatgi *et al.*, 2007) and in neoplasia (Han *et al.*, 2009; Wong *et al.*, 2009; Zhang *et al.*, 2009). Cilia are critical for hair follicle formation (Lehman *et al.*, 2008, 2009). Targeted disruption of cilia in dermal fibroblasts resulted in blockage of hair follicle formation and downregulation of Hh-responsive genes (Lehman *et al.*, 2009).

To determine if *Fuz* is required for the formation of hair follicles, we examined the skin of a mutant mouse model of *Fuz* (Gray *et al.*, 2009). Hair follicle development was remarkably impaired in the absence of *Fuz*. Both cilia formation and Hh signaling were inhibited in the skin of *Fuz* mutant mice. Interestingly, hair follicle polarization was not altered in these mutants, suggesting that core PCP genes and PCP effector genes may have unique roles during hair follicle formation.

RESULTS

Expression of *Fuz* in the skin of wild-type and mutant *Fuz* mice

Transcription levels of the gene in the skin were assessed using quantitative RT-PCR. Robust expression of *Fuz* was detected in both epidermal and dermal cells of wild-type embryos (Supplementary Figure S1 online). However, in homozygous mutants, *Fuz* transcription was undetectable (Supplementary Figure S1 online). Therefore, this mutant mouse model is considered to be a null mutation of *Fuz* ($Fuz^{-/-}$) and will be referred to as such.

Number of hair follicles is reduced in $Fuz^{-/-}$ embryonic skin

At early embryonic stages (E15.5) when the first wave of hair follicles start to form, the number of hair germs in the back skin of wild-type and $Fuz^{-/-}$ mutants was almost identical (Figure 1a, b, and e), indicating a normal induction of hair follicle formation. However, a striking hair follicle phenotype was observed in $Fuz^{-/-}$ embryos at later developmental stages (Figure 1c and d). At E18.5, the average number of hair follicles, irrespective of their differentiation stages, in the dorsal skin of $Fuz^{-/-}$ embryos was 6.7 ± 2.1 per microscopic field as compared with 12.5 ± 1.9 in wild-type littermates (Figure 1e). The skin of heterozygous ($Fuz^{+/-}$) mice appeared indistinguishable from wild-type littermates (data not shown) and only wild-type data were used as controls.

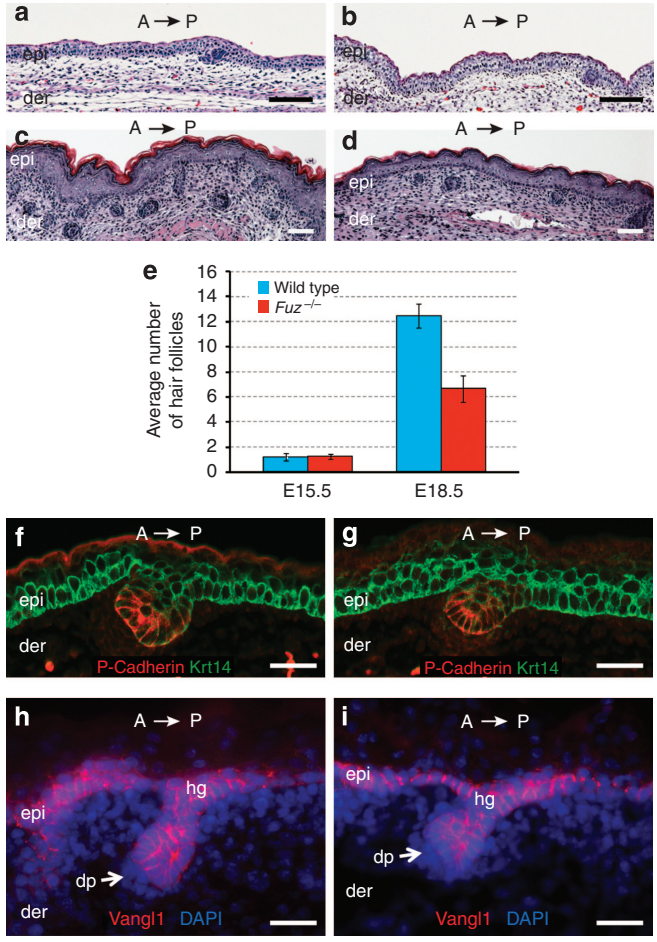

Figure 1. Developing hair follicles in dorsal skins of wild-type and $Fuz^{-/-}$ embryos. (**a**–**d**) Representative hematoxylin and eosin staining of wild-type (**a** and **c**) and $Fuz^{-/-}$ (**b** and **d**) dorsal skins at E15.5 and E18.5, respectively. The number of hair follicles is significantly reduced in the $Fuz^{-/-}$ embryos at E18.5. Of note, hair follicles maintained A-P polarization in wild-type and $Fuz^{-/-}$ skins. (**e**) Quantification of E18.5 hair follicles in $Fuz^{-/-}$ and wild-type skin showed that the average number of hair follicles of $Fuz^{-/-}$ skin was about half that of wild-type littermates per microscopic field, irrespective of their differentiation stages ($n = 15$, $P < 0.001$). (**f**, **g**) Expression of P-cadherin (red) was localized to cells on the anterior side of hair germs in both wild-type and $Fuz^{-/-}$ skin. Krt14 (green) labeling shows keratinocytes in the epidermis and hair germ. (**h**, **i**) Expression of Vangl1 (red) remained polarized to the lateral membrane of cells of the epidermis and invaginating hair germ. DAPI (blue)-stained nucleus. DAPI, 46-diamidino-2-phenyl indole; A→P, anterior–posterior orientation; der, dermis; dp, dermal papilla; epi, epidermis; hg, hair germ. Scale bar = 100 μm in **a**–**d**; 50 μm in **f**–**i**.

Hair follicle polarization of *Fuz*$^{-/-}$ mutants is similar to wild type

Histologically, dorsal hair follicles that developed in *Fuz*$^{-/-}$ embryos exhibited anterior–posterior polarization, similar to those of wild types (Figure 1c and d). At the molecular level, the expression of P-cadherin was restricted to cells at the anterior aspect of developing hair follicles (Figure 1f and g). In addition, at E15.5, keratinocytes in both wild-type (Figure 1h) and *Fuz*$^{-/-}$ mutants (Figure 1i) exhibited polarized localization of Vangl1, a core PCP component. Vangl1 was laterally localized in basal and follicular keratinocytes along the plane of the epidermis or the growth axis of developing hair follicles (Figure 1h and i). This result is consistent with findings in *Drosophila* where the disruption of a PCP effector does not normally affect the polarized location of core PCP components (Adler and Lee, 2001; Lee and Adler, 2002).

Epidermal differentiation is not altered in *Fuz*$^{-/-}$ skin

The *Fuz*$^{-/-}$ epidermis looked essentially normal, exhibiting typical embryonic skin features of the basal, spinous, and granular layers (Figure 1d). The epidermal stratification regulator p63 was examined at E14.5. The expression of p63 was restricted normally to basal cells and hair germs in wild-type and *Fuz*$^{-/-}$ skin (Supplementary Figure S2 online). At E18.5, Krt14 expression was restricted to the basal cells of both wild-type (Figure 2a) and *Fuz*$^{-/-}$ skin (Figure 2d).

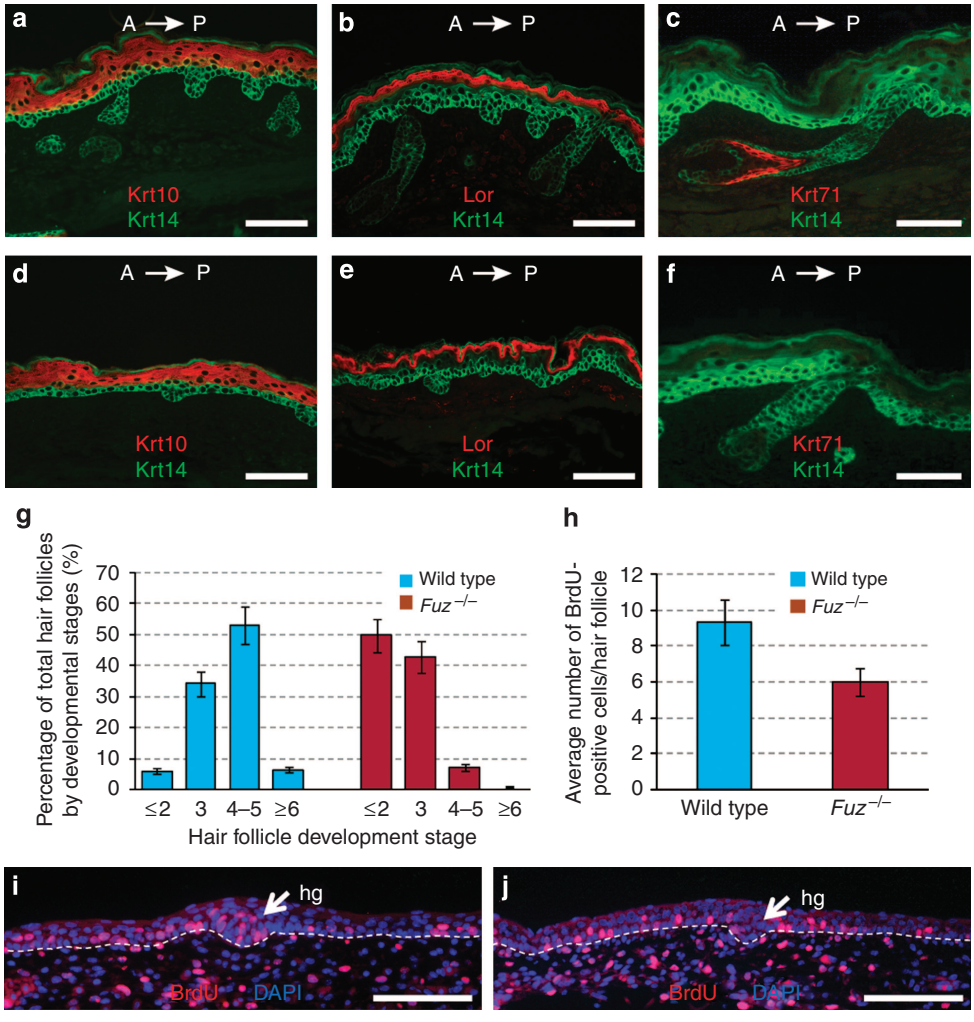

Figure 2. Differentiation and proliferation of the epidermis and hair follicle. (**a**, **b**, **d**, and **e**) Immunofluorescence labeling of Krt14, Krt10, and Lor in the epidermis of *Fuz*$^{-/-}$ skin at E18.5 (**d** and **e**) revealed almost identical expression patterns of these epidermal differentiation markers as wild-type skin (**a** and **b**). (**c** and **f**) Immunofluorescence labeling of Krt71 (red), marker of Henle's layer of the inner root sheath, showed that some hair follicles have developed past stage 5 in wild-type embryos (**c**), whereas there was no evidence of differentiation in the most developmentally advanced dorsal skin hair follicles of *Fuz*$^{-/-}$ embryos (**f**). (**g**) Distribution of hair follicles in various developmental stages in wild-type (blue) and *Fuz*$^{-/-}$ (brown) dorsal skins at E18.5 ($n = 198$ for wild type; $n = 126$ for *Fuz*$^{-/-}$). A majority (53%) of hair follicles in wild-type skin were in stage 4–5, whereas 50% of hair follicles in *Fuz*$^{-/-}$ skin were at stage 2 or below. (**i**–**j**) BrdU labeling of dorsal skin of wild-type (**i**) and *Fuz*$^{-/-}$ (**j**) embryos at E15.5. The interfollicular epidermis of both genotypes showed similar pattern of BrdU-positive cells. However, the number of BrdU-positive cells within the *Fuz*$^{-/-}$ hair germs was consistently fewer than that within wild-type hair germs. Arrows indicate early hair germs (hg). (**h**) Quantification of BrdU-positive cells in hair germs, $n = 7$. DAPI, 46-diamidino-2-phenyl indole; A→P, anterior–posterior orientation. Scale bar = 100 μm.

Early and late differentiation markers, such as keratin 10 (Krt10) and loricrin (Lor), also showed normal expression in the epidermis of $Fuz^{-/-}$ embryos at E18.5 (Figure 2d and e) when compared with wild-type littermates (Figure 2a and b).

Hair follicle morphogenesis is delayed in $Fuz^{-/-}$ embryos

Although the induction of hair follicle formation appeared normal in $Fuz^{-/-}$ mutants (described above), further development of hair follicles was affected in the $Fuz^{-/-}$ mutants. At late gestation, the majority of sparsely distributed hair follicles in the dorsal skin of the $Fuz^{-/-}$ embryos appeared to be at earlier developmental stages in comparison to wild-type littermates (Figures 1d, 2d–f). In all, 198 wild-type and 126 $Fuz^{-/-}$ hair follicles in the dorsal skin of E18.5 embryos were analyzed using morphological criteria developed by Paus *et al.* (1999). In wild-type skin, 53% of observed follicles were determined to be in stages 4 and 5. Of note, 6.6% of wild-type follicles observed had advanced to stage 6 and only 6.1% did not appear to have developed beyond stage 2 (Figure 2g). In contrast, in $Fuz^{-/-}$ skin, 50% of the observed hair follicles were at stage 2 or earlier and only 7.1% of $Fuz^{-/-}$ follicles were at stages 4 and 5. We failed to find any stage 6 hair follicles in $Fuz^{-/-}$ skin (Figure 2g). Therefore, the morphogenesis of hair follicles in the $Fuz^{-/-}$ embryos was delayed and arrested at early developmental stages (stages 2 and 3).

Immunofluorescence labeling with the hair follicle differentiation marker keratin 71 (Krt71) (Aoki *et al.*, 2001), which is expressed in the Henle's layer of the inner root sheath, showed that no hair follicle in $Fuz^{-/-}$ skin had progressed to late developmental stages at E18.5 (Figure 2f). Figure 2f shows the most developmentally advanced $Fuz^{-/-}$ hair follicle observed in this study with no sign of Krt71 expression. BrdU labeling (Figure 2i and j) showed that at E15.5, the number of proliferating cells in $Fuz^{-/-}$ hair germs was fewer than wild type (Figure 2h). This difference persisted until later stages of hair follicle formation (Supplementary Figure S3d online). TUNEL assays did not reveal any difference in the number of apoptotic cells in wild-type versus $Fuz^{-/-}$ skin (data not shown). The numbers of proliferating (BrdU-positive) cells in the basal epidermis of both genotypes were comparable (Figure 2i and j).

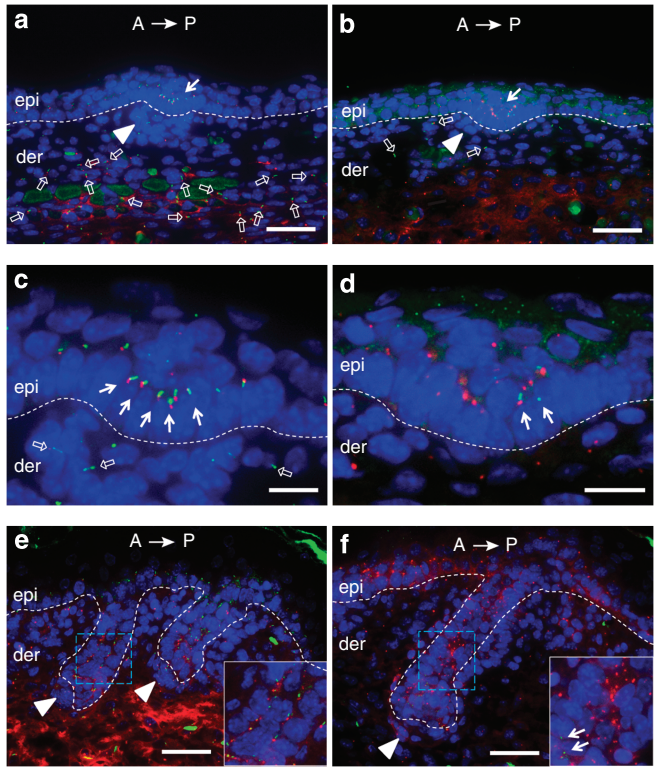

Figure 3. Primary cilia in skin of wild-type and $Fuz^{-/-}$ embryos. Primary cilia were labeled by Arl13b (green), basal bodies were labeled by γ-tubulin (red), and nuclei were labeled by DAPI (blue). (**a**, **b**) At E15.5, primary cilia were evident in both epidermal and dermal cells (open arrows) of wild-type skin (**a**). The number of cells containing primary cilia was reduced in both epidermal and dermal cells of $Fuz^{-/-}$ skin (**b**). Arrows indicate hair germs and arrowheads indicate dermal condensate. (**c**, **d**) Higher magnification of wild-type and $Fuz^{-/-}$ hair germs in (**a**) and (**b**). Arrows indicate cilia on cells in the hair germ and open arrows indicate cilia on cells comprising the dermal condensate. Of note, the majority of $Fuz^{-/-}$ germ cells and underlying dermal condensate did not have cilia but basal bodies were present. (**e**, **f**) At E18.5, cilia were evident and orientated toward the center of developing bulbous hair pegs in wild-type skin (**e**). Primary cilia were essentially absent from developing pegs of $Fuz^{-/-}$ skin (**f**). However, cells with severely truncated cilia exist (inlet in (**f**), indicated by arrows). Dotted lines highlight the epidermal–dermal boundary. DAPI, 46-diamidino-2-phenyl indole; der, dermis; epi, epidermis; hg, hair germ. Scale bar = 100 μm in (**a**, **b**, **e** and **f**), 25 μm in (**c** and **d**).

Number of primary cilia is reduced in $Fuz^{-/-}$ skin

Primary cilia were labeled with Arl13b and the basal bodies with γ-tubulin. In wild-type skin, at E15.5, cilia were located on the apical side of keratinocytes, being more prominent in basal cells (Figure 3a and c). In early hair follicles, cilia tilted toward the center of hair germs, as the downgrowing keratinocytes start to form a concentric cell mass (Figure 3c). As the embryonic skin further matures, primary cilia become restricted to the basal layer of the epidermis (Figure 3e). As the basal cells invaginate to form hair germs, which further elongate to form hair pegs, primary cilia became concentrically oriented toward the center of developing hair follicles (Figure 3e). Primary cilia were also found in dermal fibroblasts (Figure 3a and e) and cells of the dermal condensate (Figure 3a) (Lehman *et al.*, 2009). In contrast, at E15.5, the number of ciliated epidermal and dermal cells was dramatically reduced in $Fuz^{-/-}$ skin (Figure 3b and d). At E18.5, primary cilia were essentially absent in $Fuz^{-/-}$ skin (Figure 3f). However, primary cilia were occasionally observed in $Fuz^{-/-}$ skin (Figure 3d and f, inlet) that ranged from a relatively normal length to severely stunted. These observations were confirmed by labeling the primary cilia with another marker, acetylated α-tubulin (data not shown), and were consistent with defective ciliogenesis in other cell types (Gray *et al.*, 2009).

Hedgehog signaling is suppressed in the epidermis of $Fuz^{-/-}$ embryos

Significant reductions in *Ptch1* ($P = 0.017$) and *Gli2* ($P = 0.008$) expression were observed in $Fuz^{-/-}$ skin when

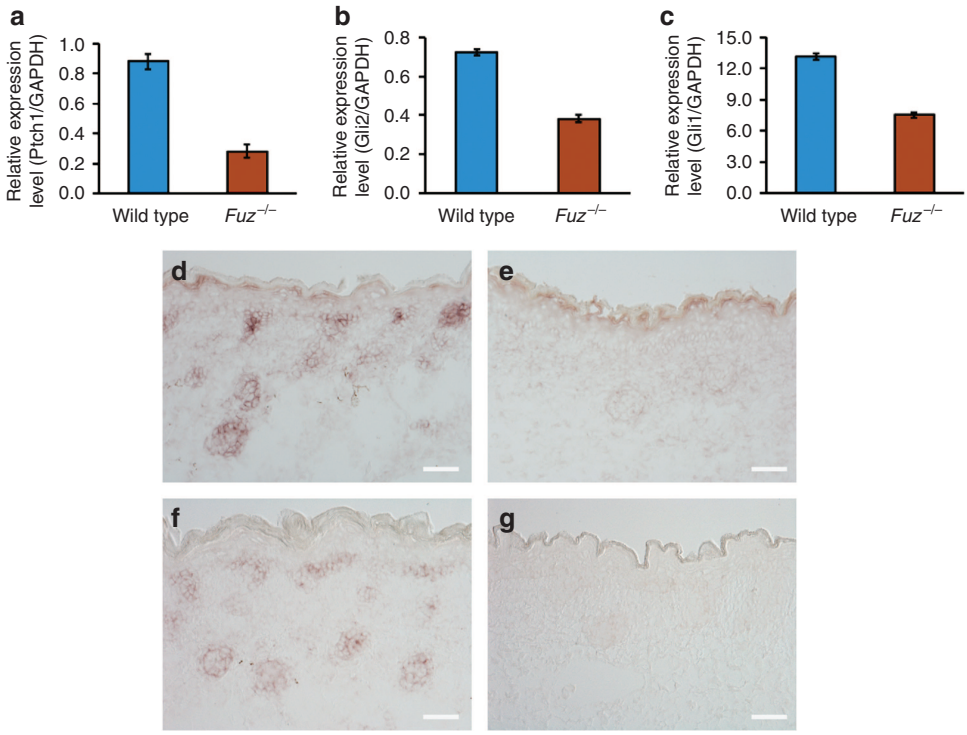

Figure 4. Expression of Shh-responsive genes in wild-type and *Fuz*$^{-/-}$ skin. (**a**, **b**) Transcription levels of *Ptch1* ($P=0.017$) (**a**) and *Gli2* ($P=0.008$) (**b**) were significantly reduced in skin of *Fuz*$^{-/-}$ embryos as compared with wild-type embryos at E15.5. (**c**) Transcription levels of *Gli1* were significantly reduced in dorsal skin of *Fuz*$^{-/-}$ embryos as compared with wild-type embryos at E18.5 ($P<0.001$). (**d**–**g**) *In situ* hybridization of *Ptch1* and *Gli2* in wild-type (**d** and **f**) and *Fuz*$^{-/-}$ skins (**e** and **g**) of E18.5 embryos, respectively. Scale bar = 50 μm.

compared with wild-type ones at E15.5 (Figure 4a and b). A pronounced reduction in *Gli1* expression ($P<0.001$) was observed at E18.5 (Figure 4c) in *Fuz*$^{-/-}$ skin. *Gli3* expression was marginally reduced in E18.5 *Fuz*$^{-/-}$ epidermis (data not shown). *In situ* hybridization showed that *Ptch1* and *Gli2* were robustly expressed in wild-type hair follicle at E18.5 (Figure 4d and f), however, their signals were almost undetectable in *Fuz*$^{-/-}$ skin (Figure 4e and g). Overall, disruption of the *Fuz* gene resulted in suppression of Hh signaling in the skin. The expression levels of two Wnt target genes, *Axin2* and *Lef1*, were unchanged in *Fuz*$^{-/-}$ skins (data not shown), suggesting a relatively intact canonical Wnt signaling pathway in *Fuz*$^{-/-}$ skin, which is consistent with normal hair follicle induction (Figure 1b).

Hair follicle development is arrested in *Fuz*$^{-/-}$ skin transplants

To answer the question whether hair follicle development is delayed or arrested in the *Fuz*$^{-/-}$ mutants, we transplanted dorsal skins of E18.5 wild-type and *Fuz*$^{-/-}$ mutants to nude mice to allow further development. At 3 weeks after transplantation, a remarkable number of hairs formed on wild-type skin transplants (Figure 5a). In contrast, there were only a few short hairs that developed at the edge of pigmented *Fuz*$^{-/-}$ transplants (Figure 5b). Histological examination showed that anagen hair follicles developed across the entire wild-type skin transplant (Figure 5c), however, most of the hair follicles present in the *Fuz*$^{-/-}$ transplants were abnormally developed with a few well-developed hair follicles present at the edge of the *Fuz*$^{-/-}$ transplants (Figure 5d; also see Discussion). Immunofluorescence examination showed that only a fraction of cells in the bulb region of these well-developed *Fuz*$^{-/-}$ hair follicles were ciliated (Figure 5g). These mutant hair follicles were able to differentiate further to express Krt71 (Figure 5h). The epidermis of *Fuz*$^{-/-}$ transplants looked normal with normal expression patterns of differentiation markers such as Lor (Figure 5j).

Both epidermal and dermal cells require *Fuz* for hair follicle formation

Because *Fuz* is expressed in both epidermal and dermal cells, it was of interest to determine whether hair follicle formation required the expression of *Fuz* in both cell types. Therefore, we generated skin grafts that were reconstituted with different combinations of primary keratinocytes and fibroblasts isolated from either wild-type or *Fuz*$^{-/-}$ skin onto nude mice. At 4 weeks after grafting, skin reconstituted with wild-type keratinocytes and wild-type fibroblasts was able to generate abundant hair (Figure 6a and d). However, hair failed to form in skin reconstituted with either *Fuz*$^{-/-}$ keratinocytes and wild-type dermal cells (Figure 6b and e) or wild-type keratinocytes and *Fuz*$^{-/-}$ dermal cells (Figure 6c and f). Interestingly, as observed in *Fuz*$^{-/-}$ transplants, there were a few hairs that formed at the edge of the grafts (Figure 6b). Hair follicles that formed in skin reconstituted with wild-type cells contained normal-appearing ciliated cells (Figure 6g and h).

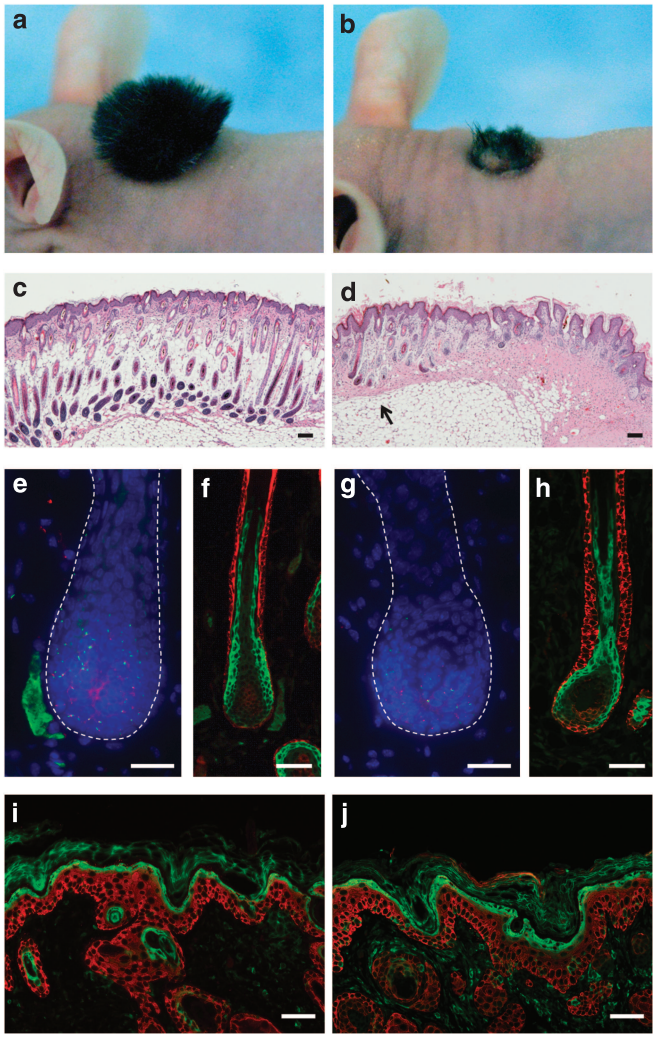

Figure 5. Hair follicles failed to grow in *Fuz*$^{-/-}$ skin transplants. (**a**, **b**) Gross appearance of E18.5 embryonic back skin of wild-type (**a**) and *Fuz*$^{-/-}$ mice (**b**) grown on nude mice for 3 weeks. Note that long and dense hair grew on the wild-type skin transplant (**a**); the *Fuz*$^{-/-}$ skin transplant was pigmented, however, only a few short hair developed at the edge of the transplant (**b**). (**c**, **d**) Hematoxylin and eosin staining of wild-type (**c**) and *Fuz*$^{-/-}$ (**d**) skin transplants. Some hair follicles at the edge of *Fuz*$^{-/-}$ skin transplant (indicated by arrow) were able to develop further (**d**). (**e** and **g**) Cilia were labeled as in Figure 3. Most cells in the hair follicle of the wild-type skin transplant contained cilia (**e**) in comparison to only a small percentage of ciliated cells were present in the *Fuz*$^{-/-}$ skin transplants (**g**). (**f** and **h**) Expression of Krt14 (red) and Krt71 (green) in anagen hair follicles of wild-type (**f**) and *Fuz*$^{-/-}$ (**h**) skin transplants. (**i** and **j**) Expression of Krt14 (red) and Lor (green) in wild-type (**i**) and *Fuz*$^{-/-}$ (**j**) skin transplants. Scale bar = 100 μm in (**c** and **d**), 50 μm in (**f**, **h**, **i** and **j**), 25 μm in (**e** and **g**).

In contrast, only the epidermal components of the hair follicle-like structures formed by wild-type keratinocytes and *Fuz*$^{-/-}$ dermal cells were ciliated (Figure 6i); and only the dermal cells of skin reconstituted by *Fuz*$^{-/-}$ keratinocyte and wild-type dermal cell were ciliated (Figure 6j). The hair follicle-like structures formed in the latter only contained a few normal-appearing ciliated cells in the dermal component (Figure 6k and l). These skin reconstitution assays showed that hair follicle formation requires the expression of *Fuz* in both epidermal and dermal cells. In addition, we show that primary cilia formed normally in the epidermal or dermal components isolated from wild-type mice, but not in the components isolated from *Fuz* mutant skin; thus documenting that the formation of primary cilia is a cell-autonomous process that does not require cross talk between the epithelia and mesenchymal compartments during hair follicle formation.

DISCUSSION

Previous studies of the PCP functions in mouse hair follicle development have focused on core PCP genes such as *Fzd6*, *Celsr1*, or *Vangl2*. Disruption of any one of these genes resulted in abnormal hair follicle orientation (Guo *et al.*, 2004; Devenport and Fuchs, 2008; Ravni *et al.*, 2009). The disruption of *Fuz*, a PCP effector gene, resulted in the delay and arrest of hair follicle development, a phenotype that has not previously been associated with PCP genes. Because vertebrate animals have acquired more sophisticated developmental processes and have even more PCP genes than *Drosophila* (Wang and Nathans, 2007), it is not surprising that different PCP components participate in distinctive developmental processes, especially in a tissue- or organ-specific manner.

Disruption of the *Fuz* gene has been shown to affect ciliogenesis in *Xenopus* and mice (Park *et al.*, 2006; Gray *et al.*, 2009; Heydeck *et al.*, 2009). In mice, cilia formation on Meckel's cartilage cells, mesenchymal cells of the notochord and limb buds, and other cell types was not completely abolished in the absence of *Fuz*, suggesting that other PCP effector genes, such as *Intu* (Zeng *et al.*, 2010) and *Frtz*, might have compensated for the loss of *Fuz*. In mouse skin, both epidermal keratinocytes and dermal fibroblasts are ciliated (Figure 3; Lehman *et al.*, 2008, 2009). The presence of cilia on dermal fibroblasts has been shown to be essential for hair follicle morphogenesis (Lehman *et al.*, 2009). In this study, we show that *Fuz* is required for primary cilia formation in both cell types. Disruption of cilia formation in either cell type blocked the formation of hair follicles. Interestingly, our skin reconstitution assays show that primary cilia form normally in the epidermal or dermal components isolated from wild-type mice, but not in the components isolated from *Fuz* mutant skin; thus documenting that the formation of primary cilia is a cell-autonomous process that does not require cross talk between the epithelia and mesenchymal compartments during hair follicle formation. Most of the cilia observed in *Fuz*$^{-/-}$ cells were stunted; however a few cells contained cilia that appeared to be of normal length. This might explain the partially penetrant hair follicle phenotype in *Fuz*$^{-/-}$ mice, which allows hair follicles to form, but ultimately arrest at different stages of morphogenesis. Because *Fuz*$^{-/-}$ epidermis exhibited normal differentiation patterns, we speculate that primary cilia may not be required for processes involved in epidermal differentiation.

Primary cilia are essential for the transduction of Hh signals during tissue morphogenesis, homeostasis, and tumorigenesis (Wong and Reiter, 2008). The decreased

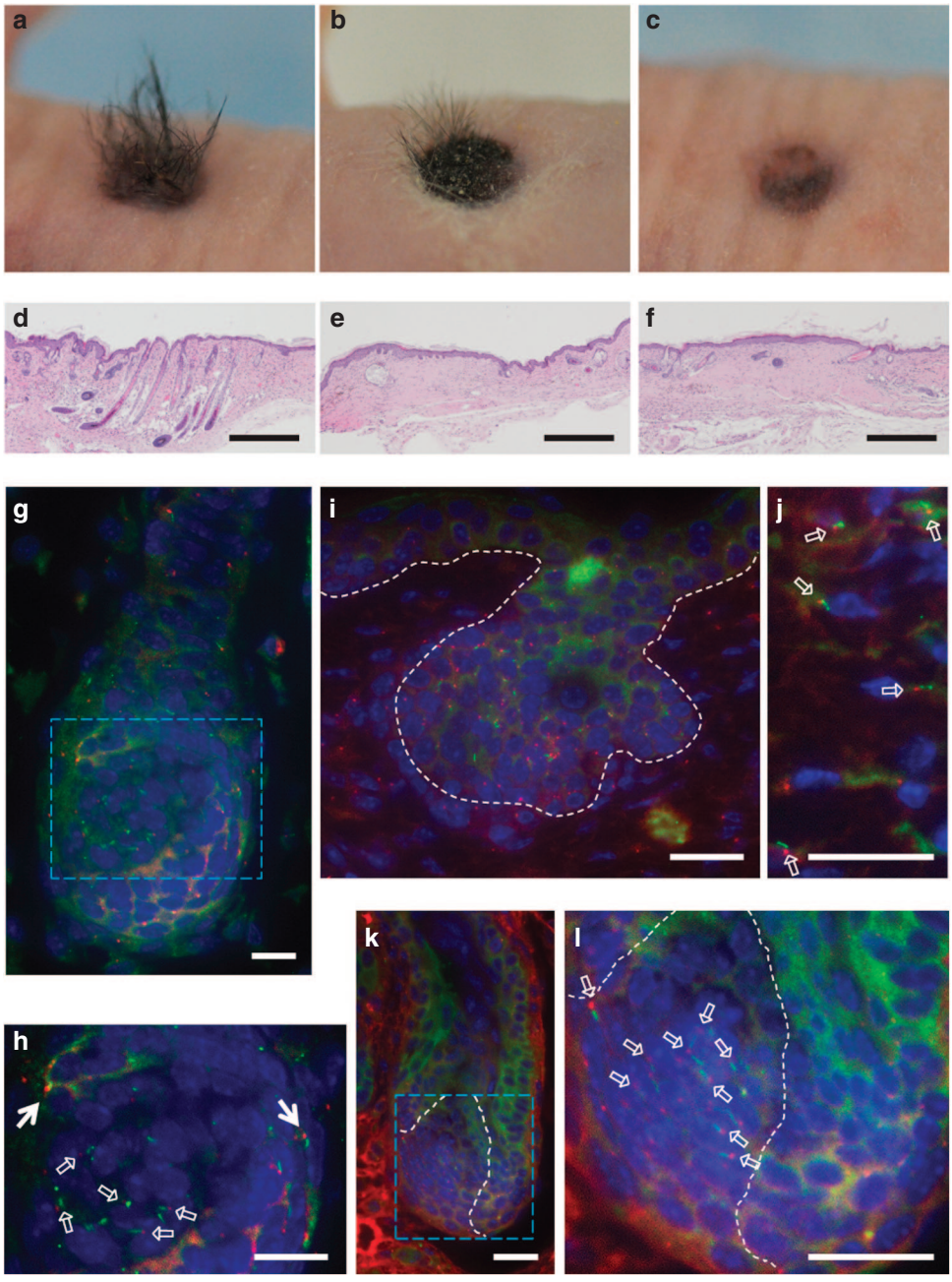

Figure 6. Hair follicle formation required the expression of *Fuz* in both epidermal and dermal cells. (**a**–**c**) Gross appearance of skin grafts reconstituted with wild-type keratinocytes and wild-type dermal cells (**a**), $Fuz^{-/-}$ keratinocytes and wild-type dermal cells (**b**), or wild-type keratinocytes and $Fuz^{-/-}$ dermal cells (**c**). (**d**–**f**) Hematoxylin and eosin staining of skin sections of corresponding grafts in (**a**–**c**). Note that the center of the skin grafts reconstituted with $Fuz^{-/-}$ cells (**e** and **f**) contained abnormally formed hair follicle structures. (**g**) Cilia were present on follicular and dermal papilla cells in hair follicles formed with wild-type cells. (**h**) Higher magnification of cropped area in (**g**). (**i**) Hair follicle-like structures that were formed by wild-type keratinocytes and $Fuz^{-/-}$ dermal cells only contained ciliated cells in the epidermal component, not the dermal component. (**j**) Dermal cells in skin grafts reconstituted by $Fuz^{-/-}$ keratinocytes and wild-type dermal cells contain cilia. (**k**) Cilia were only detectable in dermal papilla cells in a hair follicle formed by $Fuz^{-/-}$ keratinocytes and wild-type dermal cells. (**l**) Higher magnification of cropped area in (**k**). Dotted lines in **k** and **l** outline the dermal papillae. The antibodies used to detect primary cilia (Arl13b, green) and basal bodies (γ-tubulin, red) were the same as used in Figure 3. Cilia on epidermal cells are indicated by arrows; cilia in dermal cells are indicated by open arrows. Scale bar = 500 μm in (**d**–**f**), 25 μm in (**g**–**l**).

expression of *Ptch1* and *Gli2* measured in $Fuz^{-/-}$ skin could be partially explained by the overall reduction in the number of hair follicles; however, we suggest that decreased Hh signaling is primarily due to the impaired formation of primary cilia. In this regard, the arrested development of hair follicles in $Fuz^{-/-}$ mice partially phenocopied the hair phenotypes of mutants whose Shh signaling was entirely disrupted (St-Jacques *et al.*, 1998; Chiang *et al.*, 1999;

Mill *et al.*, 2003). However, in $Fuz^{-/-}$ skin, the few hair follicles were able to develop to a later stage in $Fuz^{-/-}$ skin before arresting, suggesting that the few normal-appearing cilia may be able to partially transduce Hh signals. In addition, the formation of relatively normal-appearing hair follicles at the edge of mutant skin grafts may have allowed the cilia that formed in the absence of *Fuz* to more efficiently process Hh signals as a result of the release of the Shh ligand from the adjacent host skin.

In summary, unlike members of the core PCP family, *Fuz* is important in the formation of primary cilia on epidermal keratinocytes and dermal fibroblasts, both of which are required for Hh signaling during hair follicle morphogenesis. Thus, different PCP genes may function in an orchestrated manner but exert distinctive effects during hair follicle formation. It will be intriguing to know if the simultaneous disruption of both core PCP and PCP effector genes will result in a combination of hair follicle polarization and morphogenesis defects, or reveal even more complex epistatic interactions controlled by PCP genes during skin and hair follicle development in mice.

MATERIALS AND METHODS

Animals

Mutant *Fuz* mice were generated at the Texas Institute of Genomic Medicine and housed at the Institute of Biosciences and Technology at Texas A&M Health Sciences Center and the University of Colorado Denver vivariums. Nulligravid heterozygous *Fuz* ($Fuz^{+/-}$) females were mated overnight with heterozygous males and examined for the presence of vaginal plugs the following morning. The onset of gestation was considered to be at 22:00 hours of the previous night. All animal procedures were approved by the institutional animal care and use committees at both Texas A&M Health Sciences Center and University of Colorado Denver.

Immunofluorescence labeling and microscopy

To perform immunofluorescence labeling, paraffin sections were deparaffinized, rehydrated, and heated at 100 °C in 10 mM sodium citrate (pH 6.0) for 10 minutes as an antigen retrieval step. To label primary cilia, 0.1 mM EDTA (pH 8.0) was used in place of sodium citrate. Frozen sections were used for Vangl1 labeling. Sections were incubated with primary antibodies in 10% BSA at 4 °C overnight. The following primary antibodies were used: P-cadherin (Invitrogen, Carlsbad, CA), Krt10 and Krt14 (Yuspa *et al.*, 1989), Krt71 (K6irs1, a gift from Dr Shimomura), Lor (Mehrel *et al.*, 1990), acetylated α-tubulin and Vangl1 (Sigma, St Louis, MO), and Arl13b (a gift from Tamara Caspary). Secondary antibodies were Alexa-conjugated fluorochrome 594 or 488 anti-IgG to the corresponding host species of the primary antibodies (Molecular Probes, Eugene, OR). Photographs were taken with a Nikon Eclipse 90*i* microscope in conjunction with the NIS-Elements AR 3.0 imaging software (Nikon, Melville, NY) or on a Zeiss LSM 510 META laser scanning confocal microscope and analyzed on ZEN 2009 interface (Carl Zeiss, Thornwood, NY).

Skin transplantation and skin graft reconstitution assays

Full-thickness skin transplantation was performed following procedures described elsewhere (St-Jacques *et al.*, 1998; Chiang *et al.*, 1999) except that skins were placed in silicon chambers with an interior diameter of 6 mm and analyzed 3 weeks thereafter. Skin graft reconstitution assays were performed as described by Lichti *et al.* (2008). Different portions of wild-type and $Fuz^{-/-}$ epidermal cells (1×10^6) and freshly isolated dermal cells (2×10^6) were combined and seeded into silicon chambers with a 4 mm interior diameter. Reconstituted skins were collected 4 weeks after grafting. All experiments were performed at least four times.

Statistics

The Student's *t*-test was used to calculate statistically significant differences. $P < 0.05$ was considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Dr David Norris, Dr Lee Niswander, Dr Ralf Paus, and Dr Antonio Oro for support and helpful discussions. We also thank Dr Yutaka Shimomura (Niigata University) for providing the Krt71 (K6irs1) antibody, Dr Tamara Caspary (Emory University School of Medicine) for providing the Arl13b antibody, and the Texas A&M Institute for Genomic Medicine for providing the mutant *Fuz* mice. We especially thank Hui Wei for excellent technical support; Dianne Ashby for mouse colony management; and Irene Choi for editorial assistance. This work was supported in part by a Career Development Award from the Dermatology Foundation to JC and a grant from the National Institutes of Health (AR47898) to DRR. A Skin Diseases Core Center Grant (P30 AR057212) provided support for the use of the Morphology and Phenotyping Core facility.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Adler PN, Lee H (2001) Frizzled signaling and cell–cell interactions in planar polarity. *Curr Opin Cell Biol* 13:635–40

Aoki N, Sawada S, Rogers MA *et al.* (2001) A novel type II cytokeratin, mK6irs, is expressed in the Huxley and Henle layers of the mouse inner root sheath. *J Invest Dermatol* 116:359–65

Beales PL (2005) Lifting the lid on Pandora's box: the Bardet–Biedl syndrome. *Curr Opin Genet Dev* 15:315–23

Bisgrove BW, Yost HJ (2006) The roles of cilia in developmental disorders and disease. *Development* 133:4131–43

Chiang C, Swan RZ, Grachtchouk M *et al.* (1999) Essential role for Sonic hedgehog during hair follicle morphogenesis. *Dev Biol* 205:1–9

Collier S, Gubb D (1997) *Drosophila* tissue polarity requires the cell-autonomous activity of the fuzzy gene, which encodes a novel transmembrane protein. *Development* 124:4029–37

Corbit KC, Aanstad P, Singla V *et al.* (2005) Vertebrate smoothened functions at the primary cilium. *Nature* 437:1018–21

Davis EE, Brueckner M, Katsanis N (2006) The emerging complexity of the vertebrate cilium: new functional roles for an ancient organelle. *Dev Cell* 11:9–19

Devenport D, Fuchs E (2008) Planar polarization in embryonic epidermis orchestrates global asymmetric morphogenesis of hair follicles. *Nat Cell Biol* 10:1257–68

Fuchs E (2007) Scratching the surface of skin development. *Nature* 445:834–42

Gray RS, Abitua PB, Wlodarczyk BJ *et al.* (2009) The planar cell polarity effector Fuz is essential for targeted membrane trafficking, ciliogenesis and mouse embryonic development. *Nat Cell Biol* 11:1225–32

Guo N, Hawkins C, Nathans J (2004) Frizzled6 controls hair patterning in mice. *Proc Natl Acad Sci USA* 101:9277–81

Han YG, Kim HJ, Dlugosz AA *et al.* (2009) Dual and opposing roles of primary cilia in medulloblastoma development. *Nat Med* 15:1062–5

Haycraft CJ, Banizs B, Aydin-Son Y *et al.* (2005) Gli2 and Gli3 localize to cilia and require the intraflagellar transport protein polaris for processing and function. *PLoS Genet* 1:e53

Heydeck W, Zeng H, Liu A (2009) Planar cell polarity effector gene Fuzzy regulates cilia formation and Hedgehog signal transduction in mouse. *Dev Dyn* 238:3035–42

Huangfu D, Anderson KV (2005) Cilia and Hedgehog responsiveness in the mouse. *Proc Natl Acad Sci USA* 102:11325–30

Jones C, Chen P (2007) Planar cell polarity signaling in vertebrates. *Bioessays* 29:120–32

Jones C, Roper VC, Foucher I *et al.* (2008) Ciliary proteins link basal body polarization to planar cell polarity regulation. *Nat Genet* 40:69–77

Klein TJ, Mlodzik M (2005) Planar cell polarization: an emerging model points in the right direction. *Annu Rev Cell Dev Biol* 21:155–76

Lee H, Adler PN (2002) The function of the frizzled pathway in the *Drosophila* wing is dependent on inturned and fuzzy. *Genetics* 160:1535–47

Lehman JM, Laag E, Michaud EJ *et al.* (2009) An essential role for dermal primary cilia in hair follicle morphogenesis. *J Invest Dermatol* 129:438–48

Lehman JM, Michaud EJ, Schoeb TR *et al.* (2008) The Oak Ridge polycystic kidney mouse: modeling ciliopathies of mice and men. *Dev Dyn* 237:1960–71

Lichti U, Anders J, Yuspa SH (2008) Isolation and short-term culture of primary keratinocytes, hair follicle populations and dermal cells from newborn mice and keratinocytes from adult mice for *in vitro* analysis and for grafting to immunodeficient mice. *Nat Protoc* 3:799–810

Liu A, Wang B, Niswander LA (2005) Mouse intraflagellar transport proteins regulate both the activator and repressor functions of Gli transcription factors. *Development* 132:3103–11

Mehrel T, Hohl D, Rothnagel JA *et al.* (1990) Identification of a major keratinocyte cell envelope protein, loricrin. *Cell* 61:1103–12

Mill P, Mo R, Fu H *et al.* (2003) Sonic hedgehog-dependent activation of Gli2 is essential for embryonic hair follicle development. *Genes Dev* 17:282–94

Park TJ, Haigo SL, Wallingford JB (2006) Ciliogenesis defects in embryos lacking inturned or fuzzy function are associated with failure of planar cell polarity and Hedgehog signaling. *Nat Genet* 38:303–11

Paus R, Muller-Rover S, Van Der Veen C *et al.* (1999) A comprehensive guide for the recognition and classification of distinct stages of hair follicle morphogenesis. *J Invest Dermatol* 113:523–32

Ravni A, Qu Y, Goffinet AM *et al.* (2009) Planar cell polarity cadherin Celsr1 regulates skin hair patterning in the mouse. *J Invest Dermatol* 129:2507–9

Rohatgi R, Milenkovic L, Scott MP (2007) Patched1 regulates hedgehog signaling at the primary cilium. *Science* 317:372–6

Schneider MR, Schmidt-Ullrich R, Paus R (2009) The hair follicle as a dynamic miniorgan. *Curr Biol* 19:R132–42

Simons M, Mlodzik M (2008) Planar cell polarity signaling: from fly development to human disease. *Annu Rev Genet* 42:517–40

Singla V, Reiter JF (2006) The primary cilium as the cell's antenna: signaling at a sensory organelle. *Science* 313:629–33

St-Jacques B, Dassule HR, Karavanova I *et al.* (1998) Sonic hedgehog signaling is essential for hair development. *Curr Biol* 8:1058–68

Strutt D, Warrington SJ (2008) Planar polarity genes in the *Drosophila* wing regulate the localisation of the FH3-domain protein Multiple Wing Hairs to control the site of hair production. *Development* 135: 3103–11

Veland IR, Awan A, Pedersen LB *et al.* (2009) Primary cilia and signaling pathways in mammalian development, health and disease. *Nephron Physiol* 111:p39–53

Wang Y, Nathans J (2007) Tissue/planar cell polarity in vertebrates: new insights and new questions. *Development* 134:647–58

Wong LL, Adler PN (1993) Tissue polarity genes of *Drosophila* regulate the subcellular location for prehair initiation in pupal wing cells. *J Cell Biol* 123:209–21

Wong SY, Reiter JF (2008) The primary cilium at the crossroads of mammalian hedgehog signaling. *Curr Top Dev Biol* 85:225–60

Wong SY, Seol AD, So PL *et al.* (2009) Primary cilia can both mediate and suppress Hedgehog pathway-dependent tumorigenesis. *Nat Med* 15:1055–61

Yuspa SH, Kilkenny AE, Steinert PM *et al.* (1989) Expression of murine epidermal differentiation markers is tightly regulated by restricted extracellular calcium concentrations *in vitro*. *J Cell Biol* 109:1207–17

Zallen JA (2007) Planar polarity and tissue morphogenesis. *Cell* 129: 1051–63

Zeng H, Hoover AN, Liu A (2010) PCP effector gene Inturned is an important regulator of cilia formation and embryonic development in mammals. *Dev Biol* 339:418–28

Zhang J, Lipinski RJ, Gipp JJ *et al.* (2009) Hedgehog pathway responsiveness correlates with the presence of primary cilia on prostate stromal cells. *BMC Dev Biol* 9:50