CORRECTION

Intraflagellar transport 27 is essential for hedgehog signaling but dispensable for ciliogenesis during hair follicle morphogenesis

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On p. 2200, Ng et al. (2012) was incorrectly cited in place of Liew et al. (2014). The corrected text and reference appear below. The authors apologise to readers for this mistake.

IFT27 was recently shown to play a crucial role in facilitating ciliary exit of the BBSome (Eguether et al., 2014; Liew et al., 2014), and Ift27-deficient mouse embryonic fibroblasts are unable to maintain low levels of SMO in the cilia when the Hh pathway is inactive (Eguether et al., 2014).

Intraflagellar transport 27 is essential for hedgehog signaling but dispensable for ciliogenesis during hair follicle morphogenesis

Ning Yang1, Li Li2, Thibaut Eguether3, John P. Sundberg4, Gregory J. Pazour3 and Jiang Chen1,*

ABSTRACT
Hair follicle morphogenesis requires precisely controlled reciprocal communications, including hedgehog (Hh) signaling. Activation of the Hh signaling pathway relies on the primary cilium. Disrupting ciliogenesis results in hair follicle morphogenesis defects due to attenuated Hh signaling; however, the loss of cilia makes it impossible to determine whether hair follicle phenotypes in these cilia mutants are caused by the loss of cilia, disruption of Hh signaling, or a combination of these events. In this study, we characterized the function of Ift27, which encodes a subunit of intraflagellar transport (IFT) complex B. Hair follicle morphogenesis of Ift27-null mice was severely impaired, reminiscent of phenotypes observed in cilia and Hh mutants. Furthermore, the Hh signaling pathway was attenuated in Ift27 mutants, which was in association with abnormal ciliary trafficking of SMO and GLI2, and impaired processing of Gli transcription factors; however, formation of the ciliary axoneme was unaffected. The ciliary localization of IFT25 (HSPB11), the binding partner of IFT27, was disrupted in Ift27 mutant cells, and Ift25-null mice displayed hair follicle phenotypes similar to those of Ift27 mutants. These data suggest that Ift27 and Ift25 operate in a genetically and functionally dependent manner during hair follicle morphogenesis. This study suggests that the molecular trafficking machineries underlying ciliogenesis and Hh signaling can be segregated, thereby providing important insights into new avenues of inhibiting Hh signaling, which might be adopted in the development of targeted therapies for Hh-dependent cancers, such as basal cell carcinoma.

KEY WORDS: Intraflagellar transport, Ift27, Hedgehog signaling, Cilia, Hair follicle, Skin, Mouse

INTRODUCTION
The development of the skin starts with the commitment of single-layered surface ectoderm to stratification controlled by the transcription factor TRP63 (Candi et al., 2008, 2007), followed by the terminal differentiation of epidermal keratinocytes (Blanpain et al., 2006). The induction of hair follicle formation is initiated by reciprocal interactions between epidermal keratinocytes and folliculogenic dermal papilla cells mediated by molecular signaling pathways, notably canonical Wnt signaling (Schmidt-Ullrich and Paus, 2005), which requires hedgehog (Hh) signaling (Schmidt-Ullrich and Paus, 2005). Prior studies demonstrated that disrupting genes involved in Hh signaling could result in complete arrest of hair follicle development (Chiang et al., 1999; Mill et al., 2003; St-Jacques et al., 1998; Woo et al., 2012), revealing Hh signaling as an indispensable signaling mechanism during early stages of hair follicle morphogenesis.

In vertebrates, the primary cilium, which is an antenna-like cellular protrusion, serves as the venue for Hh signaling (Corbit et al., 2005; Huangfu and Anderson, 2005; Wong et al., 2009). When the Hh signaling pathway is inactive, Hh receptor PTCH1 occupies the ciliary membrane, suppressing SMO. Upon binding of ligand, such as sonic hedgehog (SHH), the suppression by PTCH1 is relieved, permitting SMO to be activated (Rohatgi et al., 2007; Wang et al., 2009a), thereby activating Gli transcription factors and the expression of downstream target genes (Haycraft et al., 2005; Kim et al., 2009; Tukachinsky et al., 2010). In addition, the primary cilium is essential for the proteolytic processing of Gli factors into transcriptional repressors in the absence of the ligand (Haycraft et al., 2005; Huangfu and Anderson, 2005). A number of recent studies demonstrated that disrupting cilia formation results in developmental arrest of hair follicles by suppressing Hh signaling (Chen et al., 2015; Dai et al., 2013, 2011; Lehman et al., 2009). These studies confirmed the integral role of primary cilia in mediating the epithelial-mesenchymal crosstalk required for hair follicle morphogenesis. However, the loss of the ciliary axoneme in these mutants made it impossible to understand whether the attenuated Hh signaling was caused by the loss of cilia, defective trafficking of Hh components, or a combination of both.

The assembly of the ciliary axoneme and the processing of Hh signals require intraflagellar transport (IFT) (Huangfu et al., 2003). IFT is an evolutionarily conserved intracellular trafficking mechanism, which was first characterized in Chlamydomonas reinhardtii (Kozmiński et al., 1993). All IFT proteins identified in Chlamydomonas are found in mammals (Cole and Snell, 2009; Follit et al., 2009). The IFT system comprises IFT-A and IFT-B protein complexes. Both IFT-A and IFT-B are essential for cilia assembly and maintenance, as well as the transduction of molecular signals. IFT-B seems to be more fundamental for ciliary assembly, whereas IFT-A plays a role in retrograde transport and the entry of some G-protein-coupled receptors into cilia (Cole et al., 1998; Follit et al., 2009; Rosenbaum and Witman, 2002). Disrupting subunits of IFT complexes could result in the loss of the ciliary axoneme and attenuated Hh signaling (Houde et al., 2006; Huangfu et al., 2003). IFT27 belongs to the IFT-B complex (Qin et al., 2007). In contrast to other IFT proteins, IFT27 does not have homologs in Caenorhabditis elegans or Drosophila melanogaster, which do not rely on primary cilia to process Hh signals, suggesting that IFT27 might possess unique roles in ciliogenesis and Hh signaling in comparison to other IFT proteins. IFT27 is a small Rab-like GTPase
that is capable of forming a heterodimer with IFT25 (HSPB11) (Bhogaraju et al., 2011; Cole, 2003; Wang et al., 2009b). In a previous study, we discovered that IFT25 is dispensable for cilia assembly but remains essential for the trafficking of Hh signaling molecules in cilia (Keady et al., 2012). It is particularly intriguing to know whether these intracellular trafficking molecules are essential for the transduction of molecular signals in the context of epithelial-mesenchymal interaction during the development of multicellular organs.

Here, we examined the role of Ift27 during embryonic development of the skin and found that disrupting Ift27 expression blocks hair follicle morphogenesis. The hair follicle phenotype was associated with attenuated Hh signaling in Ift27 mutant skin. Accordingly, primary dermal fibroblasts isolated from Ift27 mutants were unable to respond to Hh pathway activation. Interestingly, the formation of primary cilia was unaffected in Ift27 mutant cells and we found that disrupted Hh signaling was associated with the abnormal ciliary accumulation of SMO in Ift27 mutant cells. Moreover, the ciliary localization of IFT25 was disrupted in Ift27 mutant cells, and Ift27 mutant mice displayed hair follicle phenotypes similar to those of Ift27 mutants. Thus, data obtained from this study suggest that Ift27 and Ift25 may operate in a molecularly and functionally interdependent fashion during hair follicle formation through regulating ciliary trafficking of Hh pathway components.

RESULTS

Expression of Ift27 in the mouse skin and hair follicle

To understand the relevance of Ift27 in skin development, we examined Ift27 expression at the transcriptional and translational levels. Ift27 mRNA and protein were expressed throughout embryonic skin development (supplementary material Fig. S1A; Fig. 1A,B). Interestingly, IFT27 levels decreased significantly in P28 skin (P<0.05), which is concomitant with the completion of hair follicle morphogenesis and the entrance into the first hair cycle. Furthermore, quantitative RT-PCR and in situ hybridization demonstrated that Ift27 mRNA is expressed in both epidermis and dermis (supplementary material Fig. S1B; Fig. 1C,C′), including follicular keratinocytes and dermal papilla cells of developing hair follicles (Fig. 1C,C′).

By contrast, Ift27 mRNA and protein were undetectable in hair follicles or skin of homozygous Ift27 mutants (Fig. 1C′,D,E). Thus, the homozygous Ift27 mutants are essentially Ift27-null mutants and are hereafter referred as Ift27−/−. Throughout this study, phenotypes observed in heterozygous mice (Ift27+/−) were indistinguishable from those of wild-type littermates (data not shown), and the wild-type littermates (Ift27+/+) were used as controls.

Disrupting Ift27 blocks the differentiation of follicular keratinocytes but not epidermal keratinocytes

The dorsal skin of mutant embryos was examined for developmental abnormalities. Hematoxylin and Eosin (H&E) staining of E14.5 and E15.5 skin did not reveal any apparent defects in Ift27−/− embryos (supplementary material Fig. S2A and Fig. S3A). Furthermore, immunofluorescence labeling of TRP63, KRT14, KRT1, LOR (Fig. 2B; supplementary material Fig. S2B,C and Fig. S3B), BrdU labeling (Fig. 2C; supplementary material Fig. S3G,H and Fig. S4A) and TUNEL assay (data not shown) revealed normal epidermal stratification, differentiation, proliferation and apoptosis in Ift27−/− skin throughout early (E14.5) and late (E18.5) stages of epidermal development. The expression levels of canonical Wnt and Notch target genes and the activation of β-catenin signaling pathways were also unchanged in Ift27−/− skin (supplementary material Fig. S4B-E). These observations suggest that Ift27 is unlikely to be involved in embryonic development of the epidermis. However, the E18.5 Ift27−/− skin displayed a striking hair follicle phenotype, in which the majority of hair follicles were substantially reduced in size (Fig. 2A). Therefore, we focused our investigation on the formation of hair follicles.

Classification of the developmental stages of hair follicles based on morphological features described by Paus et al. (1999) revealed that the development of hair follicles in Ift27−/− skin was significantly delayed. Specifically, in E18.5 control skin, the percentage of stage 1-2, 3-4 and stage 5-6 hair follicles was 29.3±17.9%, 41.8±9.7% and 28.8±21.3%, respectively. In the Ift27−/− skin, up to 90.1% of hair follicles remained in stage 1-2 (P<0.001), whereas only 0.7% developed beyond stage 5 (P<0.01) (Fig. 2D). Interestingly, the number of hair follicles (irrespective of their differentiation stages) was only slightly reduced in Ift27−/− skin at E18.5 (P=0.11;
Fig. 2. Skin and hair follicle phenotypes of Ift27−/− embryos. (A) Representative histology (H&E staining) of E18.5 control (Ift27+/+) and Ift27−/− dorsal skin. (B) Expression of differentiation markers of the epidermis at E18.5. There is no apparent difference in the expression profiles of KRT1 and LOR in control and Ift27−/−. (C) BrdU+ cells in the basal layer of the interfollicular epidermis of E18.5 control and Ift27−/−. (D) Distribution of the developmental stages of hair follicles of E18.5 dorsal skin. A minimum of 100 hair follicles were analyzed; n≥2. (E) Number of hair follicles in E15.5 and E18.5 control and Ift27−/− dorsal skin per microscopy field (100×); n≥4. (F) BrdU+ follicular keratinocytes in stage 2 hair follicles of E18.5 control and Ift27−/−. (G,H) Representative images (G) and statistical analysis (H) of double immunofluorescence labeling of AE13 (red) and KRT75 (green) of hair follicles in E18.5 control and Ift27−/− skin. (I,J) Representative gross appearance (I) and histology (H&E staining, J) of skin transplants at 35 days post-transplantation. *P<0.05, **P<0.01. Scale bars: 50 µm in A,B,G; 100 µm in J.

Fig. 2E), but the number of proliferating cells in Ift27−/− hair follicles was markedly reduced (P<0.01; Fig. 2F; supplementary material Fig. S4A). Thus, the data obtained so far suggested that delayed hair follicle development in Ift27−/− embryos was likely to be associated with impaired responses to mitogenic signals.

Examination of hair follicle differentiation markers, such as KRT75 (companion cell layer) and AE13 (hair cortex), demonstrated that 3.4±0.8 and 3.1±0.9 wild-type hair follicles (per microscopic field) expressed these markers, respectively (Fig. 2G,H), compared with only 0.1±0.2 and 0.2±0.2 hair follicles in Ift27−/− skin (P<0.001; Fig. 2G,H). This observation strongly suggests that delayed hair follicle development in Ift27−/− skin is caused by abnormal follicular keratinocyte differentiation.

By contrast, the induction of hair follicle formation was indistinguishable between Ift27−/− and control skin. Specifically, the number and expression pattern of KRT17+ hair germs in E15.5 Ift27−/− skin were comparable to those in wild-type littersmates (supplementary material Fig. S3C). Moreover, the expression of LEF1 (supplementary material Fig. S3D), a marker for the activation of canonical Wnt signaling, and of NGFR (formerly known as p75NTR) (supplementary material Fig. S3E), alkaline phosphatase (AP) activity (supplementary material Fig. S3F) and the number of BrdU+ follicular keratinocytes (supplementary material Fig. S3G,H) were comparable in early stage control and Ift27−/− hair follicles.

Because Ift27−/− mice die shortly after birth we could not determine whether the development of hair follicles was temporarily delayed or impermissible in these animals. To address this question, dorsal skin of E18.5 Ift27+/+ and Ift27−/− mice was transplanted onto nude (Foxn1nu) mice for further development and maturation. Ift27−/− skin was able to engraft. However, there was a drastic reduction in the number of hairs in the Ift27−/− skin (Fig. 2I). H&E staining of tissues collected 5 weeks post-grafting confirmed that the hair phenotype was due to the lack of hair follicles (Fig. 2J). Thus, disrupting Ift27 blocked hair follicle development.

Hh signaling is perturbed in Ift27−/− skin

The hair follicle phenotype of Ift27−/− mice was reminiscent of that of Hh mutants (Chiang et al., 1999; Gat et al., 1998; Mill et al., 2003; St-Jacques et al., 1998; Woo et al., 2012). Consistently, in situ hybridization and quantitative RT-PCR revealed that Ift27−/− skin (E15.5 and E18.5) had significantly reduced expression levels of Hh target genes, such as Gli1 and Ptc1, in follicular keratinocytes and dermal papilla cells (Fig. 3A-C; supplementary material Fig. S5A,B). Interestingly, the expression of Shh was essentially indistinguishable between control and Ift27−/− embryos (P=0.08; Fig. 3C; supplementary material Fig. S5C). Moreover, western blotting revealed reduced processing of full-length GLI3 (GLI3-FL) to GLI3 repressor (GLI3-R) in skin biopsied from E18.5 Ift27 mutants (Fig. 3D), which led to an increased GLI3-FL/GLI3-R ratio (Fig. 3D). These findings suggest that perturbation of the Hh pathway is likely to have occurred downstream of ligand but upstream of Gli transcription factors.
To further determine how Ift27 participates in transducing Hh signals during hair follicle development, primary dermal fibroblasts were treated with SMO agonist (SAG) and the expression of Hh-responsive genes examined. SAG treatment drastically induced the expression of Hh target genes (expression of Hh-responsive genes examined. SAG treatment drastically induced the expression of Hh target genes (Fig. 3E–G). By contrast, the expression of Smo was unaffected (Fig. 3H). IFT27 was essentially undetectable in Ift27−/− cells by western blotting (Fig. 3I). These data obtained from primary dermal fibroblasts were consistent with in vivo findings, thereby strengthening the idea that IFT27 participates in Hh signaling downstream of SMO activation. Moreover, primary dermal fibroblasts of Ift27−/− mice demonstrated increased GLI3-FL levels and an increased GLI3-FL/GLI3-R ratio (supplementary material Fig. S6), suggesting that the processing of Gli transcription factors was disrupted in Ift27−/− cells.

Formation of the ciliary axoneme is unaffected in Ift27−/− skin
Because Hh signaling requires primary cilia and the formation of cilia requires IFT, we speculated that Ift27 might be essential for Hh signaling by controlling ciliogenesis in follicular keratinocytes and dermal papilla cells. Immunofluorescence labeling with a number of ciliary markers, namely ARL13B, ADCY3 (ACIII) and acetylated α-tubulin, revealed indistinguishable patterns of primary cilia in wild-type and Ift27−/− embryos at various developmental stages (Fig. 4A; supplementary material Fig. S7; data not shown). Scanning electron microscopy of primary keratinocytes isolated from control and Ift27−/− embryos also revealed primary cilia of comparable appearance (supplementary material Fig. S8). Thus, these observations suggested that neither the formation of the ciliary axoneme nor the localization of ciliary membrane-associated proteins, such as ARL13B and ADCY3, was affected in Ift27−/− cells.

Skin transplants generated from control embryos contained an abundance of ciliated follicular keratinocytes (supplementary material Fig. S9A). Remarkably, cells in the epidermal invaginations of Ift27−/− skin transplants also contained well-ciliated keratinocytes (supplementary material Fig. S9B). These cells are proposed to be keratinocytes that are committed to the follicular fate but have failed to develop into hair follicles because of disrupted Hh signaling or primary cilia formation (Chen et al., 2015; Chiang et al., 1999; Dai et al., 2013, 2011; Gat et al., 1998; Mill et al., 2003; St-Jacques et al., 1998). Thus, the congregation of these ciliated keratinocytes in the epidermal invaginations of Ift27−/− skin transplants not only confirmed this speculation but also strengthened our previous observation that Ift27 is essential for Hh signaling (Fig. 3) but not for primary cilia formation (Fig. 4).

To further examine the ciliogenic potential of Ift27−/− cells, primary keratinocytes were treated with cytochalasin D (cyto D), a compound capable of elongating the ciliary axoneme. Twenty-four hours after cyto D treatment (0.5 µM), the number of ciliated cells remained unchanged in both wild-type and Ift27−/− keratinocytes (Fig. 4C) and the median length of the ciliary axoneme increased significantly in both genotypes (P<0.0001; Fig. 4B,D).

Thus, data obtained from in vivo and in vitro experiments strongly suggest that Ift27 does not participate in ciliogenesis or ciliary maintenance in epidermal keratinocytes.

Ciliary localization of SMO is disrupted in Ift27−/− primary dermal fibroblasts
To gain insight into how Ift27 regulates the Hh pathway, we examined the ciliary localization of Hh pathway components in primary dermal fibroblasts isolated from E18.5 mouse embryos. Similar to what was observed in Ift27−/− keratinocytes, Ift27−/− primary dermal fibroblasts were capable of forming primary cilia

Fig. 3. Hh signaling is attenuated in Ift27−/− skin. (A,B) In situ hybridization of Gli1 (A) and Ptch1 (B) in E18.5 control (Ift27+/+) and Ift27−/− skin; n=3. Arrows indicate hair follicles of similar stages; arrowheads point to dermal papilla cells; dashed line delineates the epidermal-dermal junction. (C) Relative expression levels of Gli1 and Shh in the skin of E18.5 Ift27+/+ and Ift27−/− embryos by quantitative RT-PCR; n=4. (D) Expression of full-length (GLI3-FL) and repressor (GLI3-R) forms of GLI3 in skin of E18.5 control and Ift27 mutants. (E-H) Responses of Ift27+/+ and Ift27−/− primary dermal fibroblasts to SAG treatment as determined by the relative expression levels of Gli1 (E), Ptch1 (F), Gli2 (G) and Smo (H); n=3. (I) Representative western blotting of IFT27 and GL1 in SAG-treated Ift27+/+ and Ift27−/− dermal fibroblasts and quantification of GL1; n=4. *P<0.05, **P<0.01 (two-way ANOVA and Bonferroni post-test). Scale bars: 100 µm.
in vitro (Figs 5 and 6). In control dermal fibroblasts, SMO is essentially undetectable in the ciliary axoneme when the Hh pathway is inactive (cells treated with DMSO, Fig. 5A, upper panel). Upon pathway activation (SAG treatment), control cells robustly accumulated SMO in the cilium (Fig. 5A,B). By contrast, SMO abnormally accumulated in cilia of Ift27−/− dermal fibroblasts even when the Hh pathway was inactive (Fig. 5A, lower panel). This suggested that the ciliary exit of SMO was defective in Ift27−/− cells.

In SAG-treated control fibroblasts GLI2 accumulated at the ciliary tip (Fig. 5C,D), which was consistent with an activated Hh pathway as documented previously (Kim et al., 2009). By contrast, ciliary tip accumulation of GLI2 was severely disrupted in SAG-treated Ift27−/− dermal fibroblasts (Fig. 5C,D). This result was not only consistent with the suppressed Hh pathway observed in vivo and in vitro (Fig. 3) but also suggested defective trafficking of Hh components in Ift27−/− cells in response to Hh pathway activation.

**IFT27 and the expression and localization of other IFT proteins**

Since the loss of Ift27 does not seem to impact ciliogenesis, we further determined whether the expression and ciliary localization of known ciliogenic IFT proteins are affected in Ift27−/− cells. First, double immunofluorescence labeling and western blotting confirmed the loss of expression and of the ciliary localization of IFT27 in Ift27−/− cells (Fig. 6A,E). Subsequently, we found that the expression patterns of IFT88 and IFT140, which belong to the IFT-B and IFT-A complexes, respectively, were unaffected in Ift27−/− cells (Fig. 6C,D). Specifically, IFT88 was enriched in the axoneme and basal body, whereas IFT140 was predominantly localized at the basal body (Fig. 6C,D). The expression level of IFT140 was unchanged in Ift27−/− cells as confirmed by western blotting (Fig. 6E). By contrast, in Ift27−/− cells the ciliary localization of IFT25 was essentially undetectable (Fig. 6B) and its expression was significantly reduced by 35% (Fig. 6E,F). These data suggested that IFT27 is not required for the expression and localization of IFT proteins that are involved in cilia formation but selectively regulates the stability and localization of its binding partner IFT25, which is also dispensable during cilia formation.

**Ift25 mutants phenocopy the hair follicle phenotypes of Ift27−/− mice**

To confirm the functional relevance of IFT25 during hair follicle formation, we examined the skin of Ift25-null (Ift25−/−) mice (Keady et al., 2012). As expected, Ift25−/− mice displayed a hair follicle phenotype almost identical to that of Ift27−/− mice (Fig. 7A). Specifically, although the total number of hair follicles remained unaffected (Fig. 7B), the hair follicles of E18.5 Ift25−/− embryos...
IFT27, IFT25, IFT88 and IFT140 were expressed in ANOVA. These data suggested that
IFT25, are not required for the formation and maintenance of the ciliary axoneme but are essential for processing Hh signaling
processes in the context of hair follicle morphogenesis, and
findings strongly suggest that disrupting Hh signaling or cilia
disrupting ciliogenesis results in the arrest of hair follicle
epidermal lineage (Dai et al., 2013) or in mesenchymal cells
loss-of-function mutation in IFT27 (C99Y) in Bardet-Biedl syndrome (BBS) demonstrated that IFT27 indeed plays an important role in human development and health (Aldahmesh et al., 2014).

The hair follicle phenotype observed in the germline mutant Ift27−/− mice is reminiscent of that previously reported for Hh and primary cilia germline mutants (Chen et al., 2015; Chiang et al., 1999; Dai et al., 2011; Gat et al., 1998; Mill et al., 2003; St-Jacques et al., 1998; Woo et al., 2012). Interestingly, when Hh or ciliary genes were disrupted in a tissue-specific fashion, i.e. in the epidermal lineage (Dai et al., 2013) or in mesenchymal cells (Lehman et al., 2009; Woo et al., 2012), the resultant phenotypes were almost identical to those in germline mutants (Chen et al., 2015; Chiang et al., 1999; Dai et al., 2011; Gat et al., 1998; Mill et al., 2003; St-Jacques et al., 1998; Woo et al., 2012). These findings strongly suggest that disrupting Hh signaling or cilia formation/function in either or both tissue compartments of the hair follicle will, individually or collectively, contribute to hair follicle morphogenesis defects.

IFT27 and IFT25 are subunits of the IFT-B complex (Follit et al., 2014; Rosenbaum and Witman, 2002). Direct interactions between these two proteins have been demonstrated previously (Bhogaraju et al., 2011; Keady et al., 2012; Wang et al., 2009b). In this study, we further demonstrated that the stability and subcellular localization of these proteins are required for the morphogenesis of hair follicles, such that disrupting the expression of either of them

**DISCUSSION**

Hedgehog signaling is required for the crosstalk between follicular keratinocytes and dermal papilla cells during hair follicle development. Activation of the Hh signaling pathway requires the primary cilia. A number of recent studies unequivocally demonstrated that the primary cilium is essential for hair follicle morphogenesis; disrupting ciliogenesis results in the arrest of hair follicle development due to disrupted Hh signaling (Chen et al., 2015; Chiang et al., 1999; Dai et al., 2011; Gat et al., 1998; Mill et al., 2003; St-Jacques et al., 1998; Woo et al., 2012). Interestingly, when Hh or ciliary genes were disrupted in a tissue-specific fashion, i.e. in the epidermal lineage (Dai et al., 2013) or in mesenchymal cells (Lehman et al., 2009; Woo et al., 2012), the resultant phenotypes were almost identical to those in germline mutants (Chen et al., 2015; Chiang et al., 1999; Dai et al., 2011; Gat et al., 1998; Mill et al., 2003; St-Jacques et al., 1998; Woo et al., 2012). These findings strongly suggest that disrupting Hh signaling or cilia formation/function in either or both tissue compartments of the hair follicle will, individually or collectively, contribute to hair follicle morphogenesis defects.

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**Fig. 7.** Ift25−/− embryos display defective hair follicle development similar to Ift27−/−. (A) H&E staining of E18.5 Ift25+/+ and Ift25−/− dorsal skin. (B) Statistical analysis of the number of hair follicles in the dorsal skin of E18.5 Ift25+/+ and Ift25−/− embryos per microscopy field (100×); n=4. (C) Distribution of developmental stages of hair follicles of E18.5 dorsal skin. A minimum of 100 hair follicles were analyzed. Scale bars: 100 µm.
could result in hair follicle morphogenesis arrest. It is worth noting that the Ift25 and Ift27 mutant mice were able to survive to term, whereas other IFT-B mutants, such as Ift88<sup>floxed</sup>, are unable to survive beyond mid-gestation (Huangfu et al., 2003). The relatively mild phenotypes in the former suggested that IFT25 and IFT27 might have more restricted functions, such as in transducing Hh signals as shown herein, in comparison to IFT88, which is essential for ciliogenesis in a wide range of cell types. Interestingly, disrupting Ift88 resulted in similar hair follicle phenotypes (Lehman et al., 2009) to those of the Ift25 and Ift27 mutants described herein. Because these hair follicle phenotypes were attributed to attenuated Hh signaling, these studies collectively suggest that the core function of primary cilia during hair follicle morphogenesis is the transduction of Hh signals.

The ciligenic capability of Ift27<sup>−/−</sup> cells was further examined using cyto D, which is capable of elongating the ciliary axoneme. Ift27<sup>−/−</sup> dermal fibroblasts treated with cyto D showed a comparable capacity to elongate the ciliary axoneme as control cells. Similarly, neither the expression nor localization of known ciligenic IFT proteins, such as IFT88 and IFT140, nor the trafficking of ciliary membrane-associated proteins, such as ARL13B and ADCY3, was affected in Ift27<sup>−/−</sup> cells. These data suggested that Ift27 is not involved in the machinery required for ciliary length maintenance nor the trafficking of key ciliogenic proteins in mammalian primary cells. A recent study on the flagellum of Trypanosoma brucei suggested that IFT27 is involved in both anterograde and retrograde trafficking (Huet et al., 2014). Whether IFT27 performs cellular functions beyond trafficking Hh components remains to be determined in mammalian cells.

IFT27 was recently shown to play a crucial role in facilitating ciliatory exit of the BBSome (Eguether et al., 2014; Ng et al., 2012), and Ift27-deficient mouse embryonic fibroblasts are unable to maintain low levels of SMO in the cilia when the Hh pathway is inactive (Eguether et al., 2014). In this study, we further demonstrated that suppressed Hh signaling in Ift27<sup>−/−</sup> primary dermal fibroblasts is also associated with abnormal accumulation of SMO in the cilium. However, ciliary accumulation of SMO does not necessarily correlate with pathway activation (Rohatgi et al., 2009). Thus, data obtained in this study suggest that disrupted Hh signaling in Ift27<sup>−/−</sup> cells may be associated with abnormal trafficking of Hh pathway components in the primary cilium.

The primary cilium is essential for the processing of Gli transcription factors. It is well documented that in the absence of Hh ligands GLI3-FL is processed to its repressor form GLI3-R, resulting in a relatively low GLI3-FL/GLI3-R ratio (Niewiadomski et al., 2014). Data obtained from this study demonstrated that disrupting Ift27 results in an attenuated Hh pathway and, countervintuiously, an elevated GLI3-FL level, suppressed GLI3-R level, and increased GLI3-FL/GLI3-R ratio. We speculate that these changes in Ift27<sup>−/−</sup> cells do not necessarily correspond to activation of the Hh pathway. Rather, they might merely reflect that IFT27 is required for proper processing of GLI3-FL. These data are in line with the well-documented observation that ligand-induced activation of the Hh pathway, and thus the production of Gli activators (but not GLI3-R), plays a predominant role during hair follicle morphogenesis (Mill et al., 2003). Thus, despite the elevated GLI3-FL level or increased GLI3-FL/GLI3-R ratio, the net outcome of disrupting Ift27 was Hh pathway suppression and impaired hair follicle morphogenesis. Moreover, Hh pathway activity is modulated by multiple phosphorylations of Gli transcription factors (both activators and suppressors) (Niewiadomski et al., 2014). Thus, data obtained from this study support the notion that Hh pathway activity may not be evaluated simplistically based on the levels of Gli transcription factors, and that precisely controlled processing of Gli transcription factors is an integral component of the Hh signaling pathway.

By examining key events associated with skin and hair follicle morphogenesis, we noticed that the effect of loss of Ift27 was restricted to the proliferation and cytodifferentiation of follicular keratinocytes. Stratification, proliferation and differentiation of the interfollicular epidermis and the induction of hair germ were unaffected in Ift27<sup>−/−</sup> mutants. These phenotypes correlated well with related molecular signaling pathways, such as attenuated Hh signaling and relatively normal canonical Wnt, Notch, BMP and TGFβ signaling in Ift27<sup>−/−</sup> skin. Thus, the cell fate-specific function of IFT27 can be attributed primarily to its function in processing Hh signals. Whether IFT27 participates in the transduction of other molecular signals in the skin remains to be empirically determined.

In summary, this study demonstrated an essential role of Ift27 in mediating Hh signaling during follicular keratinocyte proliferation and differentiation but not the differentiation program of the interfollicular epidermis. Although the full spectrum of Ift27 functions remains to be uncovered, the rather restricted function of IFT27 during embryonic skin development provides important insight into a novel avenue of targeting Hh signaling, such as in the targeted therapy for basal cell carcinoma (BCC), in which tumors not only originate from the hair follicles but are also dependent on hyperactive Hh signaling (Epstein, 2008; Kasper et al., 2012). Because IFT27 is likely to mediate Hh signaling through regulating the ciliary localization of SMO and the processing of Gli transcription factors, targeting IFT27 might prevent the development of drug resistance caused by acquired mutations in the SMO receptor following Vismodegib treatment (Yauch et al., 2009).

**MATERIALS AND METHODS**

**Generation of Ift27<sup>−/−</sup> mice and skin transplantation**

Ift27-targeted embryonic stem cells [Ift27<sup>tm1a(EUCOMM);Hmgu</sup>] were obtained from the EUCOMM project and used to create Ift27 mutant mice (Eguether et al., 2014). Homozygous mutants were obtained by crossing Ift27<sup>−/−</sup> mice. Genotypes of Ift27<sup>−/−</sup> mutants were determined using the following primer pairs (5′-3′): F, GGAGGATGAGGATCTTCTCC; R1, TCCAACCTGACTCCGTCCCT; R2, CCCCCCTGACGTCAGACATA. E15.5 and E18.5 embryos were obtained by timed mating. Full-thickness skin transplantation was performed as previously described (Dai et al., 2011). All procedures related to mice were approved by the IACUC of the University of Massachusetts Medical School and Stony Brook University.

**BrdU labeling and tissue processing**

Bromodeoxyuridine (BrdU) labeling was performed by intraperitoneally injecting 10 µg BrdU labeling reagent (Invitrogen) per gram body weight 2 hours prior to euthanasia. Skin specimens were obtained by removing the full-thickness dorsal skin with surgical tools and were fixed in 10% buffered formalin at 4°C overnight. Fixed skin specimens were processed and sectioned for routine histology analysis. For all analyses, a minimum of three embryos obtained from at least two different litters were examined. AP staining was performed as previously described (Tsai et al., 2010). Specifically, frozen skin sections were fixed in 4% paraformaldehyde, soaked in B3 buffer (0.1 M Tris pH 9.5, 0.1 M NaCl, 0.05 M MgCl₂) for 20 min, then in NBT (1:200)/BCIP (1:267) solution (Roche) for 20 min, and imaged.

**Cell culture and in vitro assays**

The isolation of primary skin keratinocytes and dermal fibroblasts was conducted as described (Marshall et al., 2005). Briefly, E18.5 skin was digested by dispase II (Roche) to separate epidermis and dermis. Epidermis or dermis was then digested with trypsin or collagenase, respectively, to collect keratinocytes and dermal fibroblasts. Cells were used directly for
skin reconstitution or cultured in vitro. Keratinocytes were cultured in defined keratinocyte serum-free medium (Life Technologies) on collagen I-coated tissue culture plates. Fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (4.5 g/l glucose) supplemented with 10% fetal bovine serum and 100 U/ml penicillin and 100 U/ml streptomycin. To examine cilia formation, cells were grown to near confluence and substrate starved for 24 h before being treated with SAG (100 nM, Calbiochem) or cyto D (0.5 μM, Sigma) for an additional 24 h in serum-free medium.

**Quantitative RT-PCR and western blotting**

RNA isolation and quantitative RT-PCR analyses were performed as described previously (Dai et al., 2013). The following probes were used for TaqMan analysis: Ift27, ABI Mm00508912_m1; Ptk1, Mm00436026_m1; Glil1, Mm00494635_m1; Glil2, Mm01293111_m1; Smo, Mm01162710_m1; and Actb, Mm00607939_m1 (Life Technologies). Results were analyzed using the ΔΔCT method. Relative expression levels of target genes were determined by comparison with wild-type or treatment controls after normalizing to β-actin.

Protein was extracted either by homogenizing skin in cold RIPA lysis buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 2 mM EDTA, 50 mM NaF) supplemented with protease inhibitors or by digesting cells directly in RIPA lysis buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl pH 7.4, 150 mM NaCl), or by digesting cells directly in RIPA lysis buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl pH 7.4, 150 mM NaCl) supplemented with proteinase inhibitors or by digesting cells directly in RIPA lysis buffer. Cell lysates were cleared by centrifugation at 13,000 g for 20 min at 4°C; protein concentration was determined using the BCA Protein Assay Kit (Pierce). Nuclear fractionation was conducted with the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific). Proteins were separated on a NuPage gel (Life Technologies) and transferred to Hybond nitrocellulose (Pierce).

**Immunofluorescence labeling and microscopy**

Immunofluorescence labeling and microscopy were performed as described previously (Dai et al., 2013). The following primary antibodies were used: β-actin (1:1000; Santa Cruz, sc-7457), KRT14 [1:1000 (Roop et al., 1987)], KRT1 [1:500 (Roop et al., 1987)], KRT17 [1:400; Abcam, ab111446], LOR [1:100 (Roop et al., 1987)], LIF1 [1:100; Cell Signalling, 2253S], NGRFR1 [1:200; p75NTR, Promega, G3231], BrdU (1:20; Life Technologies, 552150), cilia formation, cells were grown to near confluence and substrate starved for 24 h before being treated with SAG (100 nM, Calbiochem) or cyto D (0.5 μM, Sigma) for an additional 24 h in serum-free medium.

**Scanning electron microscopy (SEM)**

For SEM of cilia, primary keratinocytes isolated from E18.5 mice were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Cells were then post-fixed, dehydrated and gold coated through standard procedures (Bechtold, 2000). Samples were examined on a JOEL S-3000N scanning electron microscope (Hitachi).

**Statistical analyses**

All quantifications are presented as means±s.d. Student’s t-test was used unless stated otherwise. One-way ANOVA and two-way ANOVA were conducted using GraphPad Prism. P<0.05 was considered statistically significant.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

N.Y., J.P.S., G.J.P. and J.C. designed experiments and analyzed data. L.L. and T.E. performed preliminary experiments. N.Y. and J.C. carried out the experiments, collected the data and wrote the manuscript.

**References**

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**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl doi:10.1242/dev.115261/-/DC1

**Immunofluorescence labeling and microscopy**

Immunofluorescence labeling and microscopy were performed as described previously (Dai et al., 2013). The following primary antibodies were used: TRP63 (1:100; Santa Cruz Biotechnology, sc-8431), KRT14 [1:1000 (Roop et al., 1987)], KRT1 [1:500 (Roop et al., 1987)], KRT17 [1:400; Abcam, ab111446], LOR [1:100 (Roop et al., 1987)], LIF1 [1:100; Cell Signalling, 2253S], NGRFR1 [1:200; p75NTR, Promega, G3231], BrdU (1:20; Life Technologies, 552150), cilia formation, cells were grown to near confluence and substrate starved for 24 h before being treated with SAG (100 nM, Calbiochem) or cyto D (0.5 μM, Sigma) for an additional 24 h in serum-free medium.

**Scanning electron microscopy (SEM)**

For SEM of cilia, primary keratinocytes isolated from E18.5 mice were fixed overnight in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Cells were then post-fixed, dehydrated and gold coated through standard procedures (Bechtold, 2000). Samples were examined on a JOEL S-3000N scanning electron microscope (Hitachi).

**Statistical analyses**

All quantifications are presented as means±s.d. Student’s t-test was used unless stated otherwise. One-way ANOVA and two-way ANOVA were conducted using GraphPad Prism. P<0.05 was considered statistically significant.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

N.Y., J.P.S., G.J.P. and J.C. designed experiments and analyzed data. L.L. and T.E. performed preliminary experiments. N.Y. and J.C. carried out the experiments, collected the data and wrote the manuscript.

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**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl doi:10.1242/dev.115261/-/DC1

**References**


**Fig. S1.** *Ift27* mRNA expression in mouse skin. (A) *Ift27* mRNA level during skin development. n=4. (B) *Ift27* mRNA levels in epidermis and dermis of E18.5 embryos. n=3. *, P<0.05.
Fig. S2. Control (Ift27+/+) and Ift27 mutant (Ift27−/) skins at E14.5. (A) H&E staining. (B) TRP63 (red) and KRT14 (green). (C) KRT1 (red) and KRT14 (green). Note, KRT1 is absent. Scale bars: 50 μm.
Fig. S3. Control (Ift27+/+) and Ift27 mutant (Ift27−/−) skins at E15.5. (A) H&E staining. (B) KRT1 (red) and KRT14 (green). (C) KRT17 (green) and KRT14 (red). (D) LEF1 (red) and KRT14 (green). (E) NGFR (p75NTR, red) and KRT14 (green). (F) Alkaline phosphatase (AP) staining in E16.5 skins. (G) BrdU (red) and KRT14 (green). (H) Quantification of BrdU-positive cells in interfollicular epidermis (IFE) and hair follicles (HF). *, P<0.05. Scale bars: 50 µm.
**Fig S4**

(A) Ift27<sup>+/+</sup> and Ift27<sup>-/-</sup> skins at E18.5. BrdU (red) and KRT14 (green). Note that BrdU-positive cells in stage 2 hair follicles (boxed) were quantified. (B–D) Relative expression levels of Lef1, Ctnnb and Hes1 by quantitative RT-PCR. Note, the expression levels of these Wnt and Notch target genes are comparable in Ift27<sup>+/+</sup> and Ift27<sup>-/-</sup> skins. n=3. (E) Expression of phospho-SMAD2 (pSMAD2) and phospho-SMAD1/5 (pSMAD1/5) in control and mutant skin. Scale bar: 50 µm in A, 25 µm in E.

**Fig. S4.** Control (Ift27<sup>+/+</sup>) and Ift27 mutant (Ift27<sup>-/-</sup>) skins at E18.5. (A) BrdU (red) and KRT14 (green). Note that BrdU-positive cells in stage 2 hair follicles (boxed) were quantified. (B–D) Relative expression levels of Lef1, Ctnnb and Hes1 by quantitative RT-PCR. Note, the expression levels of these Wnt and Notch target genes are comparable in Ift27<sup>+/+</sup> and Ift27<sup>-/-</sup> skins. n=3. (E) Expression of phospho-SMAD2 (pSMAD2) and phospho-SMAD1/5 (pSMAD1/5) in control and mutant skin. Scale bar: 50 µm in A, 25 µm in E.
Fig. S5. Hedgehog signaling in skin of E15.5 control (Ift27+/+) and Ift27 mutant (Ift27−/−). (A–C) In situ hybridization of Gli1 (A), Ptch1 (B), and Shh (C) in E15.5 skins of control and Ift27 mutant, n=3. Dotted lines represent basement membrane. Note that the expression of Shh was comparable in hair follicles of control and Ift27 mutant. Scale bars: 50 µm.
Fig. S6. Expression of full-length GLI3 (GLI3-FL) and repressor form of GLI3 (GLI3-R) in primary dermal fibroblast isolated from control (IFT27+/+) and IFT27 mutants (IFT27−/−). (A) Representative western blotting and quantification of GLI3 in whole cell lysate. (B) Representative western blotting and quantification of GLI3 in the nuclear fraction of the cells. n=3. *, P<0.05.
**Fig. S7**

**A**

$Ift27^{+/+}$  

$Ift27^{-/-}$

**B**

$Ift27^{+/+}$  

$Ift27^{-/-}$

**Fig. S7.** Primary cilia in control ($Ift27^{+/+}$) and $Ift27$ mutant ($Ift27^{-/-}$). (A) Cilia (ARL13B, green) and basal body ($\gamma$-tubulin, red) in hair follicles of E15.5 skins. (B) Cilia (ACIII, green) and basal body ($\gamma$-tubulin, red) in stage 2 hair follicles of E18.5 skins. Lower panels are enlarged boxed areas above. Scale bars: 25 µm.
Fig. S8

**A**  
$lft27^{+/+}$  

**B**  
$lft27^{-/-}$  

**A’**  
$lft27^{+/+}$  

**B’**  
$lft27^{-/-}$  

Fig. S8. Primary cilia in control ($lft27^{+/+}$) and $lft27$ mutant ($lft27^{-/-}$) the primary keratinocytes by scanning electron microscopy. (A) Cilium in a control cell. (B) Cilium in a mutant cell. A’ and B’ are enlarged areas of cilia shown in A and B. Scale bar: 10 µm in A and B; 2 µm in A’ and B’.
Fig. S9. Primary cilia in control (Ift27+/+) and Ift27 mutant (Ift27−/−) skin transplants. (A) Cilia (ARL13B, green) and basal body (γ-tubulin, red) in hair follicles of Ift27+/+ skin transplants. (B) Cilia (ARL13B, green) and basal body (γ-tubulin, red) in the hair follicle-like invagination of Ift27−/− skin transplants. Lower panels are enlarged boxed areas above. Scale bar: 20 µm.