Kif7 regulates Gli2 through Sufu-dependent and -independent functions during skin development and tumorigenesis

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SUMMARY
Abnormal activation of Hedgehog (Hh) signaling leads to basal cell carcinoma (BCC) of the skin, the most common human cancer. Gli2, the major transcriptional activator of Hh signaling, is essential for hair follicle development and its overexpression in epidermis induces BCC formation and maintains tumor growth. Despite its importance in skin development and tumorigenesis, little is known about the molecular regulation of Gli2. Sufu and Kif7 are two evolutionarily conserved regulators of Gli transcription factors. Here, we show that Sufu and Kif7 regulate Gli2 through distinct mechanisms in keratinocytes. Sufu restricts the activity of Gli2 through Sufu-dependent and -independent regulatory functions in Hh signaling: while it promotes Hh pathway activity through the dissociation of Sufu-Gli2 complex, it also contributes to the repression of Hh target genes in the absence of Sufu. Deletion of both Sufu and Kif7 in embryonic skin leads to complete loss of follicular fate. Importantly, although inactivation of Sufu or Kif7 alone in adult epidermis cannot promote BCC formation, their simultaneous deletion induces BCC. These studies establish Sufu and Kif7 as crucial components in the regulation of Gli2 localization and activity, and illustrate their overlapping functions in skin development and tumor suppression.

KEY WORDS: Gli2, Kif7, Sufu, Basal cell carcinoma, Hedgehog, Skin, Mouse

INTRODUCTION
The sonic hedgehog (Shh) signaling pathway is crucial for growth control and patterning during embryonic development and adult homeostasis (Jiang and Hui, 2008). Inactivating mutations in the human PATCHED1 (PCTH1) gene are commonly found in basal cell carcinoma (BCC) and it is well established that abnormal hedgehog (Hh) pathway activation is the cause of BCC (Epstein, 2008; Li and Hui, 2012). Ptc1 encodes the Hh-binding membrane receptor and functions as a major negative regulator of the pathway by inhibiting the signaling membrane protein smoothened (Smo). Among the three Gli proteins, Gli2 is the principal transcriptional activator that mediates Shh signaling in skin development and tumorigenesis. Gli2−/− mice display hair morphogenesis defects similar to those of Shh−/− mice (Mill et al., 2003). Overexpression of Gli2 in epidermis is sufficient to initiate BCC formation and is required to maintain tumor growth (Grachtchouk et al., 2000; Hutchin et al., 2005). Moreover, previous studies suggested that the level of Hh pathway activation determines skin tumor phenotype (Grachtchouk et al., 2003; Grachtchouk et al., 2011). Despite the importance of Gli2 in epidermal development and tumorigenesis, little is known about the underlying mechanisms that regulate Gli2.

Sufu and Kif7 play regulatory roles in Hh signaling and interact directly with the Gli proteins (Cooper et al., 2005; Svärd et al., 2006). Although the actions of Sufu on Gli function have been extensively analyzed using immortalized cell lines (Merchant et al., 2004; Wang et al., 2010; Zeng et al., 2010), the regulation of Gli2 by Sufu in physiological settings remains poorly understood. Recent in vitro studies demonstrated that Sufu forms a complex with Gli proteins to prevent their nuclear accumulation, and Hh stimulation allows the release of Gli proteins from Sufu (Humke et al., 2010; Tukachinsky et al., 2010). Kif7 plays dual regulatory roles in Hh signaling and its action appears to be tissue specific. Kif7 regulates Gli2–/– mice display hair morphogenesis defects similar to those of Shh−/− mice (Mill et al., 2003). Overexpression of Gli2 in epidermis is sufficient to initiate BCC formation and is required to maintain tumor growth (Grachtchouk et al., 2000; Hutchin et al., 2005). Moreover, previous studies suggested that the level of Hh pathway activation determines skin tumor phenotype (Grachtchouk et al., 2003; Grachtchouk et al., 2011). Despite the importance of Gli2 in epidermal development and tumorigenesis, little is known about the underlying mechanisms that regulate Gli2.

Sufu and Kif7 play regulatory roles in Hh signaling and interact directly with the Gli proteins (Cooper et al., 2005; Svärd et al., 2006; Cheung et al., 2009; Endoh-Yamagami et al., 2009). In mice, Sufu is a potent negative regulator of the Hh pathway (Cooper et al., 2005; Svärd et al., 2006). Although the actions of Sufu on Gli function have been extensively analyzed using immortalized cell lines (Merchant et al., 2004; Wang et al., 2010; Zeng et al., 2010), the regulation of Gli2 by Sufu in physiological settings remains poorly understood. In recent in vitro studies demonstrated that Sufu forms a complex with Gli proteins to prevent their nuclear accumulation, and Hh stimulation allows the release of Gli proteins from Sufu (Humke et al., 2010; Tukachinsky et al., 2010). Kif7 plays dual regulatory roles in Hh signaling and its action appears to be tissue specific. Kif7 regulates Gli2–/– mice display hair morphogenesis defects similar to those of Shh−/− mice (Mill et al., 2003). Overexpression of Gli2 in epidermis is sufficient to initiate BCC formation and is required to maintain tumor growth (Grachtchouk et al., 2000; Hutchin et al., 2005). Moreover, previous studies suggested that the level of Hh pathway activation determines skin tumor phenotype (Grachtchouk et al., 2003; Grachtchouk et al., 2011). Despite the importance of Gli2 in epidermal development and tumorigenesis, little is known about the underlying mechanisms that regulate Gli2.

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In this report, we used mouse genetic and cell biological approaches to investigate the regulation of Gli2 by Sufu and Kif7. Molecular analysis in primary keratinocytes indicates that Sufu restricts Gli transcriptional activity by preventing the nuclear accumulation of Gli2 through cytoplasmic retention. Conversely, Kif7 promotes Hh pathway activation through the dissociation of Sufu-Gli2 complexes to allow the translocation of Gli2 into the nucleus. In addition, Kif7 contributes to repression of Hh target genes in a Sufu-independent manner. We show that knockout of both Sufu and Kif7 in embryonic epidermis leads to loss of hair follicle fate and blockage of differentiation. Furthermore, simultaneous inactivation of Sufu and Kif7 in adult epidermis results in BCC. These results demonstrate the distinct and overlapping roles of Sufu and Kif7 in Gli2 regulation during epidermal development and tumorigenesis.

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MATERIALS AND METHODS

Mouse strains and animals analyzed
K5-Cre (Tarutani et al., 1997), Ptc1flox (Ellis et al., 2003), Kif7flox and Kif7−/− (Cheung et al., 2009) mice were previously described. K14-CreESR1 transgenic mice were obtained from Jackson Laboratory. Mice were maintained in a mixed outbred background of CD1 and 129/Sv. Animals in this study were treated in accordance with protocols approved by The Hospital for Sick Children Animal Care Committee. The number of E18.5 and E16.5 embryos analyzed was (excluding animals used for keratinocyte culturing experiments): Kif7−/− (15), K5Cre;Sufu−/− (26) and K5Cre;Sufu−/−;Kif7−/− (5 at E18.5, 3 at E16.5). The number of mice with TAM-treated adult skin analyzed was: K14Cre;Kif7−/− (8), K14Cre;Sufu−/− (7) and K14Cre;Sufu−/−;Kif7−/− (7).

Skin grafts
Skin from E18.5 control (n=5), K5Cre;Sufu−/− (n=7) and K5Cre;Ptc1flox (n=9) embryos was transplanted onto a skin excision area of approximately the same size on adult nude mice.

Temporal skin-specific deletion of conditional alleles
Dorsal skin of transgenic mice (~8 weeks) was shaved and depilation cream was applied to the area to remove the whole hair shaft. Tamoxifen (100 mg total, Sigma-Aldrich) was topically applied onto the shaved area for 5 consecutive days.

Histology, immunostaining, alkaline phosphatase analysis and in situ hybridization
Samples were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin following standard procedures. Sections (5 μm) were prepared for immunostaining, in situ hybridization and endogenous alkaline phosphatase activity detection as previously described (Mill et al., 2003). Antibodies used are as follows: Ki7 (Cheung et al., 2009); K-14, K-10, K1 and Loricin (Covance); cyclin D, p63 (Santa Cruz); β-catenin (Cell Signaling); E-cadherin and P-cadherin (Invitrogen); and Ki-67 (DAKO). Generation of digoxigenin-dUTP-labeled riboprobes for Shh, Gli1 and Ptc1 (Mill et al., 2003), Ptc62 (Niewenhuys et al., 2006), keratin 17 (McGowan and Coulombe, 1998), keratin 15 (B. Morgan, Harvard Medical School, Boston, MA) and Lef1 (Kratochwil et al., 1996) have been previously described.

TUNEL labeling and β-galactosidase staining
TUNEL (Clontech) and β-galactosidase staining was performed as previously described (Mill et al., 2003).

Statistical analysis
Quantitative histomorphometry and hair follicle counts were performed according to morphological and histological criteria (Paus et al., 1999) for 15 sections of 75 mm dorsal Kif7−/− (n=4) and Kif7−/− skin (n=3) and 50 longitudinal hair follicles in sections of K5Cre;Sufu−/− and controls (n=5). Means were calculated from pooled data. Statistical significance of differences was determined using Student’s t-test or one-way ANOVA with post-hoc analysis with Student-Newman Keuls.

Keratinocytes culturing and immunofluorescence
Keratinocytes were isolated from the epidermis of E18.5 embryos as previously described (Mill et al., 2003). For immunofluorescence experiments, keratinocytes were cultured on cover slips coated with collagen I and fixed in 4% paraformaldehyde. Cells were blocked with 10% goat serum, 0.1% Triton in PBS and incubated with Gli2 antibodies (Abcam 26050) overnight at 4°C. After washing with washing solution (1% goat serum, 0.1 Triton in PBS), cells were incubated for 1 hour with appropriate Alexa-conjugated secondary antibodies (Invitrogen).

RNA isolation and qPCR
RNA was extracted from keratinocytes using Trizol (Invitrogen) according to the manufacturer’s instructions. Purified RNA was used to synthesize the first-strand cDNA using SuperScript II RT and Oligo(dT)12-18 (Invitrogen). Three or more independent RNA samples were isolated from the keratinocytes of mutants and their respective controls. Each reaction was repeated in technical duplicates. TaqMan gene expression assays were used (Applied Biosystems). Relative gene expression data were analyzed using the 2−ΔΔCT method (Schmittgen and Livak, 2008). GAPDH was used as an endogenous control and for target gene normalization.

Subcellular fractionation, co-immunoprecipitation and western blot analysis
Subcellular fractionation, western blot analysis and co-immunoprecipitation were performed as previously described (Barnfield et al., 2005). The same amount of protein was loaded in nuclear and cytoplasmic fractions. For graphic representation of subcellular fractionation, cytoplasmic to nuclear protein ratio of total cell lysate was taken into account when calculating nuclear Gli2 content using densitometry. Antibodies used are as follows: Gli1 (Cell Signaling), Gli2 (Cheung et al., 2009), Sufu (Meng et al., 2001), lamin B (Abcam), tubulin (Sigma) and β-actin (Calbiochem).

Chromatin immunoprecipitation
Chromatin immunoprecipitation was performed using the ChIP-IT kit (Active Motif) as previously described (Hu et al., 2006; Pospisilik et al., 2010). Chromatin was isolated from keratinocytes cultured from E18.5 Sufu mutant and control skin. Gli2 antibody (Abcam 26050) and negative control IgG (Active Motif) were used to immunoprecipitate the Gli2 bound-DNA complex. Gli2 bound-DNA fragments were amplified using primer sets to Gli1, Ptc1 and Hhip1 promoter region as described previously (Vokes et al., 2007).

RESULTS

Hh stimulation promotes nuclear Gli2 accumulation in keratinocytes
As Gli2 plays a crucial role in the epidermis and BCC are keratinocyte tumors, we used primary keratinocytes to decipher the molecular mechanisms of Gli2 regulation. These cultures were maintained for ~5 days and conditioned to retain keratinocytes in an undifferentiated, basal cell-like state. In mouse embryonic fibroblasts (MEFs), Hh pathway activation promotes the dissociation of cytoplasmic Sufu-Gli complexes allowing the subsequent nuclear translocation of Gli proteins to activate Gli-dependent transcription (Humke et al., 2010; Tukachinsky et al., 2010). We first examined how pathway activation affects the localization of endogenous Sufu and Gli2 in wild-type keratinocytes and found that over 90% of Gli2 and Sufu are localized to the cytoplasmic fraction (Fig. 1A, lane 1). Upon treatment with SAG, a Smo agonist, approximately a twofold increase in nuclear Gli2 was observed (Fig. 1A, compare lanes 3 and 4), although the total Gli2 level was not significantly changed (data not shown). Similar to that reported in MEFs (Humke et al., 2010), no significant change in the subcellular localization of Sufu was observed in SAG-treated keratinocytes (Fig. 1A). By contrast, we did not detect any change in the electrophoretic mobility of nuclear Gli2 upon SAG treatment, as reported by Humke et al. (Humke et al., 2010). These data demonstrate that, similar to results obtained in MEFs, Hh pathway activation promotes nuclear Gli2 localization in keratinocytes.

Sufu sequesters Gli2 in the cytoplasm to inhibit Gli activity
We performed co-immunoprecipitation to examine whether endogenous Sufu forms a complex with Gli2 in keratinocytes. Sufu was present in the Gli2 immunoprecipitates from control keratinocytes and, conversely, Gli2 was detected in the Sufu immunoprecipitates (Fig. 1B). These Sufu-Gli2 complexes are specific because they were not detected in Sufu-null lysates (Fig. 1B). Sufu−/− mice are lethal around embryonic day (E) 9 (Cooper et al., 2005; Svärd et al., 2006). To examine how Sufu controls Gli
activity in the skin, we generated conditional knockout (KO) mice of Sufu using keratin 5 (K5)-Cre (supplementary material Fig. S1) (Tarutani et al., 1997). Quantitative RT-PCR (qPCR) analysis of Hh target gene expression in Sufu KO keratinocytes showed approximately threefold upregulation of Gli1 and Ptc1, and ~1.5-fold upregulation of Ptc2 relative to the control (Fig. 1C). These results demonstrate that Sufu represses Hh signaling in keratinocytes. Western blot analysis revealed ~80% reduction in the total level of Gli2 protein in Sufu KO keratinocytes compared with the control (Fig. 1D), although Gli2 RNA level was not affected (Fig. 5A). These observations provide further support that Sufu is required for Gli2 protein stabilization, as revealed previously in MEFs (Chen et al., 2009). Sufu has been shown to inhibit Gli2-dependent transcription in MEFs via multiple mechanisms, including cytoplasmic sequestration of Gli2 (Barnfield et al., 2005). To determine how Hh pathway activation is achieved despite the low level of Gli2 protein, we performed subcellular fractionation to examine Gli2 localization in Sufu KO keratinocytes. Although Gli2 is mostly in the cytoplasmic fraction of control keratinocytes (Fig. 1E, compare lanes 1 and 3), it is undetectable in the cytoplasmic fraction of Sufu KO keratinocytes (Fig. 1E, lanes 2 and 4). Most, if not all, of Gli2 is found in the nuclear fraction of Sufu KO keratinocytes. Interestingly, Gli2 immunostaining of control keratinocytes revealed clear nuclear signals but very weak cytoplasmic staining. We reason that this may be due to masking of some of the epitopes on Gli2 in the cytoplasm. Nonetheless, consistent with the western blot results, immunostaining of Sufu KO keratinocytes clearly detected Gli2 in the nucleus and did not reveal any cytoplasmic Gli2 signals (Fig. 1F). To determine whether low Gli2 levels in Sufu KO keratinocytes are capable of binding to Hh target genes, we performed chromatin immunoprecipitation. We found that Gli2, but not the control IgG antibody enriches the Gli1, Ptc1 and Hhip1 promoter regions in Sufu KO keratinocytes (Fig. 1G), demonstrating that Gli2 is capable of binding Hh target genes in the mutant cells. As Gli2 directly induces Gli1 expression (Ikram et al., 2004) and Gli1 is a potent activator of Hh signaling in keratinocytes (Nilsson et al., 2000), we reason that elevated Hh pathway activity in Sufu KO keratinocytes is probably triggered through Gli2-dependent activation of Gli1. Subcellular fractionation indeed revealed a dramatic increase of nuclear Gli1 in Sufu KO keratinocytes when compared with the control (Fig. 1H, compare lane 1 with lane 3). Interestingly, cytoplasmic Gli1 levels are comparable (Fig. 1H, compare lane 1 with lane 2), suggesting that, in contrast to Gli2, the cytoplasmic sequestration of Gli1 is not Sufu...
dependent. These findings indicate that Sufu is required for the cytoplasmic localization and stability of Gli2. In the absence of Sufu, Gli2 translocates to the nucleus to activate Gli1 and other Hh target genes.

Deletion of Sufu in the skin leads to less hair follicles, increased proliferation and compromised epidermal differentiation

To determine the role of Sufu during embryonic skin development, we analyzed the phenotype of Sufu mutants. Sufu mutant skin exhibited a ~50% reduction in the total number of pelage hair follicles compared with control skin and the mutant follicles were in rudimentary stages of morphogenesis (Fig. 2A,B; supplementary material Fig. S2A,B). Keratin14 (K14) staining showed an expansion of the basal cell layer beyond its normal one-cell layer thickness (Fig. 2A,B,C,D), and analysis of proliferation markers Ki67 and p63 revealed that these cells were hyperproliferative (Fig. 2C,D,G,H; supplementary material Fig. S2C). Increased proliferation is probably the cause of basal cell expansion, as very few or no apoptotic cells were detected (supplementary material Fig. S2D,E). Expression of epidermal differentiation markers K1 and Loricrin was reduced in the mutants, suggesting compromised epidermal stratification (Fig. 2E-H). Next, we determined the expression pattern of Hh target genes in Sufu mutants. Sufu mutant skin showed ectopic expression of Gli1, Ptc1 and Ptc2 in the interfollicular epidermis (IFE), where the pathway is normally inactive (Fig. 2I-N, arrows) indicating that the function of Sufu in the skin is to represses Hh pathway activity in the IFE. These results illustrate that, Sufu is required for the proper formation of hair follicles, and the control of proliferation and differentiation in the IFE.

Inactivation of Sufu in the epidermis is not sufficient to induce BCC formation

The function of Ptc1 as a tumor suppressor in the skin is well established; whether Sufu acts as a tumor suppressor is, however, unclear. Sufu mutations were found in several cases of sporadic BCC but other mutations such as PTC1 and TP53 were also present, making it difficult to discern whether Sufu mutations are directly involved in the transformation or simply consequences of the transformation event (Reifenberger et al., 2005). As K5Cre;Sufu–/– mutants die immediately after birth, we performed skin grafting experiments to study the post-embryonic development of Sufu-deficient epidermis. Sufu mutant skin grafts developed disorganized hair follicles and showed epidermal hyperplasia with the expansion of the basal cell layer compared with control skin grafts (Fig. 3A,B,D,E). As a positive control, K5Cre;Ptc1–/– mutant skin was grafted onto the back of nude mice. Consistent with the role of Ptc1 as a tumor suppressor, histological analysis revealed both nodular and infiltrative BCC-like lesions in Ptc1 mutant skin grafts (Fig. 3C). Importantly, Sufu mutant skin grafts did not exhibit elevated expression of the proliferation marker p63 and the BCC markers, such as K14, Gli1, K17 and K15, compared with Ptc1 mutant skin grafts (Fig. 3G-R). These findings indicate that inactivation of Sufu alone is not sufficient to induce BCC formation, and suggest that additional genetic alterations may be required for tumorigenesis.

Kif7 is a positive regulator of Gli activity in hair follicles

Similar to Sufu, Kif7 also binds to Gli proteins and acts as a negative regulator of Shh signaling in MEFs (Cheung et al., 2009; Endoh-Yamagami et al., 2009; Liem et al., 2009). However, we showed that Kif7 promotes Hh signaling by restricting the inhibitory activity of Sufu in growth plate chondrocytes, suggesting a tissue-specific role of Kif7 (Hsu et al., 2011). Interestingly, Sufu KO keratinocytes show increased Kif7 protein expression (supplementary material Fig. S3A,B), suggesting that Kif7 may perform a specific function in the skin. To determine the regulatory function of Kif7 in the skin, we used Kif7–/– mice. In embryonic skin, Kif7 is predominantly expressed in proliferating keratinocytes of the basal layer and hair follicles. Kif7 shows higher expression in the keratinocytes residing in the hair follicles when compared with those in the IFE (supplementary material Fig. S3C). At E18.5, Kif7–/– skin showed a significant reduction in hair follicle numbers and these follicles were developmentally delayed when compared with control skin (Fig. 4A,B,A,B); supplementary material Fig. S4A,B). Whole-mount in situ analysis of Kif7, an early marker of epidermal appendage development revealed a reduced number of follicles in the mutants as early as E15.5 (supplementary material Fig. S4C,D). In contrast to Sufu mutant skin, differentiation and proliferation of the IFE is not affected in Kif7–/– skin (supplementary material Fig. S4E-K). These results indicate that Kif7 is required for the development of follicular epidermis, but not IFE.

![Fig. 2. Sufu is required for hair follicle development and epidermal stratification in the embryonic skin.](image-url)
To determine whether Kif7 is required for Hh pathway activation in the follicular epithelium, we examined the expression of Hh target genes in Kif7–/– skin. In situ hybridization analysis detected weaker Ptch1 and Gli1 expression in Kif7–/– follicles though Shh expression appeared largely normal (Fig. 4C-H). Unlike Sufu mutant skin, there was no ectopic expression of Gli2. Consistent with this, immunostaining of Gli2 is consistently lower in the amount of Sufu protein immunoprecipitated by Sufu antibodies compared with the control (Fig. 4M). Interestingly, we found that mutant skin, there was no ectopic expression of Gli2 expression appeared largely normal (Fig. 4C-H). Unlike Kif7–/– skin. qPCR analysis revealed a ~60% reduction of Ptch1 and Gli1 expression in Kif7–/– keratinocytes (Fig. 4I). Consistent with reduced Hh pathway activity, Kif7–/– keratinocytes displayed reduced levels of Gli2 mRNA and in Gli2 protein (Fig. 4J,K). These results demonstrate that Kif7 promotes Hh pathway activity in the hair follicles during skin development.

**Kif7 promotes the dissociation of Sufu-Gli2 complexes in keratinocytes**

To gain insight into the molecular action of Kif7 on Gli-dependent transcription in keratinocytes, we focused on the nuclear translocation of Gli2 and the formation of Sufu-Gli2 complexes. Subcellular fractionation revealed that nuclear Gli2 level is reduced by 50% in Kif7–/– keratinocytes compared with control (Fig. 4K, compare lanes 3 and 4), suggesting that Kif7 promotes nuclear localization of Gli2. Consistent with this, immunostaining of Gli2 also revealed a dramatic reduction of nuclear signals in Kif7–/– keratinocytes (Fig. 4L). We consistently detected a doublet of Gli2 in keratinocytes and found only the faster migrating Gli2 species in the nuclear fraction of Kif7–/– keratinocytes (supplementary material Fig. S5A). However, the faster mobility of this species is probably due to post-translational modifications other than phosphorylation as treatment with lambda phosphatase did not affect the mobility of the Gli2 doublet in wild-type keratinocytes (supplementary material Fig. S5B and data not shown). As Sufu sequesters Gli2 in the cytoplasm of keratinocytes, we examined whether Kif7 influences the formation of Sufu-Gli2 complex. Using cytoplasmic lysates from Kif7–/– and control keratinocytes, which contain comparable amount of Sufu and Gli2 proteins, we detected more Sufu-Gli2 complexes in Kif7–/– keratinocytes when compared with the control (Fig. 4M). Interestingly, we found that the amount of Sufu protein immunoprecipitated by Sufu antibodies is consistently lower in the Kif7–/– lysates than control. Quantification of these results revealed an approximately twofold increase of Sufu-Gli2 complexes in Kif7–/– keratinocytes (Fig. 4M).

In keratinocytes, Kif7 regulates Sufu stability by a proteasome-dependent mechanism (Hsu et al., 2011). However, we found that neither Sufu mRNA nor Sufu protein levels change in Kif7–/– keratinocytes (supplementary material Fig. S6A,B; data not shown). These observations suggest that Kif7 reduces the inhibitory action of Sufu by promoting the dissociation of Sufu-Gli2 complex and that this action is independent of the stability of Sufu. As Kif7 can form complexes with Gli2 and Sufu (supplementary material Fig. S6C), it is possible that Kif7 may act by competing with Sufu for Gli2 interaction. Together, these results illustrate that Kif7 acts as a positive regulator of Hh signaling in keratinocytes by promoting the dissociation of Sufu-Gli2 complexes and Gli2 nuclear localization.

**Sufu and Kif7 play overlapping functions in embryonic skin**

Our molecular analysis suggests that Kif7 acts through Sufu to maintain Hh pathway activity. To test whether Kif7 also regulates Hh signaling independently of Sufu, we generated skin-specific deletion of Sufu in the Kif7–/– background (K5Cre:Sufufl/fl;Kif7–/– or DKO). Loss of Sufu in Kif7–/– keratinocytes results in a further upregulation of Hh target genes, Gli1 and Hhip1, compared with Sufu KO keratinocytes. Gli2 expression was also significantly higher in DKO keratinocytes compared with that observed in Sufu KO keratinocytes (Fig. 5A). These results indicate that Kif7 contributes to the repression of Gli2 and its transcriptional activity in the absence of Sufu, and suggest that Kif7 performs additional functions besides the regulation of Sufu-Gli2 complex formation. To determine the functional significance of elevated Hh signaling in DKO keratinocytes, we examined the skin of DKO mutants. All DKO skin examined displayed irregularly shaped and invaginations of densely packed epithelial cells that histologically resemble basaoid lesions (Fig. 5B,C). DKO skin displayed impairment in IFE differentiation that is more severe than the Sufu mutants with an increase in proliferation (compare Fig. 5D-I and Fig. 2C-H).

Strikingly, DKO skin lacks hair follicle-like structures, as revealed by the loss of follicular marker P-cadherin and the presence of E-cadherin (Fig. 5J-M). The Wnt/β-catenin pathway is crucial for hair follicle specification (Huelsken et al., 2001; Andl et al., 2002). Nuclear β-catenin expression was used as a marker to determine whether the follicular fate is initiated in the skin lesions of DKO mice. In control skin, canonical Wnt signaling is active in the placodes as revealed by nuclear β-catenin immunostaining; however, nuclear β-catenin was not detected in the epithelial lesions of the DKO mice (Fig. 5L-O). Lack of hair follicle initiation in the DKO skin was further illustrated by the loss of the placode markers Left1 and Shh (Fig. 5P,Q; data not shown). These results indicate that hair follicle fate is lost in the DKO skin and that the DKO epithelial cells have adopted an undifferentiated
interfollicular fate. Thus, Sufu and Kif7 possess overlapping roles in the specification of hair follicle fate and epidermal differentiation.

**Inactivation of Sufu and Kif7 in adult epidermis results in superficial BCC**

To investigate the function of Sufu and Kif7 in the adult skin, we generated tamoxifen-inducible (TAM) epidermis-specific KO mice for Sufu (K14CreESR1;Sufuf/–), Kif7 (K14CreESR1;Kif7f/–), as well as Sufu and Kif7 together (K14CreESR1;Sufuf/–;Kif7f/–). Tamoxifen was administered through topical application to activate CreERtam specifically in postnatal skin (Vasioukhin et al., 1999). The efficiency of deletion in these experiments was examined using the ROSA26-lacZ reporter line (supplementary material Fig. S7). No β-galactosidase activity was detected in mice that were treated with ethanol (supplementary material Fig. S7B). By contrast, β-galactosidase was detected throughout the epidermis and hair follicles by 12 weeks, demonstrating that the deletion strategy is robust (supplementary material Fig. S7F-H). We harvested control and TAM-treated skin from mice between 7 and 12 weeks after TAM application. No observable skin lesions were detected in ethanol-treated transgenic mice or TAM-treated Kif7f/– keratinocytes, as revealed by qPCR. Data are means with error bars representing 95% confidence intervals (**P<0.01, t-test). (J) The expression of GlI2 is significantly reduced in Kif7f/– keratinocytes, compared with control. This is quantified in the adjacent graph. Lamin B and tubulin serve as control nuclear and cytoplasmic proteins, respectively, to assess the quality of the fractionation. Cell fractionation lysates are pooled from seven independent sets of control and Kif7f/– keratinocytes samples. (K) Immunostaining shows that GlI2 signal is reduced in the nuclear compartment of Kif7f/– keratinocytes compared with control. Dashed lines represent cell periphery. (M) Co-immunoprecipitation experiment reveals an increase in the amount of Sufu-GlI2 interaction in lysates from Kif7f/– keratinocytes compared with control. "\( \text{J}\)-test). Mean GlI2 protein levels normalized to actin±s.e.m. (n=3) in Kif7f/– keratinocytes and control (*P<0.05, t-test).

**Fig. 4. Kif7 acts as a positive regulator of Hh signaling in the skin by promoting the dissociation of Sufu-GlI2 complexes.**

(A,A',B,B') Hematoxylin and Eosin staining of Kif7f/– dorsal skin at E18.5. (A',B') Higher magnification. (A',B') K14 immunohistochemistry reveals that the basal cell layer remains unchanged in Kif7f/– skin. AP staining reveals presence of dermal papilla in Kif7f/– hair follicles. (C-H) In situ hybridization analysis shows that, although Shh expression is not affected, GlI1 and Ptch1 expression was reduced in Kif7f/– hair follicles. (I) qPCR analysis demonstrates a reduction in Ptch1 and GlI1 expression in Kif7f/– keratinocytes. Data are means with error bars representing 95% confidence intervals (**P<0.01, t-test). (J) The expression of GlI2 is significantly reduced in Kif7f/– keratinocytes, as revealed by qPCR. Data are means with error bars representing 95% confidence intervals (**P<0.01, t-test). (J) Mean GlI2 protein levels normalized to actin±s.e.m. (n=3) in Kif7f/– keratinocytes and control (*P<0.05, t-test). (K) Subcellular fractionation reveals a severe reduction in nuclear (N) GlI2 in Kif7f/– keratinocyte compared with control. This is quantified in the adjacent graph. Lamin B and tubulin serve as control nuclear and cytoplasmic proteins, respectively, to assess the quality of the fractionation. Subcellular fractionation lysates are pooled from seven independent sets of control and Kif7f/– keratinocytes samples. (L) Immunostaining shows that GlI2 signal is reduced in the nuclear compartment of Kif7f/– keratinocytes compared with control. Dashed lines represent cell periphery. (M) Co-immunoprecipitation experiment reveals an increase in the amount of Sufu-GlI2 interaction in lysates from Kif7f/– keratinocytes compared with control. Graphic representation of co-immunoprecipitation experiments whereby the amount of Sufu-GlI2 complex was determined by normalizing Sufu that co-immunoprecipitated with GlI2 and vice versa. Scale bars: 50 μm. See also supplementary material Figs S3-S6.
BCC, these skin lesions were highly proliferative and expressed BCC markers, including K14 and cyclin D (Fig. 6J,O,T). In both mice and human, BCC is characterized by elevated Hh signaling and a selective upregulation of cyclin D, whereas BFH display lower expression of Hh target genes and cyclin D compared with BCC (Grachtchouk et al., 2003). Previous studies suggested that the level of Hh pathway activation determines the tumor phenotype (Grachtchouk et al., 2003; Grachtchouk et al., 2011). As Sufu KO keratinocytes do not display high Hh target gene expression as DKO keratinocytes and only BFH is observed in keratinocytes, we hypothesized that Sufu plays a key role in suppressing the Wnt/β-catenin pathway in superficial BCC in humans (Yang et al., 2008). Consistent with these observations, we found increased cytoplasmic and nuclear β-catenin in BFH and BCC of the Sufu;Kif7-TAM treated mice (Fig. 6U-Y). Interestingly, increased β-catenin was also observed in the skin lesions of our Sufu;Kif7-TAM treated mice. Furthermore, non-phosphorylated (inactive) β-catenin was detected in the protein lysates from Sufu;Kif7-TAM treated skin, while little or no expression was present in the lysates from Kif7-TAM or Sufu;Kif7-TAM treated skin. Higher magnification of β-catenin staining in insets: U-Y. bud, budding; hf, hair follicle. Scale bars: 50 μm. See also supplementary material Figs S7-S9.

activated Wnt/β-catenin pathway is observed in superficial BCC in humans (Yang et al., 2008). Consistent with these observations, we found increased cytoplasmic and nuclear β-catenin in BFH and BCC of the Sufu;Kif7-TAM treated mice (Fig. 6U-Y). Interestingly, increased β-catenin was also observed in the skin lesions of our Sufu;Kif7-TAM treated mice. Furthermore, non-phosphorylated (active) β-catenin was detected in the protein lysates from Sufu;Kif7-TAM treated skin, while little or no expression was present in the lysates from Kif7-TAM or Sufu;Kif7-TAM treated skin. Higher magnification of β-catenin staining in insets: U-Y. bud, budding; hf, hair follicle. Scale bars: 50 μm. See also supplementary material Figs S7-S9.
DISCUSSION

In this study, we demonstrate the distinct and overlapping functions of Sufu and Kif7 during skin development and tumorigenesis. We provide molecular insight into the roles of Sufu and Kif7 on Gli2 regulation using the skin as a model system. Our results illustrate that Sufu and Kif7 play opposing roles in Shh signaling through the regulation of Gli2 subcellular distribution, and that Kif7 performs distinct Sufu-dependent and –independent functions. These two regulators of Shh signaling are pivotal to skin development and tumorigenesis. In their absence, hair follicle fate is completely lost in embryonic epidermis and keratinocytes in adult epidermis are transformed to form BCC.

Sufu represses Gli proteins via distinct mechanisms

The output of mammalian Hh signaling is determined by the activity of Gli proteins, which are regulated at both the transcriptional and post-transcriptional levels (Hui and Angers, 2011). Sufu acts as a negative regulator of Hh signaling by forming complexes with all three Gli proteins and inhibiting their transcriptional activator function (Ding et al., 1999; Barnfield et al., 2005). Gli3 is processed by the proteasome into an N-terminal Gli3 repressor when the Hh pathway is inactive, and Gli3 repressor formation is largely compromised in Sufu−/− embryos. In addition, Sufu also plays a role in the stabilization of Gli2 and Gli3 by antagonizing the action of Spop, a nuclear speckle protein and an adapter of the cullin 3-based ubiquitin E3 ligase that promotes the degradation of full-length Gli2 and Gli3, but not Gli1 (Chen et al., 2009; Wang et al., 2010). Thus, Sufu regulates the activity of Gli proteins through multiple mechanisms, including processing and stabilization.

Here, we illustrate another important regulatory function of Sufu, which is to control the subcellular distribution of Gli2. In cultured mesenchymal cells, Hh pathway stimulation promotes the dissociation of cytoplasmic Sufu-Gli complexes and the subsequent nuclear translocation of Gli activators (Hunke et al., 2010). However, the function of these cytoplasmic Sufu-Gli complexes and the role Sufu plays in the nuclear entry of Gli activators are largely unexplored. Gli2 activator is pivotal for Shh signaling in the skin. We show that endogenous Sufu forms inhibitory cytoplasmic complexes with Gli2 in keratinocytes to prevent the nuclear entry of Gli2 (Fig. 7A). We propose that the Sufu-Gli2 complex is crucial for limiting the transcriptional activity of Gli in the IFE where the Hh pathway is normally repressed (Fig. 7B). Despite the potent inhibitory role of Sufu on Hh pathway activity in the skin, tumor studies here indicate that loss of Sufu alone is not sufficient for robust Gli activation to induce BCC formation.

In vitro studies have suggested that Sufu represses the activity of Gli transcription factors through cytoplasmic retention of Gli1 and Gli2 (Ding et al., 1999; Barnfield et al., 2005), and by recruiting co-repressor complexes in the nucleus (Cheng and Bishop, 2002). Our data here revealed that the regulatory actions of Sufu on Gli1 and Gli2 are distinct. First, although the cytoplasmic sequestration of Gli2 is Sufu dependent, Sufu plays a less crucial role in the cytoplasmic localization of Gli1. Our finding is consistent with the results from a previous report that the majority of overexpressed Gli1 was localized in the cytoplasm of Sufu−/− MEFs, similar to those found in wild-type MEFs (Svärd et al., 2006). Previous studies indicate that Spop is involved in the degradation of Gli2 and Gli3, but not Gli1, and suggest that Sufu opposes the activity of Spop to stabilize Gli proteins (Chen et al., 2009; Wang et al., 2010). Consistent with this model, Gli2 protein was significantly reduced in Sufu KO keratinocytes, suggesting that Sufu is involved in the stabilization of Gli2, but not of Gli1.

Kif7 promotes nuclear entry of Gli2

We and others have identified kinesin motor protein Kif7 as another key regulator of Gli transcription factors (Cheung et al., 2009; Endoh-Yamagami et al., 2009; Liem et al., 2009). Similar to its Drosophila counterpart Costal 2 (Cos2), Kif7 plays dual regulatory roles in Hh signaling: it acts negatively in controlling Hh target gene expression during early neural tube development and in MEFs, whereas it promotes Shh-dependent floor-plate development and Ihh-dependent chondrocyte development (Hsu et al., 2011). Hh signaling promotes the translocation of Kif7 to the tip of primary cilium in cultured mesenchymal cells, and Gli2 and Gli3 do not accumulate at the ciliary tip of Kif7−/− MEFs, suggesting that Kif7 might act as a ciliary motor in the transport of Gli proteins along the primary cilium (Endoh-Yamagami et al., 2009; Liem et al., 2009). However, our recent study demonstrates that Kif7 is not required for the ciliary localization of Gli2 and Gli3 in chondrocytes (Hsu et al., 2011). In fact, both Gli2 and Gli3 accumulate at the ciliary tip of Kif7−/− chondrocytes when compared with wild-type chondrocytes, which show little or no Gli2 and Gli3 ciliary staining. Although this discrepancy remains to be addressed, it is probably related to the opposing roles of Kif7 in Hh signaling in MEFs versus chondrocytes. In chondrocytes, Kif7 promotes Ihh signaling through negative regulation of Sufu: Kif7−/− chondrocytes show elevated level of Sufu protein and, importantly, reduction of Sufu gene dose alleviates the reduction of Hh pathway...
activity in these cells (Hsu et al., 2011). The role of Kif7 on Sufu-Gli2 interaction in chondrocytes is unknown.

Our results here indicate that Kif7 is also a positive regulator of Hh signaling in keratinocytes. In contrast to chondrocytes, there is no obvious change in the level of Sufu protein in Kif7−/− keratinocytes. Despite this, we found a consistent increase of Sufu-Gli2 complexes in Kif7+/− keratinocytes. In addition, there is a drastic reduction of nuclear Gli2 in these cells. These observations are consistent with the notion that the association/dissociation of cytoplasmic Sufu-Gli2 complexes are involved in the regulation of the nuclear entry of Gli2 and that Kif7 plays a crucial role in these processes (Fig. 7A). How does Kif7 modulate Sufu-Gli2 complexes? In Drosophila, Cos2 binds to both the N-terminal and C-terminal domains of Ci, and Sufu competes with Cos2 for binding to the N-terminal region of Ci (Wang and Jiang, 2004). We speculate that, in the absence of Kif7, more Gli2 will be accessible to bind Sufu, leading to the increase of Sufu-Gli2 complexes in Kif7+/− keratinocytes. Intriguingly, Kif7 can perform a negative regulatory role in Hh signaling when Sufu function is absent. Hh pathway activity is further augmented in Sufu KO keratinocytes when Kif7 is deleted. Thus, Kif7 clearly possesses both Sufu-dependent and Sufu-independent functions in Hh signaling in chondrocyte as well as in keratinocytes. Future experiments will be necessary to determine how Kif7 suppresses Hh target gene expression independently of Sufu.

Spatial requirement of Kif7 and Sufu during skin development

Studies in MEFS demonstrated that Kif7 localization is dependent on the presence or absence of Hh ligand, suggesting that the function of Kif7 function may be regulated by Hh stimulus (Endoh-Yamagami et al., 2009; Liem et al., 2009). We speculate that the function of Kif7 in keratinocytes is spatially dependent on the location of Shh, which is normally restricted to the distal tip of the developing hair follicle (Fig. 7B). In these Shh-receiving cells, Kif7 promotes Hh signaling through the dissociation of Sufu-Gli2 complex. In IFE cells, which do not receive Shh signals, Sufu is present to maintain the Shh pathway in an ‘off’ state. In these cells, Kif7 acts cooperatively with Sufu as a negative regulator (Fig. 7B). We propose that the negative regulatory action of Sufu masks that of Kif7 and that only in the absence of Sufu can the inhibitory function of Kif7 be revealed. Consistent with the role of Kif7 as an inhibitor of the Hh pathway, overexpression of Kif7 could block the actions of a constitutively activated Smo in vitro and in vivo (Cheung et al., 2009). Although we do not observe a drastic effect of Kif7 on Gli2 protein levels in Kif7+/− keratinocytes, Kif7 appears to play a role in the degradation of Gli2 as Kif7+/− embryos exhibit elevated Gli2 protein levels (Cheung et al., 2009; Endoh-Yamagami et al., 2009; Liem et al., 2009). Further analysis will be needed to decipher the molecular mechanism underlying the dual regulatory activities of Kif7 on Gli2.

Deletion of Sufu is not sufficient to induce BCC

The role of Sufu in tumorigenesis is complex. It has been reported that whereas Sufu+/− mice are not prone to tumorigenesis, Sufu−/− mice in the p53-null background results in robust formation of medulloblastoma in the cerebellum, highlighting a key role for Sufu in tumor suppression in the cerebellum (Lee et al., 2007). However, loss of p53 has minimal effects on the latency, cellular phenotype and proliferation capacity of basaloid skin lesions in Sufu−/− mice (Lee et al., 2007; Heby-Henriscon et al., 2012). These observations illustrate that the different thresholds of sensitivity to transformation between cerebellum and skin. We show here convincingly that loss of Sufu in embryonic as well as adult epidermis does not transform keratinocytes into BCC. Histological and marker gene analyses indicate that loss of Sufu only results in BFH. Our results suggest that the level of Hh pathway activation in Sufu KO keratinocytes can only drive BFH formation but is not robust enough to induce BCC. Importantly, additional ablation of Kif7 in Sufu KO keratinocytes drastically increases the incidence of BFH and the majority of double knockout mice succumb to BCC. Thus, our studies indicate a pivotal role for Kif7 in determining tumor outcome in the skin and illustrate the novel overlapping functions of Sufu and Kif7 in BCC suppression (Fig. 7C). Whether the overlapping functions of Sufu and Kif7 in tumor suppression is specific to the skin or can be observed in other tissues awaits future inducible tissue-specific KO mouse studies.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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References


Gli2 regulation by Sufu and Kif7


