Ptch1 Overexpression Drives Skin Carcinogenesis and Developmental Defects in K14Ptch\textsuperscript{FVB} Mice

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Ptch1 is a key regulator of embryonic development, acting through the sonic hedgehog (SHH) signaling pathway. Ptch1 is best known as a tumor suppressor, as germline or somatic mutations in Ptch1 lead to the formation of skin basal cell carcinomas. Here we show that Ptch1 also acts as a lineage-dependent oncogene, as overexpression of Ptch1 in adult skin in K14Ptch\textsuperscript{FVB} transgenic mice synergizes with chemically induced Hras mutations to promote squamous carcinoma development. These effects were not because of aberrant activation of SHH signaling by the K14Ptch\textsuperscript{FVB} transgene, as developmental defects in the highest expressing transgenic lines were consistent with the inhibition of this pathway. Carcinomas from K14Ptch\textsuperscript{FVB} transgenic mice had only a small number of nonproliferative Ptch1 transgene-positive cells, suggesting that the Ptch1 transgene is not required for tumor maintenance, but may have a critical role in cell-fate determination at the initiation stage.

INTRODUCTION

Ptch1 is a key signaling receptor in the sonic hedgehog (SHH) signaling pathway, which is critical in embryonic development, tissue patterning, and cell-fate decisions (Ingham et al., 1991; Chen and Struhl, 1996; Marigo et al., 1996). Mutations resulting in loss of Ptch1 function or aberrant expression of genes in the SHH pathway lead to developmental defects and a cancer-prone phenotype. Germline mutations in the human PTCH1 gene are responsible for Gorlin syndrome (also called nevoid basal cell carcinoma syndrome), which is characterized by various developmental malformations and a high predisposition to skin basal cell carcinoma development (Hahn et al., 1996; Johnson et al., 1996). Mice partially deficient in functional Ptch1 exhibit developmental abnormalities that are comparable to those of nevoid basal cell carcinoma syndrome patients. In addition, these mice develop tumors such as medulloblastomas, rhabdomyosarcomas, and basal cell carcinomas following UV or ionizing radiation exposure, demonstrating a critical role for Ptch1 in the regulation of developmental homeostasis and tumor suppression (Goodrich et al., 1997; Hahn et al., 1998; Asztterbaum et al., 1999).

We have previously identified a polymorphic variant in the mouse Ptch1 gene (Ptch\textsuperscript{FVB}) that conferred susceptibility to early postnatal squamous cell carcinoma (SCC) development in transgenic mice expressing a mutant H-ras gene under the control of the keratin 5 promoter (K5Hras mice) (Wakabayashi et al., 2007). In this model, both Ptch1 and activated Ras are expressed under the control of constitutive keratin promoters during skin development (Byrne et al., 1994), and it remained possible that this paradoxical effect of Ptch1 in promoting cancer may be linked to developmental abnormalities related to this specific model. Therefore, we tested the role of Ptch1 in adult-onset skin tumor formation using the classical DMBA (7,12-dimethylbenz[a]anthracene)/TPA (12-O-tetradecanoylphorbol-13-acetate) model involving sporadic mutation of the endogenous H-ras gene, rather than the high levels of mutant H-ras expressed during development in the K5Hras transgenic mice. Our data demonstrate that even low levels of Ptch1 transgene expression that do not perturb expression of Gli transcription factors promote formation of malignant SCCs. We conclude that the SHH pathway can have either positive or negative roles in the development of alternative tumor types within the same tissue.

RESULTS

Developmental abnormalities in K14Ptch\textsuperscript{FVB} mice are consistent with diminished Hedgehog signaling

We previously generated four different transgenic lines (lines 6, 7, 8, and 10) expressing Ptch\textsuperscript{FVB} under the control of the keratin 14 (K14) promoter at variable levels, with line 6 showing the highest Ptch1 transgene expression (Wakabayashi et al., 2007). Initially, these transgenic lines were generated...
and maintained on the FVB/N background. Continuous breeding to the same background resulted in poor litter sizes, particularly in line 6 with the highest PtchFVB levels. To maintain this line, we crossed the line 6 K14PtchFVB mice to wild-type C57BL/6 mice and obtained litters on this hybrid background. We observed major phenotypic abnormalities in line 6 K14PtchFVB mice on both FVB/N and hybrid backgrounds. First, when compared with their wild-type littermates, line 6 K14PtchFVB mice exhibited shorter mean body lengths (5.6 ± 0.48 vs. 7.9 ± 0.25 cm, \(P = 0.00065\) by t-test) and lower mean body weights (5.9 ± 0.74 vs. 14.7 ± 0.68 g, \(P < 0.00001\) by t-test) at 4 weeks of age (Figure 1a). This smaller body size was not visible in the other transgenic lines apart from a slight size difference in line 8 K14PtchFVB mice (data not shown). Furthermore, although the wild-type littermates showed normal eye development, line 6 K14PtchFVB mice clearly displayed ocular defects involving bilateral and unilateral underdevelopment of the ocular structures (Figure 1b). Newborn line 6 K14PtchFVB mice exhibited either aphakia with microphthalmia (Figure 1b, a–c) or display hypoplastic ocular development. Adult K14PtchFVB mice exhibit permanently closed eyes (lower center and right panels). (c) Abnormal limb development in line 6 K14PtchFVB mice. WT mice have five digit hindlimbs and four digit forelimbs, whereas K14PtchFVB mice have four digit hindlimbs and three digit forelimbs.
Average number of papillomas per mouse is comparable between K14PtchFVB type (WT) mice (line 7 (L7) and line 8 (L8)). 

dimethylbenz[a]anthracene; TPA, 12-

with mutations in Gas1, a membrane-bound glycoprotein that 

panel). Similar ocular defects have been described in mice 

upper middle panel) or anophthalmia (Figure 1b, upper right 

and WT mice at 20 weeks (P

Kaplan–Meier method). 

incidence in WT mice (two lines: L7 and L8) and WT mice 

rare survival at 50 weeks (Figure 2c, P

= 0.0027, Kaplan–Meier method). 

K14PtchFVB mice develop a higher frequency of chemically induced skin carcinomas than wild-type mice 

To explore the oncogenic function for Ptch1 in the setting of adult-onset skin carcinoma development, we performed two-stage skin carcinogenesis by treating DMBA followed by TPA. Because of the difficulties in breeding the line 6 transgenic mice, we carried out the carcinogenesis studies with line 7 and 8 transgenic mice on the FVBxC57BL/6 F1 genetic background (Wakabayashi et al., 2007). By 20 weeks after treatment, both lines of K14PtchFVB transgenic mice and wild-type mice developed a similar number of papillomas, (Figure 2a, P = 0.89 by Kruskal–Wallis test). It is known that a subset of benign papillomas induced by DMBA/TPA progress to malignant SCCs or spindle cell carcinomas (Burns et al., 1991; Oft et al., 2002). Thus, we monitored the mice for carcinoma development for up to 50 weeks after TPA treatment. At this time point, 71% of line 7 K14PtchFVB mice and 84% of line 8 K14PtchFVB mice had developed carcinomas, compared with only 35% of wild-type mice (Figure 2b; P = 0.023 for line 7, P = 0.0011 for line 8 by Fisher’s exact test). The carcinoma latency period was significantly shortened in K14PtchFVB mice compared with wild-type mice, which translated to lower carcinoma-free survival at 50 weeks (Figure 2c, P = 0.0027 by Kaplan–Meier method). 

The skin carcinomas were either SCCs or spindle cell carcinomas in both K14PtchFVB and wild-type mice, with no difference in terms of the frequency of histological subtypes (Supplementary Table S1 online). A hallmark of DMBA-initiated skin tumors is activating H-ras mutations at codon 61 (CAA to CTA, Gln to Leu) (Balmain et al., 1984; Quintanilla et al., 1986). A similar codon 61 CTA mutation frequencies was detected in carcinomas from K14PtchFVB (24/27; 89%) and wild-type (10/11; 91%) mice (Supplementary Table S1 online). Taken together, these data support a positive role of Ptch1 expression in promoting sensitivity to carcinoma development initiated by the mutation of Ras in adult mice.

**Figure 2. Two-stage skin carcinogenesis in line 7 and 8 K14PtchFVB and wild-type (WT) mice (line 7 (L7) and line 8 (L8)). (a) Papilloma development in K14PtchFVB and wild-type mice after DMBA/TPA treatment. DMBA, 7,12-dimethylbenz[a]anthracene; TPA, 12-O-tetradecanoylphorbol-13-acetate. Average number of papillomas per mouse is comparable between K14PtchFVB and WT mice at 20 weeks (P = 0.89, Kruskal–Wallis test). (b) Carcinoma incidence in K14PtchFVB and WT mice. By 50 weeks after treatment, a higher percentage of L7 and L8 K14PtchFVB mice develop skin carcinomas than WT mice (P = 0.023 for L7; P = 0.0011 for L8; Fisher’s exact test). (c) Carcinoma-free survival in K14PtchFVB (two lines: L7 and L8) and WT mice (P = 0.0027, Kaplan–Meier method).**

is known to antagonize SHH signaling (Lee et al., 2001a, b; Seppala et al., 2007). Adult line 6 K14PtchFVB mice demonstrated complete and permanent closure of the developmentally abnormal eyes (Figure 1b, lower panel). Finally, just as limb deformities have been linked to defects in both mouse and human genes involved in the SHH pathway, the line 6 K14PtchFVB mice displayed abnormal limb development (Figure 1c). Both forelimbs and hindlimbs of line 6 K14PtchFVB mice showed oligodactyly with fewer digits, consistent with a general inhibition of SHH signaling during mouse development. The defective eyes and limb deformities were not observed in line 7 and 8 K14PtchFVB mice. These phenotypic abnormalities in Ptch1 transgenic mice are clearly opposite to those observed in Ptc1+/− mouse or animals expressing transgenic Shh (Goodrich et al., 1997; Oro et al., 1997). We conclude that the Ptch1 transgene, although expressed in a subset of tissues under the control of the K14 promoter, acts in the expected manner as an inhibitor of SHH signaling during mouse development.
We previously showed that the product of the mouse Tid1 tumor-suppressor gene showed differential binding to PtchFVB and Ptch B6 in 293T cells (Wakabayashi et al., 2007). To explore the possible mechanisms by which Ptch1 expression may interact with H-Ras signaling, we investigated the effects of H-RAS transfection or TPA treatment on Tid1/Ptch1 binding and on the activation of the mitogen-activated protein kinase signaling pathway.

Figure 3a shows that transfection of mutant HRAS into 293T cells led to the accumulation of the GTP-bound form of RAS, but surprisingly had only a minor effect on activation of phospho-extracellular signal–regulated kinase (p-ERK) (Figure 3a, lanes 1 and 2). Co-transfection of Ptch1 (PtchFVB or PtchB6) in this assay led to only a modest but reproducible stimulation of p-ERK, and this was increased slightly in the presence of oncogenic V12HRAS.

In parallel experiments, oncogenic HRAS had little influence on the differential binding of Tid1 to PtchFVB or PtchB6 by immunoprecipitation (IP) with anti-Tid1 and anti-HA antibodies. IB, immunoblotting. (c) IP showed that TPA treatment, which had a very strong effect on the activation of p-ERK, destabilized the strong binding between Tid1 and Ptch1, especially for the PtchB6 variant.

**Effect of oncogenic H-Ras and TPA treatment on Ptch1 binding to Tid1**

We previously showed that the product of the mouse Tid1 tumor-suppressor gene showed differential binding to PtchFVB and PtchB6 in 293T cells (Wakabayashi et al., 2007). To explore the possible mechanisms by which Ptch1 expression may interact with H-Ras signaling, we investigated the effects of H-RAS transfection or TPA treatment on Tid1/Ptch1 binding and on the activation of the mitogen-activated protein kinase signaling pathway. Figure 3a shows that transfection of mutant HRAS into 293T cells led to the accumulation of the GTP-bound form of RAS, but surprisingly had only a minor effect on activation of phospho-extracellular signal–regulated kinase (p-ERK) (Figure 3a, lanes 1 and 2). Co-transfection of Ptch1 (PtchFVB or PtchB6) in this assay led to only a modest but reproducible stimulation of p-ERK, and this was increased slightly by co-transfection of oncogenic V12HRAS. In parallel experiments, oncogenic HRAS had little influence on the binding of Tid1 to PtchFVB or PtchB6 as shown by co-immunoprecipitation assays (Figure 3b). However, TPA treatment, which had a very strong effect on the activation of p-ERK (Figure 3c, second to bottom panels), destabilized the strong binding between Tid1 and Ptch1, especially for the PtchB6 variant (Figure 3c, lanes 3 and 4). This reduced level of binding, seen both at 30 minutes and 1 hour after TPA treatment, was more similar to that seen with the FVB variant protein, suggesting that activation of HRAS/mitogen-activated protein kinase signaling may directly affect the stability of the Ptch1/Tid1 complex.
Gene expression analysis of SHH signaling targets in K14PtchFVB mouse skin and skin carcinomas

Loss of the inhibitory function of Ptch1 in the SHH signaling pathway results in upregulation of Gli1, Gli2, and Ptch1 itself (Dahmane et al., 1997; Aszterbaum et al., 1999). To investigate whether Ptch1 overexpression in skin and skin SCCs gives rise to perturbations in SHH signaling, we performed quantitative real-time reverse transcriptase–PCR for the major target genes Ptch1, Gli1, Gli2, as well as Ccnb1 (cyclin B1), which was proposed to act together with Ptch1 in regulating the cell cycle (Barnes et al., 2001). Although the average transcript level of total Ptch1 was elevated in the skin (Figure 4a, P < 0.0001 by t-test), and to a lesser extent also in skin cancers from K14PtchFVB mice (Figure 4b, P = 0.0014 by t-test), Gli1 and Gli2 levels in skin (Figure 4a) and skin carcinomas (Figure 4b) were comparable between K14PtchFVB and wild-type mice. The average Ccnb1 transcript level was not different between the two genotypes, both in normal skin and in skin tumors (Figure 4a and b). We conclude that all of these known SHH pathway candidate genes are not significantly disrupted in expression levels, at least at the whole tissue level, in Ptch1 transgenic skins.

To investigate other possible consequences of Ptch1 overexpression in K14PtchFVB mice, we chose candidate genes on the basis of correlation in transcript levels with Ptch1 in normal skin from interspecific backcross mice (Quigley et al., 2009; additional data not shown). We performed quantitative real-time reverse transcriptase–PCR analysis for eight candidate genes—Bmp6, Bnc2, Hdgfrp3, Nt5e, Sox4, Gli3, Lphn1, and Ncdn—all of which were significantly correlated with Ptch1 expression in normal skin. The expression analysis showed that only the Sox4 transcript level was significantly decreased in adult skins of K14PtchFVB mice compared with controls (Figure 4c, P = 0.001 by t-test), but no difference was seen in skin carcinomas (Figure 4b). This reduced level of Sox4 was also seen, but to a lesser extent, in newborn skins from line 6 and 8 K14PtchFVB mice (Figure 4d, P = 0.023 by t-test).

Presence of Ptch1 transgene-positive cells in skin carcinomas

To examine Ptch1 transgene expression at the cellular level in skin and skin carcinomas from transgenic mice, we carried out an immunohistochemical analysis using antibodies against the hemagglutinin (HA) tag present at the C-terminus of the Ptch1 transgene. Cells positive for anti-HA were simultaneously positive for anti-Ptch1 by co-staining. First, we evaluated normal skin from the K14PtchFVB transgenic lines for the presence of HA/Ptch-positive epithelial cells. Of the three K14PtchFVB transgenic lines, a small number of HA/Ptch-positive cells was only detectable in skin from line 6 K14PtchFVB mice (Figure 5a). These HA/Ptch-positive cells were clustered in small foci rather than being scattered as single cells at the basal layer of the epidermis (Figure 5a). On the other hand, no HA-positive cells were identified in skin from line 7 and 8 K14PtchFVB mice. It appears that although the Ptch1 mRNA level remains elevated in adult skin from K14PtchFVB mice compared with wild-type mice, significant protein expression is only detectable in clusters of epidermal cells during development.

Next we examined the presence of HA/Ptch-positive cells in skin carcinomas from K14PtchFVB mice. Of the 24 transgenic mouse carcinomas tested, 20 (83%) showed small clusters of HA/Ptch-positive cells, but none were detected in carcinomas from wild-type mice (Supplementary Table S1 online). These cells were mainly detected in SCCs, and if present in spindle cell carcinomas, they were predominantly in areas of the tumor that showed squamous differentiation (Figure 5b and c). In the SCCs, the HA/Ptch-positive cells were located at the superficial aspect of the spinous layer, near the interface with the keratinized/cornified cells (Figure 5b), particularly in tumor regions with strong K14 expression (Figure 5a). The cell proliferation marker, Ki67, was frequently expressed in the skin cancer cells, but the HA/Ptch-positive cells (78–100%) were largely negative for Ki67 expression (Figure 5b), indicating that these cells are likely nonproliferative. We also performed co-staining of the known skin stem cell markers, Sox2, Pax6, or Cd34, with the HA/Ptch tag (Li et al., 2005; Takahashi and Yamanaka, 2006; Malanchi et al., 2008). Some HA/Ptch-positive cells showed co-staining with Sox2 and Pax6, but the pattern was essentially random (Figure 5c), and these cells were largely negative for Cd34 (Figure 5c). We conclude that any HA/Ptch-positive cells that are found in skin carcinomas are predominantly terminally differentiated cells without significant proliferative and stem cell–like activity.

DISCUSSION

In this study, we have explored the paradoxical role of exogenous Ptch1 expression in promoting Ras-driven SCC development. Line 6 K14PtchFVB mice with the highest Ptch1 overexpression had severe developmental defects, including growth retardation, loss of digits in the forelimbs and hindlimbs, and abnormalities of the eyes. The similarity between these phenotypes and those caused by germline mutations leading to loss of the SHH pathway function (Chiang et al., 1996; Chiang et al., 2001; Zhang et al., 2001) indicates that the expression of Ptch1 driven by the K14 promoter during development (Kopan and Fuchs, 1989; Byrne et al., 1994) results in perturbation of Shh signaling by either cell autonomous or non-cell autonomous mechanisms, leading to the observed major developmental abnormalities in developing epithelia.

Skin tumor susceptibility studies were conducted on two transgenic lines (lines 7 and 8), in which Ptch1 transgene expression was relatively low. Nevertheless, these lines were found to be highly susceptible to chemically induced carcinomas of the skin in a two-stage carcinogenesis study. Gene expression analysis detected no changes in the major down-stream effectors of SHH signaling, such as Gli1 or Gli2, in adult skin, although it remains possible that single cells or clusters of Ptch1-positive cells in transgenic skin could express the altered levels of these markers. Of the other candidate genes selected on the basis of correlations with Ptch1 expression in heterogeneous mouse populations (Quigley et al., 2009), only Sox4 was expressed at lower level in the skins of K14PtchFVB mice. A previous study suggested that PTC1 is one of the SOX4 transcriptional target genes (Scharer et al., 2009), and coexpression of Sox4 and Shh in the developing
hair germ was observed (Kobiela et al., 2007). Therefore, it is possible that one consequence of Ptch1 overexpression is deregulation of stem cell dynamics through a feedback leading to altered Sox4 expression in K14PtchFVB mice. At the molecular level, in vitro studies using human 293T cells indicated that one possible mechanism by which Ptch1 and the Ras pathway interact is through the modulation of mitogen-activated protein kinase signaling. Strong activation of p-ERK by TPA led to disruption of the previously reported strong binding of the Ptch1 PtchB6 variant to mTid1 (Wakabayashi et al., 2007), a tumor-suppressor protein originally discovered in Drosophila (Canamasas et al., 2003). It is possible that activation of Ras/mitogen-activated protein kinase signaling alleviates the tumor-suppressor functions of Tid1 through reduction of its interaction with Ptch. Further studies using conditional knockout alleles will be required to confirm this hypothesis.

Figure 4. Quantitative real-time reverse transcriptase–PCR (qRT–PCR) analysis of the candidate sonic hedgehog (SHH) signaling targets. (a) Ptch1, Gli1, Gli2, and Ccnb1 transcript levels in skins from K14PtchFVB (n = 14) and wild-type (WT; n = 6) mice. Ptch1 expression is significantly elevated in the skins of K14PtchFVB mice (P < 0.0001 by t-test). (b) Average Ptch1, Gli1, Gli2, Ccnb1, and Sox4 transcript levels in skin cancers from K14PtchFVB and WT mice. Ptch1 expression is also elevated in skin carcinomas from K14PtchFVB mice (P = 0.0014 by t-test). (c) Analysis of Bmp6, Bnc2, Hdgfrp3, Nt5e, Sox4, Gli3, Lphn1, and Ncdn transcript levels in adult skins from K14PtchFVB and WT mice. The average Sox4 transcript level is significantly decreased in adult skins of K14PtchFVB mice (P = 0.001 by t-test). (d) Average Bmp6, Bnc2, Hdgfrp3, Nt5e, Sox4, Gli3, Lphn1, and Ncdn transcript levels in newborn skins from K14PtchFVB (n = 5) and WT (n = 5) mice. The average Sox4 transcript level is also slightly lower in newborn skins of K14PtchFVB mice (P = 0.023 by t-test). Statistical significance is indicated as **P < 0.001; *P < 0.05 by t-test.
required to determine whether similar mechanisms have a role in promoting the growth of Ras-initiated cells in mouse skin.

We also tested the possibility that the subpopulation of cells within carcinomas that continued to express the Ptch1 transgene might be involved in tumor maintenance, or express markers of skin stem cells. However, these cells were only found in very well-differentiated tumors, or in highly differentiated cells within more aggressive tumors. They were negative for expression of cell cycle marker, suggesting that they do not display proliferative activity and were also negative for markers, such as Cd34, Pax6, and Sox2, which have been implicated in control of skin stem cells. These data are compatible with the interpretation that the Ptch1 transgene expression, although clearly acting to promote SCC development, is no longer required for the maintenance of these tumors and is silenced during tumor progression.

Our data are compatible with the hypothesis that the SHH pathway, when activated through loss of Ptch1 or activation of downstream effectors, such as Smoothened (Smo) or Gli1/2, leads to increased susceptibility to basal cell carcinoma formation by promoting the cell-fate decision leading to the appropriate cell of origin for these tumors. The overexpression of Ptch1, however, promotes an alternative epidermal cell-fate decision leading to increased SCC formation. The concept that
developmental regulators that promote one particular lineage can also simultaneously suppress an alternative lineage is well known (Davidson, 2010). Such a mechanism may have an important influence on susceptibility to different types of tumors in the same tissue, conferring susceptibility to one type but resistance to an alternative tumor arising from a different lineage. Polymorphisms in genes that influence alternative cell-fate decisions may therefore have an important role in determining individual tumor susceptibility at an early stage of carcinogenesis.

**MATERIALS AND METHODS**

**Mice, tumor induction, and histological analysis**

All animal experiments were conducted under the approval of the Institutional Animal Care and Use Committee of the University of California, San Francisco. The original $K14Ptch^{FVB}$ mice from lines 6,
7, and 8 were previously described (Wakabayashi et al., 2007). Lines 7 and 8 were maintained on the FVB/N background and crossed with C57BL/6 mice to generate F1 wild-type and K14PtchFVB mice for two-stage chemical carcinogenesis. A total of 23 line 7 K14PtchFVB and 26 line 8 K14PtchFVB mice along with 35 wild-type mice (combined from both lines) were used to induce chemical carcinogenesis. Mice were genotyped using PCR primers previously described (Wakabayashi et al., 2007). To induce skin carcinogenesis, a single dose of DMBA was topically applied at 8 weeks of age followed by twice-weekly application of TPA for 20 weeks as described (Balmain et al., 1984; Quintanilla et al., 1986). Papilloma number was recorded from 10 weeks after DMBA up to 20 weeks and carcinoma development was monitored up to 50 weeks after TPA treatment. Mice were killed if they were moribund, if there were excessive tumor loads, or if any single tumor exceeded 1.5 cm in diameter, or at the termination of the experiment. Tumors removed by surgical dissection were immediately snap-frozen in liquid nitrogen or fixed in 10% buffered formalin solution for further analysis. Paraffin-embedded tumor sections were stained with hematoxylin and eosin for histopathological analyses.

DNA and RNA extraction
Frozen skin and tumor specimens were powdered in liquid nitrogen with pestle and mortar and homogenized for DNA and RNA preparation. For RNA extraction, specimens were homogenized and dissolved in TRIzol Reagent (Invitrogen, Carlsbad, CA) and purified for RNA isolation according to the manufacturer’s instructions. Genomic DNA was isolated using standard phenol/chloroform extraction following an overnight incubation at 55 °C with Proteinase K (Sigma, St Louis, MO) in a lysis buffer (50 mM Tris at pH 8.0, 100 mM EDTA, 100 mM NaCl, and 1% SDS).

H-ras mutation analysis
To determine a specific H-ras mutation, CAA to CTA at codon 61, exon 2 of the H-ras gene was amplified using primers previously described (Nagase et al., 2003). Amplified PCR fragments were digested with restriction enzyme XbaI (NEB, Ipswich, MA) at 37 °C for 2 hours and electrophoresed in a 4% Nusieve 3:1 agarose gel (Lonza, Walkersville, MD).

Cell culture and transfection
293T cells were grown at 37 °C in DMEM supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glucose, and maintained in a humidified atmosphere of 5% CO2. HA-tagged PtchFVB and PtchB6 constructs in pcDNA3.1 and pLXSP3 retrovirus constructs in pLXSP3 were previously described (Wakabayashi et al., 2007). For transient transfection or co-transfection, Lipofectamine 2000 (Invitrogen) was used according to the manufacturer’s instructions (Invitrogen). 2007). For transient transfection or co-transfection, Lipofectamine 2000 (Invitrogen) was used according to the manufacturer’s instructions (Invitrogen). 2007). For transient transfection or co-transfection, Lipofectamine 2000 (Invitrogen) was used according to the manufacturer’s instructions (Invitrogen). 2007). For transient transfection or co-transfection, Lipofectamine 2000 (Invitrogen) was used according to the manufacturer’s instructions (Invitrogen).

Western blotting, immunoprecipitation, and Ras-GTP assays
Western blotting was performed as previously described (Wakabayashi et al., 2007). Antibodies ERK1/2 (137F5), p-ERK1/2 (20G11), Tid1 (RS13 and RS-11), H-Ras (C-20, sc-520), HA-tag (mouse monoclonal 6E2 and goat polyclonal ab9134), and β-actin (from Sigma) were used. For TPA treatment, transfected 293T cells were incubated with 500 nM of TPA for 30 minutes and 1 hour. For immunoprecipitation, 500 µg of lysates were processed with HA Tag IP/Co-IP kit (Thermo Scientific, Waltham, MA) and Dynabeads Protein G IP kit (Invitrogen) according to the manufacturers’ instructions. For Ras-GTP assay, cells were washed with ice-cold phosphate-buffered saline and lysed in 1 x MLB (Magnesium-containing Lysis Buffer) with protease and phosphatase inhibitor cocktails. Raif-RBD pull down was performed with Ras Activation Assay kit containing Raf-1 RBD agarose beads according to the manufacturer’s manual (EMD Millipore, Biella, CA).

Expression analysis for SHH signaling targets
Single-strand complementary DNAs were synthesized from 1 µg of DNase-treated total RNA from skins and skin carcinomas using SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufacturer’s manual. Predesigned TaqMan probes and primers for Ptch1 (Mm01306905), Glil (Mm00494654), Glil (Mm01293111), Ccnb1 (Mm00838401), Sox4 (Mm00486317), Bmp6 (Mm01332882), Bnc2 (Mm01266537), Nfse (Mm00501917), Hdpfrp3 (Mm01324333), Glil (Mm00492435), Lpbn1 (Mm00492435), Ncdn (Mm00445929), Actb (β-actin, 4352933E), and glyceraldehyde-3-phosphate dehydrogenase (Mm99999915) were used for quantitative real-time reverse transcriptase–PCR analysis (ABI, Foster City, CA). Amplification of complementary DNAs was carried out in triplicate for each sample in the ABI 7900HT system according to the manufacturer’s protocol. The normalized transcript level was determined by calculating ΔCt values by subtracting mean Ct values of target genes with mean Ct values of housekeeping genes. Then, 1/ΔCt values were used to obtain representative values indicating higher values to higher transcript levels.

Immunofluorescence
Skin and carcinoma specimens were fixed in buffered formalin solution and processed for paraffin embedding. Sections (5 µm in thickness) were deparaffinized with xylene two times for 10 minutes and processed in a pressure cooker for antigen retrieval using Trilogy solution (Cell Marques, Rocklin, CA). The sections were blocked in 10% donkey serum supplemented with 0.3% Triton X-100 for 1 hour and incubated with primary antibody (HA tags, goat; Abcam, Cambridge, MA) for 1 hour at room temperature. After rinse with phosphate-buffered saline, the sections were incubated with FITC-conjugated secondary antibody for 1 hour at room temperature. For double staining, HA-tag incorporated sections were further blocked in 10% goat serum supplemented with 0.3% Triton X-100 for 1 hour and incubated with rabbit Ptch1 (Abcam), Ki67 (Neomarkers, Fremont, CA), K14 (Covance, Princeton, NJ), Sox2 (Abcam), Pax6 (Abcam), or Cd34 (rat, BD Pharmingen, San Diego, CA) antibodies. After rinse with phosphate-buffered saline, the sections were incubated with Alexa 555-conjugated anti-rabbit or anti-rat secondary antibodies followed by 4,6-diamidino-2-phenylindole staining. The section images were examined under the fluorescent microscope (Olympus BX60, Center Valley, PA).

CONFLICT OF INTEREST
The authors state no conflict of interest.

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**SUPPLEMENTARY MATERIAL**

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

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