

# Sonic hedgehog-induced type 3 deiodinase blocks thyroid hormone action enhancing proliferation of normal and malignant keratinocytes

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**The Sonic hedgehog (Shh) pathway plays a critical role in hair follicle physiology and is constitutively active in basal cell carcinomas (BCCs), the most common human malignancy. Type 3 iodothyronine deiodinase (D3), the thyroid hormone-inactivating enzyme, is frequently expressed in proliferating and neoplastic cells, but its role in this context is unknown. Here we show that Shh, through Gli2, directly induces D3 in proliferating keratinocytes and in mouse and human BCCs. We demonstrate that Gli-induced D3 reduces intracellular active thyroid hormone, thus resulting in increased cyclin D1 and keratinocyte proliferation. D3 knockdown caused a 5-fold reduction in the growth of BCC xenografts in nude mice. Shh-induced thyroid hormone degradation via D3 synergizes with the Shh-mediated reduction of the type 2 deiodinase, the thyroxine-activating enzyme, and both effects are reversed by cAMP. This previously unrecognized functional cross-talk between Shh/Gli2 and thyroid hormone in keratinocytes is a pathway by which Shh produces its proliferative effects and offers a potential therapeutic approach to BCC.**

basal cell carcinoma | thyroxine | differentiation | cancer

Thyroid hormone action is regulated by the activity of the deiodinases. Type 2 deiodinase (D2) activates the prohormone thyroxine (T4) by converting it to thyroid hormone (T3), whereas D3, by inactivating T3, terminates thyroid hormone action (1). All vertebrates express D2 and D3 that, in adults, contribute to plasma T3 and T4 homeostasis by their concerted actions with the hypothalamic–pituitary feedback axis. This homeostatic mechanism is possible because the *Dio3* gene is transcriptionally stimulated by T3, whereas D2 is inhibited by two thyroid hormone-mediated effects, a transcriptional down-regulation of *Dio2* as well as protein inactivation by ubiquitination (for review, see ref. 2). During development, preprogrammed changes in D2 and D3 expression are thought to regulate intracellular T3 concentrations essential to the normal development of the central nervous system, including the retina and the inner ear (3–6). However, the signals governing the changes in D2 and D3 expression during these complex processes are largely unknown.

New insight into the developmental regulation of deiodinase expression has recently been obtained in the chicken growth plate, where Indian hedgehog induces WSB-1, an E3 ubiquitin ligase adaptor that inactivates D2 (7). The hedgehog pathway, acting through the Gli family of transcription factors, determines patterns of cell growth and differentiation in a wide variety of developmental settings (8–13). Given that, in general, signals regulating D2 expression affect D3 in a reciprocal fashion (2), we hypothesized that hedgehog proteins could up-regulate D3 while suppressing D2 expression. To explore this possibility, we turned to skin, a system in which Sonic hedgehog (Shh) is known to play

dominant physiological as well as pathological roles (14). Both D2 and D3 are present in skin, a well recognized target of thyroid hormone (15), although their specific cellular distribution and functional roles remain unknown. Here we report that while Shh promotes WSB-1-induced D2 degradation in normal and malignant keratinocytes, D3 expression is highly increased in the same setting. These coordinated changes in deiodinase expression reduce thyroid hormone action in the skin microenvironment, a pathway by which Shh exerts its actions. Our data also support a potential therapeutic application for T3 in basal cell carcinoma (BCC), the most common type of cancer in light-skinned individuals, caused by a hyperactivated Shh signaling pathway.

## Results and Discussion

D3 first appears in the mouse embryonal epidermis at embryonic day (E) 15.5 and is highly expressed in the epidermal layers and in hair follicle keratinocytes by E17.5 [supporting information (SI) Figs. 4 and 5]. After birth, in the growing phase of the hair follicle cycle (anagen), D3 expression is primarily localized in the hair follicle and more specifically is intense in the hair matrix and in the surrounding outer root sheath, weakly detectable in the epidermis and in the skeletal muscle layer (panniculus carnosus). It progressively decreases during catagen and is almost absent in telogen (Fig. 1*a* and data not shown). This pattern, consistently observed during consecutive hair cycles (data not shown), parallels that of Shh target proteins, which are expressed in anagen and cease in telogen (16).

To evaluate whether Shh induces D3 in keratinocytes, we treated HaCaT cells (a human keratinocyte cell line) with recombinant Shh. Shh increased D3 mRNA expression (3.8- to 4.6-fold) and, to a similar extent, WSB-1 mRNA, as well as the canonical Shh targets Ptch and Foxe-1 (Fig. 1*b*) (17, 18). D3 mRNA was also increased by the Shh transcriptional effector Gli2 and the activated Gli2ΔN mutant (data not shown), which confirms that *Dio3* responds to the Shh/Gli2 cascade. The

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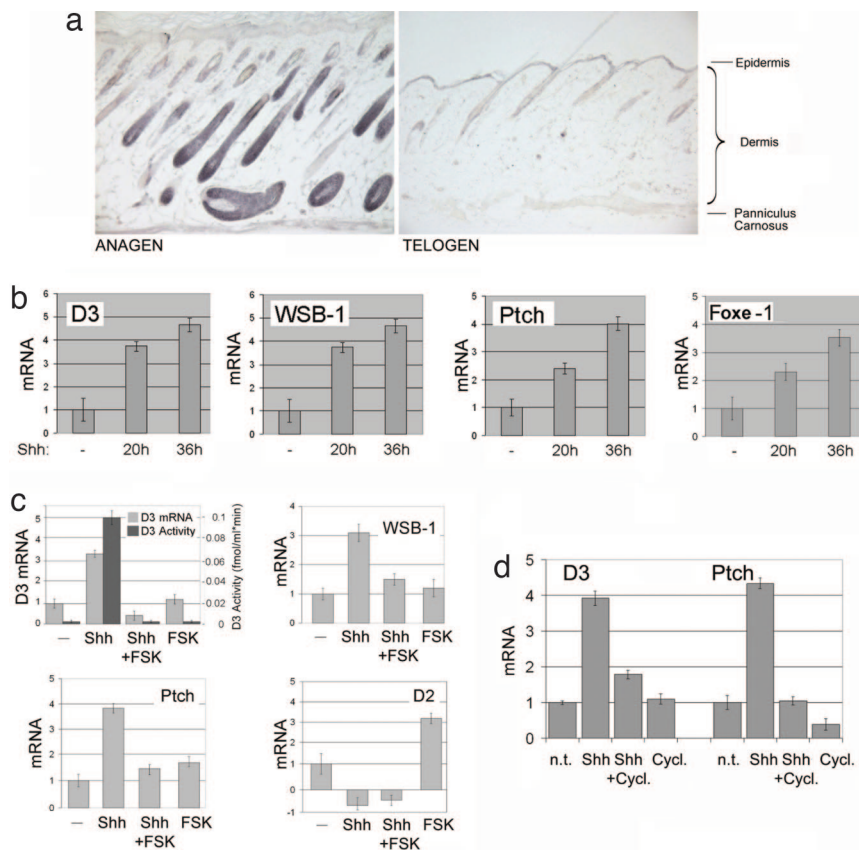
The authors declare no conflict of interest.

Abbreviations: BCC, basal cell carcinoma; D2, type 2 iodothyronine deiodinase; D3, type 3 iodothyronine deiodinase; Shh, Sonic hedgehog; T3, thyroid hormone; T4, thyroxine.

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**Fig. 1.** Shh induces D3 expression and activity. (a) D3 expression during the hair follicle cycle is time- and cell type-specific and overlaps Shh targets. Paraffin sections of mouse skin at different stages of the hair follicle cycle were probed with D3-718 antibody. During anagen (P5), D3 was highly expressed in the hair follicle matrix and absent from the dermal papilla. In telogen (P21), D3 expression was almost absent from the hair follicles. (b) mRNA expression of D3 and WSB-1 as well as of the Shh targets Ptch and Foxe-1 was measured by real-time PCR with cDNAs from HaCaT cells treated for 20–36 h with 4  $\mu$ g/ml recombinant Shh. (c) Expression and activity of D3 were measured in HaCaT cells treated with 4  $\mu$ g/ml Shh or vehicle, supplemented 12 h later with 50  $\mu$ M forskolin (FSK), and harvested 24 h thereafter. WSB-1, Ptch, and D2 mRNA expression was similarly assessed. (d) D3 mRNA (and Ptch used as an internal control) expression was measured in HaCaT cells treated with 4  $\mu$ g/ml Shh, Shh plus 10  $\mu$ M cyclopamine, or vehicle for 36 h. (b–d) Data represent the mean of at least three independent experiments in duplicate. SD are indicated.

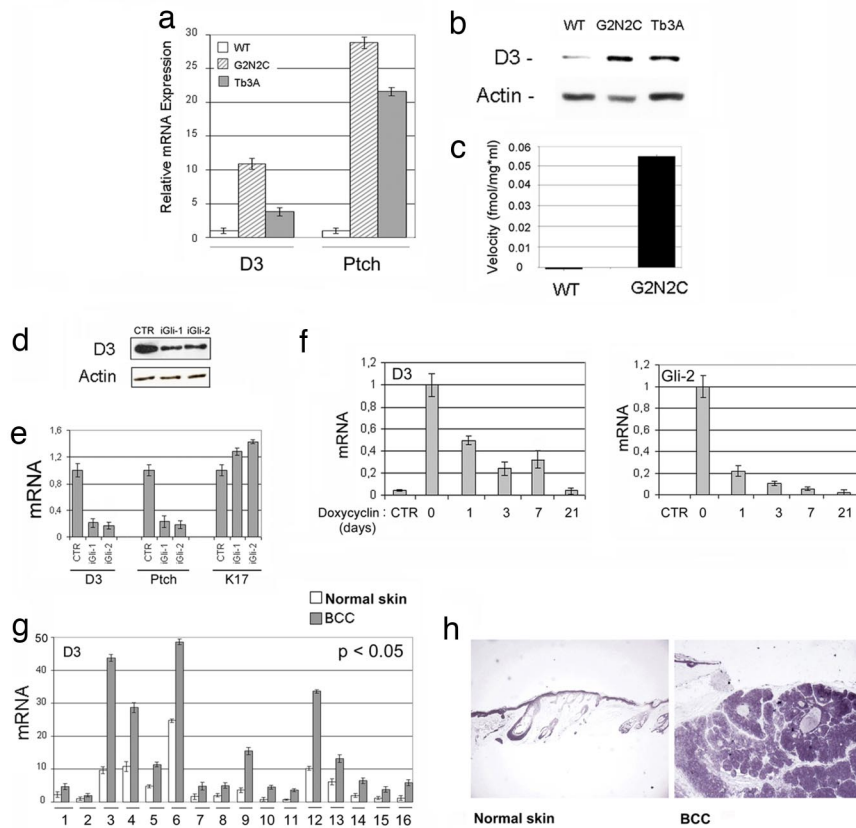
increase in D3 enzymatic activity was even greater than that of D3 mRNA (Fig. 1c). Shh induction of D3, and of WSB-1 and Ptch, was completely reversed by treatments with forskolin or with the specific smoothed inhibitor cyclopamine, which both inhibit Shh signaling (19, 20) (Fig. 1c and d and data not shown). In contrast, D2 mRNA was not increased by Shh signaling but was induced 3.2-fold by forskolin (Fig. 1c), consistent with the presence of a cAMP-regulating element in the *Dio2* promoter (21).

Shh is known to be constitutively active in BCCs and related skin tumors (13, 22). We found that D3 and Ptch mRNAs are significantly higher in two BCC cell lines (G2N2C and Tb3A) derived from Gli2 $\Delta$ N-expressing transgenic mice than in WT-7 (WT), a control keratinocyte cell line (Fig. 2a) (23). D3 protein paralleled D3 mRNA (Fig. 2b) as did D3 enzymatic activity (Fig. 2c). To test the hypothesis that active Gli2 is responsible for elevated D3 expression in G2N2C cells, we inactivated Gli2 by RNAi, using specific Gli2 RNAi oligonucleotides (SI Fig. 6a and b). D3 expression was significantly reduced at both mRNA and protein levels upon Gli2 knockdown, similarly to the Gli2 direct target Ptch (Fig. 2d and e), suggesting a crucial role for active Gli2 in sustained D3 expression.

To verify the dependence of D3 expression from Gli2 in an *in vivo* setting, we took advantage of a mouse model that conditionally expresses Gli2 in the skin generating multiple BCCs. Doxycycline administration causes Gli2 transgene inactivation,

and reduced hedgehog targets gene expression, associated with tumor regression (24). Time-dependent analysis of D3 mRNA levels after doxycycline-treatment showed that elevated D3 expression before treatment was followed by a time-dependent D3 reduction (Fig. 2f), in agreement with the concept of D3 as a Gli2 target gene. Interestingly, after 21 days of doxycycline treatment, the persistent nonproliferating cell population expresses very low D3 levels (Fig. 2f), suggesting a functional link between D3 expression and cell proliferation. Next, we analyzed two BCC samples from Gli2 mice (23) and found that they also expressed elevated D3 levels compared with normal skin (data not shown). We then measured D3 mRNA in 16 human BCC samples and found that it was significantly higher in BCCs versus surrounding normal skin (Fig. 2g). Furthermore, D3 levels correlated with the functional status of the Shh pathway as demonstrated by comparison (Bonferroni multiple comparison test) with Ptch and Foxe-1 mRNA expression (SI Fig. 7a and b). Similar results were also obtained by normalizing D3 expression with a keratinocytes marker (K14) to take into account possible differences in the tissue samples between BCCs versus normal skin (SI Fig. 7c). Fig. 2h shows D3 immunostaining from a representative human BCC sample.

To identify the molecular mechanisms underlying D3 regulation by Shh/Gli2, we investigated whether Gli2 directly controls the *Dio3* promoter. HeLa cells were transfected with a subset of h*Dio3* promoter deletion constructs together with a wild-type



**Fig. 2.** D3 expression is markedly increased in mouse and human basal cell carcinoma. (a) Real-time PCR analyses of D3 and Ptch expression in mouse BCC (G2N2C and Tb3A) and in control WT-7 (WT) cells. *c-Abl* mRNA level served as reference. mRNA expression levels in WT cells are set as 1. (b) Western blot analysis of D3 expression in G2N2C, Tb3A, and WT cells. Thirty micrograms of total cell lysates were subjected to SDS/PAGE and immunoblotted with a polyclonal anti-D3 antibody (1:500). (c) D3 activity was measured in the indicated protein lysates as described under "Materials and Methods." (d) Western blot analysis of D3 expression in G2N2C transiently transfected with a control RNAi oligonucleotide (CTR) or with two Gli2 RNAi oligonucleotides (iGli-1 and iGli-2). (e) D3, Ptch, and K17 mRNA expression in the same cells as in d. (f) D3 mRNA expression in regressive BCCs from doxycycline-treated bitransgenic mice. Efficient transgene inactivation was confirmed by the potent Gli2 mRNA down-regulation in the same samples. (g) D3 mRNA levels were analyzed by real-time PCR in 16 human BCC samples and in the corresponding tumor-free skin from the same patients. Values were normalized to  $\beta$ -actin mRNA levels. All samples were run in triplicate and referred to normal skin in sample 1 set arbitrarily as 1. *P* values were calculated by one-way ANOVA. (h) D3 immunostaining of a representative human BCC sample (sample 12 in d) and the normal counterpart skin.

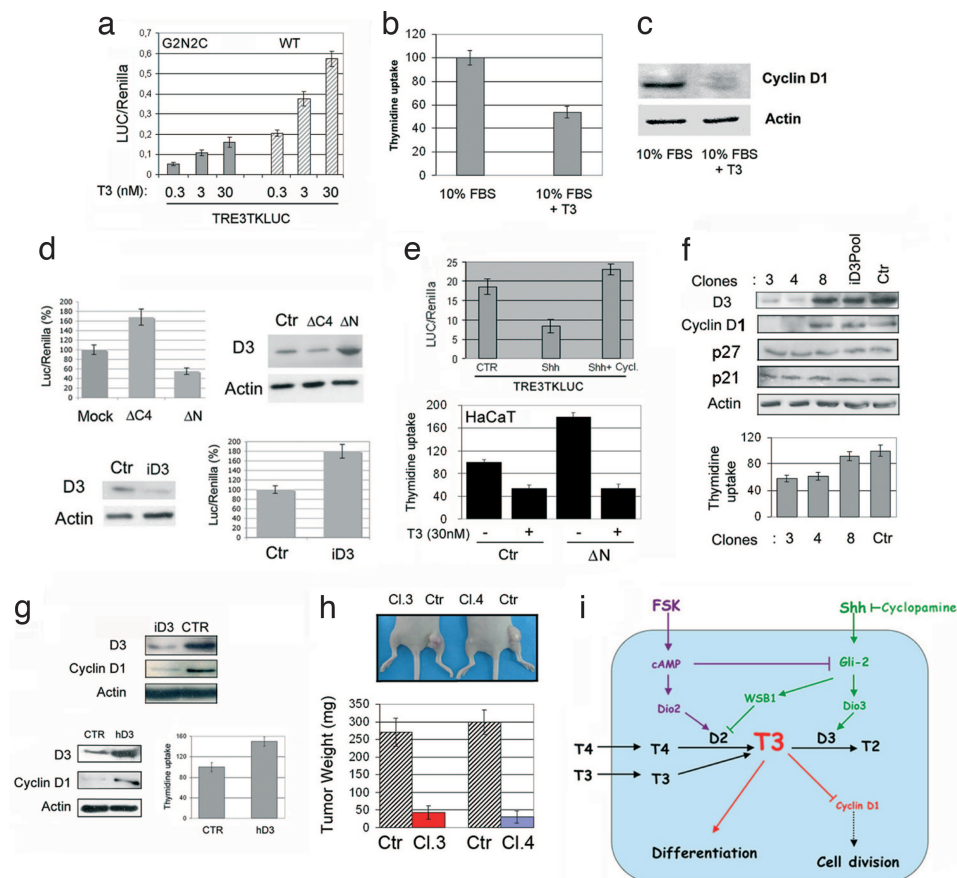
(WT), a dominant-negative ( $\Delta$ C4), or a constitutively active ( $\Delta$ N) Gli2 mutant (SI Fig. 8a). A 749-bp *hDio3* region was strongly induced in the presence of WT and  $\Delta$ N, whereas a minimal *hDio3* promoter region ( $-90$  bp from the ATG codon) was unresponsive. The 3,180-bp 5'-UTR of WSB-1 was also strongly induced by both WT and  $\Delta$ N Gli2 (SI Fig. 8a). These findings show that *Dio3* and WSB-1 respond transcriptionally to Gli2. Similar results were obtained in normal and malignant keratinocytes. In G2N2C cells, the  $-749$  *hDio3* promoter was further induced by an exogenous  $\Delta$ N mutant and inhibited by  $\Delta$ C4 (SI Fig. 8b). Gli2 responsiveness required DNA sequences spanning  $-224/-90$  from the initiator ATG. Interestingly, the  $-749$  construct was at least 20-fold less active under basal conditions in WT cells than in G2N2C cells (SI Fig. 8b and c), which coincides with the lower D3 mRNA level in the absence of active Gli2.

We examined the binding of Gli2 to a putative binding site (D3-A; GATCACCCA) highly homologous to a canonical Gli2-binding site (GACCACCCA) located  $-191$  bp 5' to the ATG (SI Fig. 9a). A specific shifted band appeared when nuclear extracts from WT or  $\Delta$ N-transfected HEK293 cells were added to the radiolabeled D3-A probe (SI Fig. 8d and e). Moreover, D3-A formed a complex with nuclear extracts from G2N2C cells but not from WT cells (SI Fig. 8f and g), again suggesting that the Gli2 protein is required for binding. A change from CACC to

TCTT ( $-749$ mut) within the 749-bp *hDio3* promoter markedly reduced the response to Gli2 in HeLa (SI Fig. 8a) and G2N2C cells (SI Fig. 8b). Thus, D3-A is critical for the *hDio3* response to Gli2. The D3-A site, which is evolutionarily conserved in rodents, is present in the putative 5'-UTR of the *hDio3* mRNA. Using 5'-RACE and reverse transcription PCR analyses, we identified two alternative transcription start sites in mouse keratinocytes, the major one being downstream from the D3-A site (SI Fig. 9).

Thyroid hormone affects many physiological processes, including the balance between cell proliferation and differentiation (25, 26). In many cell types, T3 induces differentiation, a process linked to growth arrest and exit from the cell cycle. We postulated that D3 could enhance BCC proliferation by inactivating T3. We analyzed BCC cells and WT keratinocytes after expression of a thyroid hormone-sensitive promoter (TRE3-TK-Luc) and found that, although both responded to T3 in a dose-dependent fashion (Fig. 3a), G2N2C cells are less sensitive to T3 than the WT cells at various T3 concentrations, suggesting that thyroid hormone signaling is attenuated in the BCC cell line versus the WT keratinocytes. In G2N2C cells, an excess of T3 (30 nM) reduced thymidine incorporation by  $\approx 40\%$  (Fig. 3b). Cyclin D1 is a common target of Shh (27) and thyroid hormone receptor (25). In G2N2C cells treated with 30 nM T3, cyclin D1 expression was much lower than in untreated control cells (Fig. 3c), which





**Fig. 3.** D3 modulates proliferation and tumorigenicity of Gli2 $\Delta$ N-expressing tumor cells. (a) G2N2C and WT cells were transiently cotransfected with a T3-responsive TRE3TKLUC construct and RSV-*Renilla* as internal standard, in charcoal-stripped medium, supplemented for 24 h with 0.3 nM (physiological range), 3 nM, and 30 nM T3. (b) G2N2C cells, supplemented with 30 nM T3 or not for 7 days, were incubated for 1 h with [*methyl*-<sup>3</sup>H]thymidine to a final concentration of 0.05  $\mu$ Ci/ml. [<sup>3</sup>H]Thymidine incorporation in untreated cells was arbitrarily set as 100%. (c) Western blot analysis for cyclin D1 protein levels in G2N2C cells treated or not with 30 nM T3 for 7 days. (d) *Upper*) G2N2C cells were transiently cotransfected with TRE3-TK-Luc, the indicated Gli2 mutants, and RSV-*Renilla* as internal reference. The Luc/*Renilla* value from mock-transfected plasmid was arbitrarily set as 100% (*Right*). (*Left*) Western blot analysis of D3 protein levels from the same samples. (*Lower*) G2N2C cells were cotransfected with TRE3-TK-Luc/RSV-*Renilla* constructs and the D3 RNAi oligonucleotide or the control RNAi (see “Materials and Methods”), and total lysates were analyzed for D3 protein levels (*Right*) or Luc/*Renilla* values as above. (e) *Upper*) HaCaT cells were transiently cotransfected with a T3-responsive TRE3-TK-Luc construct and RSV-*Renilla* as internal standard and treated for 24 h with vehicle, recombinant Shh, or Shh plus 10  $\mu$ M cyclopamine as indicated. (*Lower*) A large T3 excess completely abrogates Gli2 $\Delta$ N-induced proliferation. [<sup>3</sup>H]Thymidine incorporation in HaCaT cells transiently transfected with or without the Gli2 $\Delta$ N mutant and treated with 30 nM T3 or vehicle for 48 h is shown. (f) *Upper*) Western blot analyses of D3, cyclin D1, p27, and p21 protein levels in the indicated clones, iD3 pool, and control cells. (*Lower*) Thymidine-uptake assay of the iD3 G2N2C clones in normal serum condition. (g) *Upper*) Western blot analysis of D3, cyclin D1, and actin levels in G2N2C cells transiently transfected with a D3 (iD3) or a control (CTR) RNAi oligonucleotide. (g) *Lower*) D3, cyclin D1, and actin levels and thymidine incorporation in D3-depleted clone 4 stably transfected with a resistant hD3 plasmid (hD3) or a control empty vector (CTR). (h) Control G2N2C cells (Ctr) and clones 3 and 4 were injected ( $2 \times 10^6$  cells in 200  $\mu$ l of low-calcium medium) s.c. into nude mice [Ctr cells (*Right*) and Cl.3 or Cl.4 (*Left*)]. Growth of tumors from Cl.3 and Cl.4 cells was significantly decreased compared with the corresponding tumors from Ctr cells ( $n = 15$  animals for each clone). Results are shown as mean  $\pm$  SD. (i) Shh and cAMP exert opposite effects on the deiodinase-mediated regulation of intracellular T3 in the keratinocytes.

suggests that T3-mediated inhibition of cell cycle progression involves cyclin D1.

To evaluate whether Gli2 activity affects thyroid hormone signaling in transformed keratinocytes, we transfected different Gli mutants and measured the thyroid status thereafter. Whereas constitutively active Gli2 $\Delta$ N ( $\Delta$ N) reduced TRE3-TK-Luc activity, the dominant-negative Gli2 $\Delta$ C4 ( $\Delta$ C4) enhanced it, with an accordingly opposite change in D3 expression (Fig. 3*d Upper*). A similar increase in thyroid hormone action was observed by D3 knockdown (Fig. 3*d Lower*). Accordingly, in HaCaT cells, Shh treatment reduces the thyroid hormone signaling (Fig. 3*e Upper*) an effect dependent on D3 activity in these cells because no T4 was present in the medium to avoid a possible effect of T3 locally generated by D2-mediated T4 to T3 conversion. Furthermore, proliferation of HaCaT cells, nearly doubled by  $\Delta$ N expression, is reversed by excess T3 (Fig. 3*e Lower*). All

of these data indicate that the Shh/Gli2 signaling attenuates thyroid hormone action in normal and malignant keratinocytes via D3 activity thus facilitating proliferation.

To examine the effect of D3 depletion on cell proliferation, we transfected a specific D3 shRNAi construct (iD3) into BCC cells. Stable transfected iD3 clones were selected by using neomycin resistance. D3 levels were not significantly reduced in the pool (iD3-Pool) of neomycin-resistant cells from two independent transfections (Fig. 3*f Upper*), suggesting that a negative selection was acting against the growth of D3-depleted cells. Therefore, we selected individual clones and isolated two clones (clones 3 and 4) that showed greatly reduced D3 mRNA and protein levels (SI Fig. 10*a* and Fig. 3*f*) for further study. Thymidine uptake experiments showed that, consistent with the increased intracellular thyroid status (SI Fig. 10*b*), D3 depletion significantly reduced proliferation (Fig. 3*f Lower*). In contrast to the stable

levels of cell cycle inhibitors (p21 and p27), cyclin D1 levels were reduced after D3 depletion (Fig. 3*f Upper*), an effect also observed in two other clones (clones 14 and 16) selected to demonstrate the internal consistency of the phenotype (SI Fig. 10*c*). Accordingly, by transient transfection of a D3-specific RNAi oligonucleotide, cyclin D1 levels were also significantly reduced in the mass cell population (Fig. 3*g Upper*).

To further prove that it is D3 expression that is essential for cell proliferation, we performed a rescue experiment by reintroducing, in D3-depleted cells, the human D3 gene, which is resistant to mouse RNAi-mediated degradation (SI Fig. 10*d*). As expected, restoring D3 activity resulted in increased cyclin D1 levels and sustained thymidine uptake (Fig. 3*g Lower*), indicating a functional correlation between D3 activity and proliferation. To assess whether D3 depletion affects tumorigenicity, we injected D3-depleted and control cells into nude mice. Three weeks later, the tumor growth of control cells was >5-fold greater than that of D3-depleted clones in the same mice (Fig. 3*h*), suggesting that D3 protein is required *in vivo* for the tumorigenic potential of transformed keratinocytes.

The present work reveals a mechanism by which Shh can influence the balance between cell proliferation and differentiation in the tissue microenvironment by inactivating T3 as well as blocking its production (Fig. 3*i*). Importantly, the Shh-mediated effects can occur *in vivo* in a time- and tissue-specific fashion independently from the constant levels of circulating thyroid hormone. Shh-induced D3 mRNA and activity in human and mouse keratinocytes is blocked by forskolin as is the Shh induction of both Ptc1 and WSB-1, consistent with the recognized cAMP inhibition of Gli2 effects (Fig. 1*b* and *c*). This blockade of D3 and WSB-1 actions is accompanied by an increase in *Dio2* mRNA, thus helping reverse the T3-suppressive effects of Shh (Fig. 1*c*).

It is interesting to note that Shh alters the deiodinase balance with dual convergent mechanisms (transcriptional up-regulation on D3 and posttranscriptional down-regulation of D2). Previously characterized regulators of D3 at transcriptional levels included T3, the ERKs, and the TGF- $\beta$  pathways (28). Gli proteins are expressed in gliomas (29, 30), and it is not surprising that D3 is also expressed in glial cells (31). The role played by Gli proteins in glial D3 expression has not been yet explored. However, the expression of D3 in embryonic epidermal keratinocytes and in different areas of the hair follicle suggests that other agent(s), together with Shh/Gli2, control D3 expression in the skin.

Shh induces proliferation in a wide variety of cellular systems including the skin (13). In contrast, T3 is a differentiating agent, reducing cyclin D1 and permitting cellular differentiation (25, 26). The convergence of two Shh-induced mechanisms to reduce intracellular T3 implies that thyroid hormone inhibits Shh-dependent effects in the skin. Thyrotoxicosis and hypothyroidism have well recognized effects on this organ, and thyroid hormone receptors are present in the epidermis and in hair follicle (15). However, specific T3-regulated events in normal (or malignant) keratinocytes have not been elucidated. We demonstrated directly that, in G2N2C cells, the expression of a T3-dependent reporter plasmid is regulated by T3 (Fig. 3*a*) and that a D3 knockdown enhances reporter expression caused by the endogenous thyroid hormones (Fig. 3*d*). The Shh-mediated induction of D3 significantly extends the concept that Shh induces a local thyroid hormone attenuation in the microenvironment, already independent of, but synergizing with its reduction of D2 activity (7). The fact that Shh induces D3, thereby preventing T4 activation as well as directly inactivating T3, whether it is generated from T4 or comes from the thyroid gland through the circulation, suggests that abrogating thyroid hormone signaling in a time- and cell type-specific fashion is essential to the biological effect of this morphogen.

The Shh-induced D3 expression observed in normal keratinocytes also occurs in a pathological context. BCCs are associated with hyperactivity of the Shh pathway, and here we show that D3 and Ptc1 (a bona fide Shh target) are increased to a similar degree in BCCs, further supporting the link between D3 expression and Shh/Gli2 pathway (Fig. 2*g* and SI Fig. 7). In addition, either blocking D3 synthesis by RNAi or opposing its action by excess T3 results in a dramatic impairment of proliferation in normal and malignant keratinocytes. These findings suggest that not only is D3 up-regulated in BCC but its action, i.e., induction of hypothyroidism at a cellular level, is required for proliferation. The reduced proliferation produced by D3 knockdown is likely to occur also *in vivo* as demonstrated by the 5-fold reduced tumor growth rate relative to the parent cell in the same nude mice (Fig. 3*h*). Previous data showed that thyroid hormone topically applied on human skin did not alter plasma T3 levels (32), which suggests that the Shh-induced hypothyroidism is likely to be restricted to the skin, not systemic.

In conclusion, it is likely that the normal concentration of plasma thyroid hormones is not permissive for Shh-induced keratinocyte proliferation. The T3-mediated blockade of the proliferation and the reduced tumorigenic potential of D3-depleted cells support this notion. Accordingly, the increased D2 mRNA levels recently observed in the stem cell-enriched human hair follicle bulge cells (33) suggest that intracellular T3 concentrations even greater than those in plasma are appropriate for the low proliferation state of these cells. It is of interest that D3 is expressed in many different neoplastic cells as well as in placenta and human fetal epithelium, with an as yet unknown function (2). We speculate that the resulting reduced intracellular T3 provides a metabolic advantage for these rapidly proliferating cells. Reversing this T3 deficiency may be a reasonable therapeutic approach for BCCs and perhaps other D3-expressing tumors.

## Materials and Methods

**Cell Cultures, Transfections, and Reagents.** G2N2C, Tb3A, and WT keratinocytes are derived from transgenic mice expressing a constitutively active form of Gli2 under the control of the keratin 5 promoter (23). They were isolated from BCC-like tumors called trichoblastomas, which are referred to as BCCs throughout this work. Transient transfections were performed by using Lipofectamine 2000 (Life Technologies, Ltd., Paisley, Scotland) according to the manufacturer's instructions. Recombinant human Shh was purchased from R&D Systems (Minneapolis, MN); forskolin and cyclopamine were from Sigma (St. Louis, MO). Monoclonal anti-cyclin D1,  $\beta$ -actin, p21, and p27 antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA).

**Animals.** For the analysis of hair follicle morphogenesis and cycling, embryonic and neonatal skin from 3-week-old Swiss CD1 female mice was harvested at various times of estimated gestational age. All experiments involving mice were conducted according to Institutional Animal Care and Use Committee procedures.

**Immunohistochemistry.** The rabbit polyclonal D3 antibody D3-718 was affinity-purified as described previously (28). D3 antibody 676 (34) was kindly provided by T. Visser. For immunohistochemistry, sections were incubated with primary D3 antibody diluted 1:400 (the same antibody concentration was also used for preimmune control; SI Fig. 4) and processed with the Vectastain Elite ABC immunoperoxidase kit (Vector Laboratories, Burlingame, CA).

**Plasmids and Expression Constructs.** The reporter plasmids for the *hDio3* promoter constructs (28) and plasmids Gli2 WT,  $\Delta$ C4, and  $\Delta$ N (35) are reported elsewhere. We used a genomic human

