FOXE1, A New Transcriptional Target of GLI2, Is Expressed in Human Epidermis and Basal Cell Carcinoma

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Sonic hedgehog (Hh) signaling plays a key role in epidermal development and skin cancer. Mutational inactivation of the tumor suppressor gene patched (*PTCH*) leads to constitutive activation of the Hh signaling pathway, resulting in activation of target gene transcription by the zinc finger transcription factors GLI1 and GLI2. Recent experiments in mice point to GLI2 as the key mediator of Hh signaling in skin. We have concentrated on the identification of candidate mediators of GLI2 function in the human epidermis. We show here that the forkhead/winged-helix domain transcription factor *FOXE1* is likely to be a direct GLI2 target gene. The kinetics of *FOXE1* induction are similar to the known direct target *PTCH*, and a 2.5 kb upstream fragment containing five GLI-binding sites activates transcription in a reporter assay. We show by *in situ* hybridization that *FOXE1* is expressed in the outer root sheath of the hair follicle, where murine Gli2 is also expressed. *FOXE1* expression is also found in basal keratinocytes of the human epidermis and basal cell carcinoma (BCC). These data point to a putative role of FOXE1 in mediating Hh signaling in the human epidermis downstream of GLI2.

Key words: forkhead transcription factor/GLI transcription factor/hair follicle/hedgehog signaling
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Hedgehog (Hh) signaling plays a key role in vertebrate development, where it is involved in a multitude of biological processes such as cell differentiation, proliferation, and growth (reviewed in Ingham and McMahon, 2001; Nybakken and Perrimon, 2002). Inappropriate activation of Hh signaling has been implicated in developmental defects and a predisposition to various malignancies such as basal cell carcinoma (BCC), medulloblastoma, and rhabdomyosarcoma (reviewed in Toftgard, 2000; Bale, 2002; Mullor *et al*, 2002). Analysis of hereditary and sporadic BCCs revealed that ligand-independent activation of Hh signaling in epidermal cells is the primary event in tumorigenesis. Uncontrolled activation is caused by inactivating mutations in the human tumor suppressor gene patched (*PTCH*) or, in rare cases, by gain-of-function mutations in the Smoothened gene (*SMOH*) (Gailani *et al*, 1996; Hahn *et al*, 1996; Johnson *et al*, 1996; Aszterbaum *et al*, 1998; Xie *et al*, 1998).

Normally, transduction of the HH signal is triggered by binding of secreted HH protein to the 12 pass transmembrane receptor protein (PTCH). Binding of HH to PTCH is thought to relieve the inhibitory effect of PTCH on the seven pass transmembrane protein Smoothened (SMOH), leading to transduction of the signal to the nucleus (reviewed in (Ingham and McMahon, 2001; Nybakken and Perrimon, 2002). The zinc finger transcription factors (TF) *GLI1* and *GLI2* have been identified as key mediators of HH signal transduction and like *PTCH* are themselves HH target genes. Consequently, BCCs express elevated levels of *GLI1*, *GLI2*, and *PTCH*, and other HH target genes (Gailani *et al*, 1996; Dahmane *et al*, 1997; Ghali *et al*, 1999; Regl *et al*, 2002).

Support for a critical role of *GLI1* and *GLI2* in BCC development has come from transgenic mouse models showing that overexpression of either protein in epidermal cells can induce the formation of tumors, some of which display BCC-like characteristics (Grachtchouk *et al*, 2000; Nilsson *et al*, 2000; Sheng *et al*, 2002). More recently, studies of knockout mice have pointed to a role of Gli2 rather than Gli1 as the primary mediator in Hh signal transduction. Firstly, mice deficient in *Gli1* are viable and have no obvious developmental defects (Park *et al*, 2000). In contrast, *Gli2*$^{-/-}$ mice are characterized by severe developmental malformations, including the lack of the floor plate of the neural tube and of the anterior pituitary, and by numerous lung, foregut, skeleton, and skin defects (Ding *et al*, 1998; Matise *et al*, 1998; Mill *et al*, 2003). This *Gli2* phenotype broadly overlaps with that described for *Shh*- or *Ihh*- deficient mice (Chiang *et al*, 1996, 1999; St-Jacques *et al*, 1999). Secondly, removal of *Gli2* but not of *Gli1* leads to partial rescue of the *ptc*$^{-/-}$ phenotype, where Hh signaling is hyperactivated (Bai *et al*, 2002). Thirdly, *Gli2* but not *Gli1* is required for Hh signaling in hair follicle development (Mill *et al*, 2003). Together, these findings suggest that Gli2 rather than Gli1 is the primary transducer of the Hh signal.

To elucidate the mechanism by which GLI2 controls HH-mediated epidermal development and skin cancer, we

Abbreviations: BCC, basal cell carcinoma; Hh, hedgehog; PTCH, 12 pass transmembrane receptor protein; *PTCH*, human tumor suppressor gene patched; Tet, tetracycline

focused on the identification of GLI2-regulated genes with a putative role in mediating Hh signaling downstream of GLI2 in epidermal cells. Here, we present data that the forkhead transcription factor *FOXE1* may be a direct GLI2 target gene. Like *GLI2, FOXE1* is expressed in normal epidermis and BCC, suggesting an involvement of *FOXE1* in executing the transcriptional program triggered by *GLI2*.

Results

Overexpression of GLI2 in human keratinocytes increases *FOXE1* mRNA levels To identify GLI2-regulated genes, we have expressed GLI2 in human keratinocytes followed by cDNA array analysis of GLI2 expressing cells and controls. We have genetically modified the human keratinocyte cell line HaCaT (Boukamp *et al*, 1988) to allow tetracycline (tet)-inducible expression of human *GLI2* (referred to as HaCaT-GLI2His) (Regl *et al*, 2002). Tet-inducible cell lines offer the advantage of precise temporal control of GLI2 expression, which has proved invaluable for the identification of early target genes. Expression profiling of samples treated with tet for 24 and 72 h, respectively, was carried out on filter arrays containing approximately 2200 human expressed sequence tag (EST) (unpublished data). To eliminate differences between clonal HaCaT-GLI2His lines, RNA from four independently isolated lines was pooled and used for cDNA array analysis. As shown in Fig 1, the forkhead transcription factor *FOXE1* was highly upregulated in a GLI2 concentration-dependent manner compared with controls, with a (5.4 $\pm$ 0.94)-fold increase after 24 h and a (10 $\pm$ 1.15)-fold increase after 72 h of tet treatment.

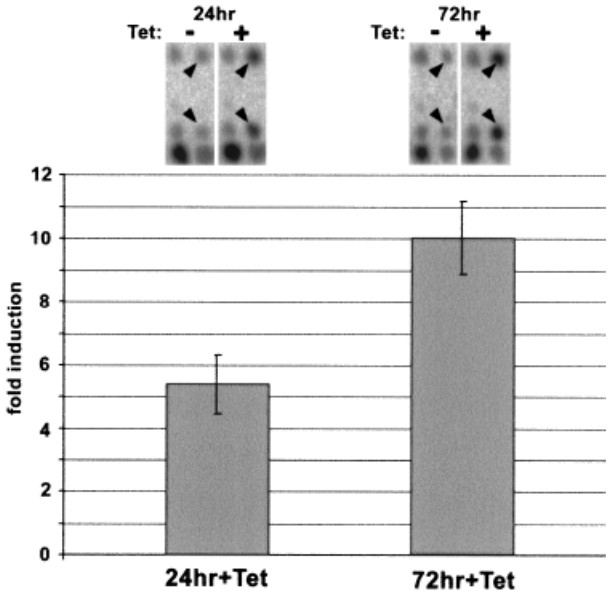

Figure 1
***FOXE1* is upregulated in response to *GLI2* expression in HaCaT and primary human keratinocytes.** cDNA array screen for GLI2 target genes in HaCaT-GLI2His cell lines. The upregulation of *FOXE1* transcript is shown as fold induction compared with uninduced samples. Tetracycline was added at the times indicated. Average induction and standard deviation (SD) were calculated from eight data points. Spots representing *FOXE1* are shown in the upper panel (black arrowheads).

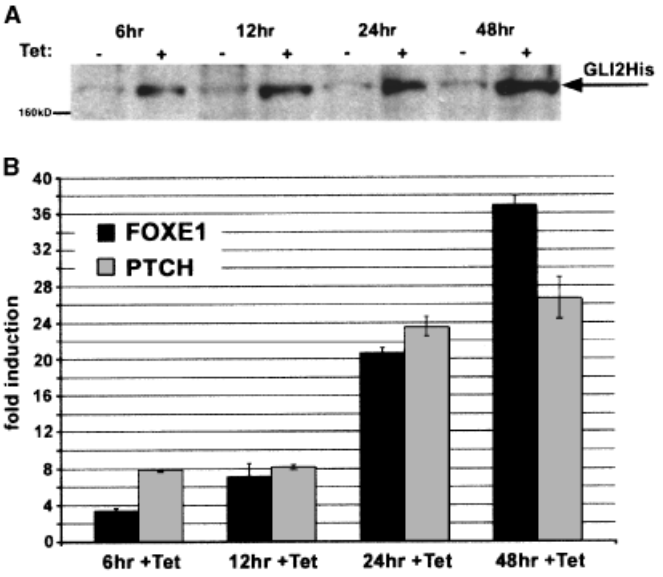

Figure 2
***FOXE1* mRNA expression is an early response to GLI2.** Time-course analysis of *FOXE1* expression in HaCaT-GLI2His cells treated with tetracycline for the times indicated is shown. (*A*) Western blot of GLI2His transgene expression. (*B*) Real-time RT-PCR measurements of *FOXE1* mRNA levels. For comparison, patched (PTCH) is shown. Expression levels are given as fold induction compared with uninduced samples. Data represent the average of three independent PCR runs carried out in duplicate. Error bars are given as standard deviation (SD). All results were normalized for total RNA amount using large ribosomal protein P0 (RPLP0) as reference (Martin *et al*, 2001).

Since cell lines derived from the spontaneously immortalized HaCat cell line may not always reflect the situation in primary cells, the induction of *FOXE1* in response to *GLI2* was confirmed in primary human keratinocytes infected with either GLI2 expressing retrovirus (SIN-GLI2-EGFP) or retrovirus expressing EGFP (SIN-EGFP) only as reference (Regl *et al*, 2004). Expression of FOXE1 and the direct target PTCH as control was measured by real-time RT-PCR in cells 72 h after infection. Similar to HaCaT-GLI2His cell lines, a strong induction of FOXE1 transcription ((16.6 $\pm$ 0.3)-fold) and PTCH ((19.78 $\pm$ 2.45)-fold) was detected in retrovirally infected primary keratinocytes expressing GLI2 (data not shown).

Real-time RT-PCR was also used to validate *FOXE1* induction levels as measured by cDNA arrays in HaCaT cell lines expressing *GLI2*. Additionally, the kinetics of the transcriptional response of *FOXE1* and the appearance of the GLI2 protein were simultaneously analyzed in detail (Fig 2*A*, *B*). RNA and protein samples were taken from HaCaT-GLI2His cells induced for 6 h, 12, 24, and 48 h with tet and compared with untreated controls from the same time points. GLI2 protein was clearly increased over background expression level, also seen in uninduced cells 6 h after tet addition, and levels further increased with prolonged treatment (Fig 2*A*). Similarly, *FOXE1* transcript levels were already elevated (3.42 $\pm$ 0.25)-fold at 6 h of tet treatment and further increased in a GLI2 concentration-dependent manner ((7.17 $\pm$ 1.39)-fold at 12 h, (20.61 $\pm$ 0.57)-fold at 24 h up to (36.9 $\pm$ 1.02)-fold at 48 h). The kinetics of *FOXE1* mRNA accumulation were compared with those of the known GLI target *PTCH* and a similar

Table I. *FOXE1* transcription is preferentially activated by GLI2

	***FOXE1* mRNA (± SD)**			***PTCH* mRNA (± SD)**		
+Tet (h)	**12**	**24**	**72**	**12**	**24**	**72**
HaCaT-GLI1	2.15	5.83	11.97	8.67	13.27	20.96
	(± 0.09)	(± 0.19)	(± 2.40)	(± 1.05)	(± 0.28)	(± 1.12)
HaCaT-GLI2His	6.16	19.65	39.60	13.48	13.95	24.57
	(± 0.52)	(± 1.90)	(± 5.19)	(± 1.14)	(± 1.99)	(± 5.81)

Tet, tetracyline; HaCaT-GLI2His, tet-inducible expression of human *GLI2*.

response was observed ((7.82 ± 0.17)-fold at 6 h, (8.15 ± 0.24) at 12 h, (23.57 ± 1.1) at 24 h and (26.63 ± 2.3)) (Fig 2*B*). The rapid increase in *FOXE1* mRNA transcription in response to GLI2, which was also observed by northern blot (data not shown), suggests that *FOXE1* represents an early GLI2 target gene, which like *PTCH* may be directly regulated by GLI2.

Since GLI1 and GLI2 recognize the same consensus-binding sequence and are known to have overlapping functions (Bai and Joyner, 2001), we compared the induction of *FOXE1* by GLI1 and GLI2 in the respective HaCaT cell lines. Expression levels for *FOXE1* and *PTCH*, given as fold induction compared with uninduced samples, are shown in Table I.

In contrast to the strong induction of *FOXE1* mRNA levels in response to GLI2, a significantly reduced activation of *FOXE1* was detected in HaCaT cells expressing GLI1, pointing to *GLI2* as the main activator. No such differences were observed for *PTCH*, which was upregulated to comparable mRNA levels in HaCaT-GLI1 as well as in HaCaT-GLI2His cell lines. Since, however, there exists a positive feedback loop between *GLI1* and *GLI2* expression (Regl *et al*, 2002), it is difficult to say what part of *FOXE1* activation by GLI1 may actually be due to GLI2.

The promoter region of FOXE1 contains GLI-binding sites The early response of FOXE1 to GLI2 prompted us to investigate whether *FOXE1* might be directly regulated by *GLI2*. We analyzed the putative *FOXE1* promoter region encompassing from −2000 to +688 (translational start site) relative to the transcriptional start site (+1) for the presence of potential Gli-binding sites. Since the transcription start for *FOXE1* was not exactly known, we mapped it by 5′-RACE to a position 28 bp upstream of the known mRNA 5′ end (Acc. no.: NM_004473) (data not shown).

Five sites differing from the GLI-binding consensus motif GACCACCCA (Kinzler and Vogelstein, 1990) by two nucleotide substitutions were found (Fig 3*A*, *B*). All were shown to interact specifically with recombinant purified GLI2 Zn protein (amino acids 1–332, truncated after the Zn finger DNA-binding domain) in electrophoretic mobility shift assays (EMSA) (data not shown).

In a luciferase reporter gene assay, a 2470 bp fragment (−1934 to +536) (Fig 3*A*) containing all five GLI-binding sites was shown to activate transcription 3-fold in the presence of GLI2 compared with control (Fig 3*C*). The early response of *FOXE1* transcription to *GLI2*, and the presence of GLI-binding sites in the FOXE1 promoter region, together

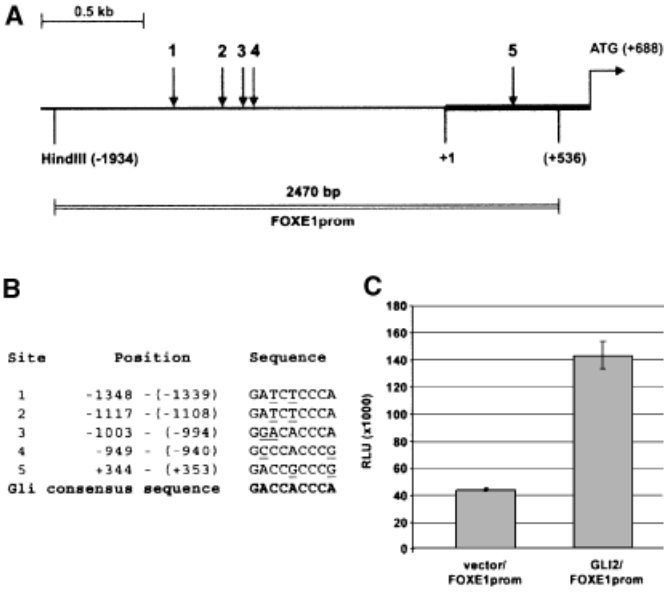

Figure 3
The 5′ upstream region of *FOXE1* contains potential GLI binding sites. (*A*) Location of five GLIi-binding sites (1–5), which were identified by EMSA analyses. (*B*) Sequence and position of the binding sites relative to the transcriptional start site (+1) as determined by 5′RACE. Bases that differ from the published GLI binding consensus sequence (GACCACCCA (Kinzler and Vogelstein, 1990)) are underlined. *C*) Luciferase reporter assay showing activation of the putative *FOXE1* promoter (−1934 to +536) by GLI2. HaCaT cells were transfected with FOXE1 promoter reporter construct (FOXE1prom), together with either control vector or GLI2 expression vector. Data represent average value of three independent experiments. RLU, relative luciferase units.

with the activation of the *FOXE1* promoter in a reporter assay, indicate that *FOXE1* is a likely direct target of the *GLI2* transcription factor in epidermal cells.

FOXE1 is overexpressed in BCCs We have previously shown that *GLI2* mRNA levels are highly elevated in BCC samples compared with normal skin (Regl *et al*, 2002). Thus, if *FOXE1* acts as a downstream target of *GLI2 in vivo* and has a relevant role in *GLI2* induced processes such as BCC development, *FOXE1* should also be present in these tumors. We have, therefore, analyzed BCC samples with known *GLI2* and *GLI1* levels (Regl *et al*, 2002) for *FOXE1* transcript levels (Table II). In all 15 BCC samples tested, *FOXE1* mRNA was detected at levels higher than in normal skin. The normal skin sample, used as standard in all experiments, consisted of a pool of four independently isolated human skin biopsies to reduce site-specific and

Table II. Comparison of *FOXE1, GLI2,* and *GLI1* mRNA levels in BCC[a]

BCC ID	*FOXE1* (± SD)[b]	*GLI2*[c]	*GLI1*[c]	Type	Origin
25	59.21 (± 1.03)	67.28	1861.82	Nodular	Neck
37	32.41 (± 6.00)	16.14	53.02	Nodular	Back
6	25.66 (± 4.58)	5.63	16.51	Nodular	Head
32	22.78 (± 2.28)	18.22	591.81	Nodular	Pool of 3
38	22.59 (± 0.55)	18.61	128.80	Nodular	Upper arm
27	15.54 (± 1.82)	8.62	225.03	Nodular	Head
12	12.37 (± 1.49)	8.02	267.43	Nodular	Head
23	10.72 (± 2.36)	13.70	814.63	Nodular	Head
31	10.11 (± 2.12)	5.65	135.96	Nodular	Back
30	8.73 (± 0.66)	12.32	206.64	Nodular	Thorax
18	7.32 (± 0.42)	7.89	3024.54	Nodular	Head
2	7.29 (± 0.27)	9.74	116.48	Nodular	Head
35	5.58 (± 0.19)	2.81	17.82	Nodular	Thorax
8	4.84 (± 0.72)	6.69	95.50	Nodular	Thorax
9	4.02 (± 0.28)	3.35	157.53	Nodular	Head

[a]The Pearson correlation (two-tailed) was calculated pairwise using SPSS for all combinations. Only *FOXE1 versus GLI2* was significant at 0.01 level with a Pearson coefficient of 0.883.
[b]Values for *FOXE1* expression represent the ratio of *FOXE1* mRNA in tumor to that in normal skin. Ratios and SD were calculated from the average of four real-time RT-PCR measurements.
[c]Data for *GLI2* and *GLI1* expression are taken from Regl *et al* (2002) and are shown here for comparison. The same tumor samples were used for *FOXE1* mRNA.
BCC, basal cell carcinoma.

individual variations. It should be noted that GLI2 and FOXE1 are expressed at a much higher level in normal skin than GLI1. These ratios shown do not reflect absolute concentration. Upregulation of *FOXE1* mRNA levels ranged from 4-fold (BCC9) to 59.2-fold (BCC25) with an average fold increase of 15.8-fold. Pearson's correlation was calculated pairwise for GLI1 *versus* FOXE1, GLI2 *versus* FOXE1 and GLI1 *versus* GLI2 using SPSS (SPSS, Chicago, IL). Only FOXE1 *versus* GLI2 showed significant correlation ($p < 0.01$) (Table II). Since upregulation of *FOXE1* expression is GLI2 dependent in a cultured human keratinocyte line as well as in primary human keratinocytes, it is likely that the elevated levels of *FOXE1* reflect increased GLI2 activity in BCC.

***In situ* localization of FOXE1 in BCC and normal skin** We next investigated *FOXE1* expression in BCC by *in situ* hybridization. In agreement with the results of real-time RT-PCR, all tested BCC ($n = 6$) showed intense staining throughout the tumor islands (Fig 4*A*, *C*). No specific signal was detected in the surrounding stroma cells. The specificity of tumor staining was demonstrated by hybridizing adjacent sections with *FOXE1* sense RNA probes (Fig 4*B*, *D*). Thus, the results show that the elevated *FOXE1* transcript level detected with real-time RT-PCR in BCC is specific for the epithelial component of the BCC samples and does not result from expression in surrounding mesenchymal tissues.

Next, we analyzed follicular and interfollicular human epidermis for *FOXE1* mRNA expression. In interfollicular epidermis, specific staining was detected in the basal and suprabasal layers but not in the underlying mesoderm (Fig 5*A*, *C*). It should be pointed out that the quantitative RT-PCR data in Table II indicate that expression must be much lower than in BCC. In hair follicles, the highest level of *FOXE1* mRNA was present in the outer root sheath (Fig 5*C*, *E*). Occasionally, staining of mesenchymal cells of the perifollicular connective tissue sheath was observed.

Discussion

HH/GLI signaling has been implicated in the regulation of hair follicle development and skin tumorigenesis (Iseki *et al*, 1996; Oro *et al*, 1997; St-Jacques *et al*, 1998; Chiang *et al*, 1999; Oro and Higgins, 2003). A growing body of data points to a decisive role of the zinc finger transcription factor GLI2 in mediating these processes in response to HH signaling (Bai *et al*, 2002; Sheng *et al*, 2002; Mill *et al*, 2003). In a search for potential downstream mediators of GLI2 function in epidermal cells, we have identified a new GLI2 target gene, *FOXE1*, whose expression is strongly enhanced in response to GLI2. The presence of five GLI-binding sites in the putative promoter region of *FOXE1*, together with the activation of reporter gene expression by this region in the presence of GLI2, points to direct regulation of FOXE1 by GLI2. The largely overlapping expression domains of *FOXE1* and *GLI2* in human hair follicles, epidermis, and BCC Ikram *et al*,[1] suggest a role of

[1]Ikram SI, Neill GW, Regl G, *et al*: *GLI2* is expressed in normal human epidermis and BCC and induces *GLI1* expression by binding to its promoter. Accepted for publication, J Invest Dermatol (MS JID-2003-0435)

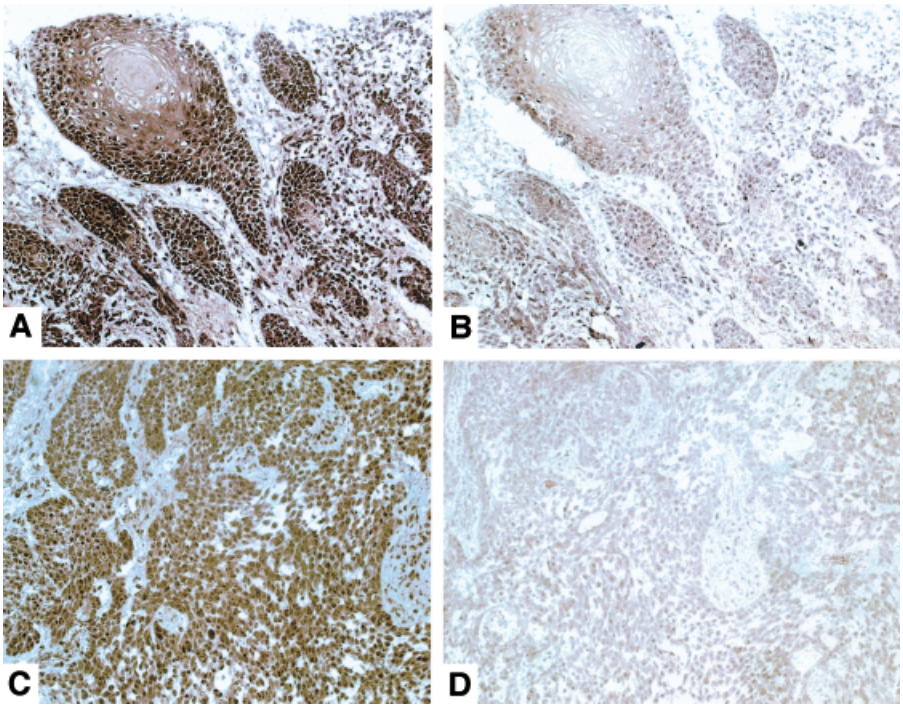

Figure 4
***FOXE1* mRNA localizes to BCC tumor islands.** *FOXE1* expression is shown in two representative tumors by *in situ* hybridization. (*A* and *C*) Only BCC tumor islands but not surrounding stroma cells are stained. (*B* and *D*) Adjacent sections were hybridized with sense RNA as negative controls to show the specificity of hybridization.

FOXE1 in epidermal development and carcinogenesis downstream of HH/GLI signaling. Whether expression of FOXE1 in interfollicular epidermis is dependent on HH signaling is unclear since expression of well-characterized Hh targets such as GLI1 and PTCH is mainly restricted to follicular regions of the skin. Co-expression of FOXE1 and GLI2 in interfollicular epidermis may therefore be controlled by signals other than Hh, a situation that has been described for Gli2 in ventroposterior mesoderm formation of Xenopus, where Gli2 expression is induced by FGF rather than Hh signaling (Brewster *et al*, 2000).

FOXE1 (also named *FKHL15/TTF2*) belongs to a large family of transcription factors characterized by their DNA-binding domain—the forkhead/winged-helix motif. *In vitro* analysis of the mechanism of *foxe1* action suggested a role for Foxe1 as a transcriptional repressor (Zannini *et al*, 1997; Perrone *et al*, 2000). Forkhead proteins display a remarkable functional diversity and are implicated in a variety of biological processes ranging from embryonic development to regulation of cell growth and proliferation in adult tissues (Kaufmann and Knochel, 1996; Carlsson and Mahlapuu, 2002). Notably, expression of several mammalian forkhead genes has been shown to be controlled by the Hh/Gli signaling pathway: *foxa2* (*HNF-3*β), expressed in the floor plate of the notochord via direct transcriptional regulation by GLI1 (Sasaki *et al*, 1997), *foxc2* (*Mfh1*) and *foxd2* (*FREAC-9/Mf2*) (Wu *et al*, 1998; Furumoto *et al*, 1999), both involved in sclerotome cell proliferation, *foxf1* (*FREAC-1/HFH-8*) in lung and foregut mesenchyme (Mahlapuu *et al*, 2001), and *FOXM1* (*Trident/HFH-11/INS1*) in human BCC (Teh *et al*, 2002).

Although *FOXE1* was originally isolated from keratinocytes (Chadwick *et al*, 1997), its role in epidermal development and homeostasis is not understood. FOXE1/TTF2 function has primarily been investigated in the context of thyroid development (Zannini *et al*, 1997; Clifton-Bligh *et al*, 1998; De Felice *et al*, 1998; Damante *et al*, 2001; Castanet *et al*, 2002). Mice lacking both copies of *foxe1* suffer from neonatal hypothyroidism characterized by a sublingual small ectopic or completely missing thyroid gland and cleft palate (De Felice *et al*, 1998). Patients with Bamforth syndrome, who are heterozygous for *FOXE1*, display in addition to thyroid agenesis a spiky hair phenotype (Clifton-Bligh *et al*, 1998; Castanet *et al*, 2002). A corresponding hair phenotype was not observed in *foxe1*$^{-/-}$ mice since they die before hair formation (De Felice *et al*, 1998). Expression of *foxe1* was, however, observed in embryonic whiskers of wild-type mice (Dathan *et al*, 2002). Together with the spiky hair phenotype of Bamforth patients, this points to a role of Foxe1 in hair follicle development where Gli2 is essential (Mill *et al*, 2003).

In summary, we have characterized *FOXE1* as a novel GLI2 target gene in human keratinocytes. Given the critical role of GLI2 in the proliferation of hair follicle cells and tumorigenesis, co-expression of *FOXE1* and *GLI2* in normal skin and BCC suggests that *FOXE1* may be involved in mediating the proliferative effect of GLI2 *in vivo*. Functional analysis of FOXE1 in epidermal development and disease will thus be a major aim of future studies to shed new light on the molecular mechanisms downstream of the Hh/GLI signaling pathway.

Materials and Methods

RNA isolation and real-time RT-PCR analyses BCC and normal skin were snap-frozen after surgical removal and stored at -70°C until use. Ethical committee permission was obtained from the East London and City Area Health Authority and all biopsies and skin samples were taken with full patient consent. Total RNA was isolated after homogenization with a high-speed dispersing tool

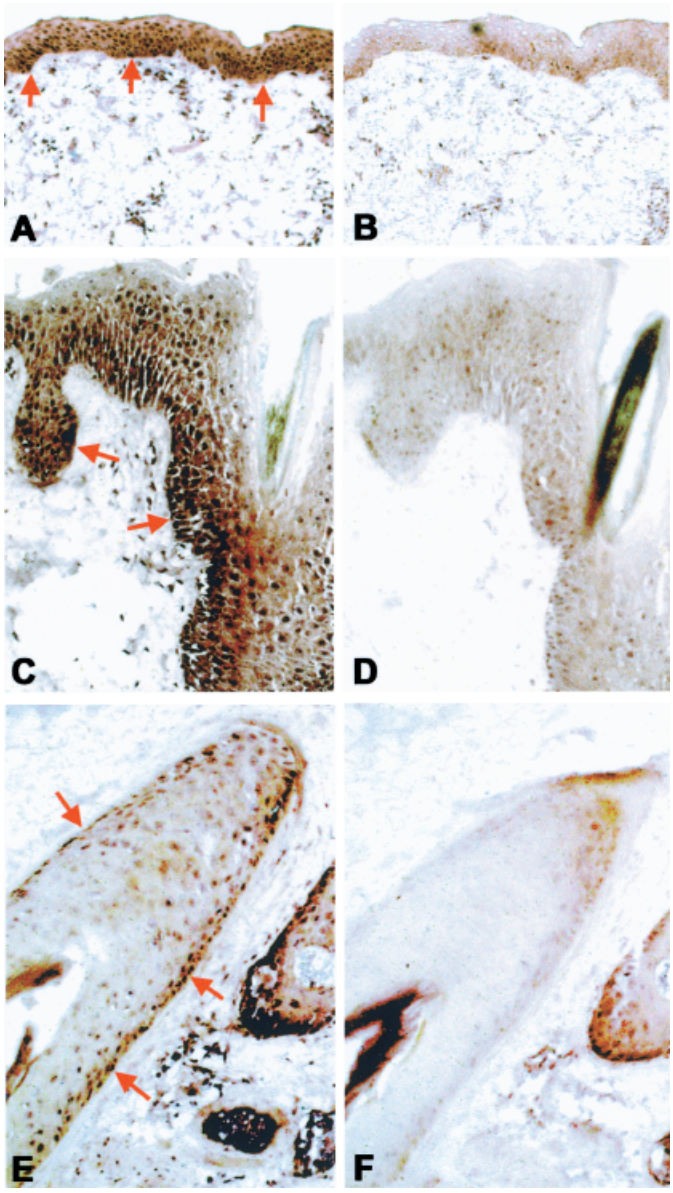

Figure 5
***FOXE1* is expressed in the human epidermis and the outer root sheath of the hair.** *FOXE1* mRNA expression was visualized by *in situ* hybridization (*A* and *C*) Specific staining for FOXE1 was observed in basal keratinocytes of a normal human epidermis (red arrows). *E*) Sections of a hair follicle show that *FOXE1* transcript is strongly expressed in cells of the outer root sheaths of the hair. (*B*, *D*, and *F*) Adjacent sections were hybridized with a sense RNA probe (control) to demonstrate the specificity of the hybridization.

(ULTRA TURRAX T18 basic, IKA Labortechnik, Staufen, Germany) using TRI-reagent (Molecular Research Center, Cincinnati, Ohio) followed by further purification using the High Pure RNA Isolation Kit (Roche, Basel, Switzerland), which includes a DNAase treatment to remove genomic DNA. RNA quality and quantity were assayed on a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, California). cDNA was synthesized with Superscript II (Rnase H-) reverse transcriptase (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. Real-time RT-PCR analysis was carried out on a Rotorgene 2000 (Corbett Research, Sydney, Australia) using iQ SYBR Green Supermix (BIORAD, Hercules, California). Fold-induction values (x) were calculated using the following formula: $x = 2^{-\Delta\Delta C_t}$, where C_t represents the mean threshold cycle of all replicate analyses of a given gene and ΔC_t represents the difference between the C_t values of the gene in question (target) and the C_t value of the reference gene (RPLP0) (Martin *et al*, 2001). $\Delta\Delta C_t$ is the difference between ΔC_t values of the samples for each target (e.g. ΔC_t for FOXE1 in tumor) and the mean ΔC_t of the calibrator (e.g. ΔC_t for FOXE1 in normal skin).

Primer sequences for real-time RT-PCR studies were as follows: RPLPO forward primer (5′–3′) GGCACCATTGAAATCCTGAGTGATGTG, reverse primer (5′–3′) TTGCGGACACCCTCCAGGAAGC; GLI2 forward primer (5′–3′) TGGCCGCTTCAGATGACAGATGTTG, reverse primer (5′–3′) CGTTAGCCGAATGTCAGCCGTGAAG; PTCH forward primer (5′–3′) TCCTCGTGTGCGCTGTCTTCCTTC, reverse primer (5′–3′) CGTCAGAAAGGCCAAAGCAACGTGA; FOXE1 forward primer (5′–3′) CATCTTGGATGCTGCCCTGCGTATT, reverse primer (5′–3′) CCAGCACGTCCTGCTCAAAAGTTCA.

Screening of cDNA arrays A non-redundant set of 2200 sequence verified EST-clones obtained from the human-UniGem V2.0 clone set (Incyte Genomics, Cambridge, UK) was amplified by PCR and products were spotted in quadruplicate on a 22 × 11cm nylon membrane (Hybond N+, Amersham Bioscience, Uppsala, Sweden) using the MicroGridII arrayer (BioROBOTICS, Huntingdon, UK). The membranes were hybridized with probes obtained from total RNA prepared from GLI2 expressing HaCaT cells and controls using TRI-reagent (Molecular Research Center, Cincinnati, Ohio) followed by a LiCl precipitation step. Fifteen micrograms total RNA from each sample were reverse transcribed with Superscript II (Rnase H-) reverse transcriptase (Invitrogen) in the presence of 70 μCi [^{33}P]dCTP (specific activity 3000 Ci per mmol) (Amersham Bioscience). Labeled cDNA was purified with GFX DNA Purification Kit (Amersham Bioscience). Hybridization was carried out for 36 h in 5 × SSC, 5 × Denhard's, 1% SDS followed by extensive washing with 2 × SSC, 0.1% SDS; 0.2 × SSC, 0.1% SDS and 0.1% SSC, 0.1% SDS. Filters were exposed for 4 d onto a phosphoimager screen (Fuji, Japan) and scanned with BAS-1800II (Fuji, Tokyo, Japan). The images were analyzed using the AIDA Metrix suite (RAYTEST, Straubenhardt, Germany).

Western blot analysis Western blot analysis of GLI2 protein expression in HaCaT-GLI2His cells was performed according to standard procedures using a monoclonal mouse anti-HIS peroxidase-conjugated antibody (A7058, SIGMA-Aldrich, Vienna, Austria) followed by ECL detection (Amersham Bioscience). Proteins were blotted on Hybond-ECL (Amersham Bioscience) and exposed to Hyperfilm ECL (Amersham Bioscience).

Cell culture and generation of tet-inducible HaCaT lines HaCaT cells were cultured in Dulbecco's modified Eagle medium (DMEM pH 7.2, high glucose, Invitrogen Life Technologies, Carlsbad, California) supplemented with 10% fetal calf serum (FCS) (Invitrogen), 100 mg per liter streptomycin and 62.5 mg per liter penicillin at 37°C, 5% CO_2 atmosphere. The T-Rex system (Invitrogen) was used to generate double stable inducible HaCaT lines expressing human *GLI1* and *GLI2* tagged with a 6 × HIS epitope under the control of the bacterial tet repressor (HaCaT-GLI2His). Double stable lines were established and grown in the presence of Zeocin (Invitrogen) and Blasticidin-S (ICN-Biomedica, Costa Mesa, California). Transgene expression was induced by adding 1mg per liter tet to the cell culture medium.

To generate the retroviral bicistronic GLI2-EGFP expression construct, N-terminally His-tagged *GLI2* was cloned into pI2E-A, a modified version of pIRES2-EGFP plasmid (Clontech, Hampshire, UK) (Regl *et al*, 2002). PI2E-A-GLI2 was digested with *Sal*I and *Not*I to excise CMV-GLI2-IRES-EGFP. The resulting fragment was cloned into *Xho*I- and *Not*I-digested retroviral SIN-IP plasmid (a gift from Prof. P. Khavari) to create SIN-GLI2-EGFP. Retrovirus production and transduction of primary keratinocytes were carried out as previously described (Regl *et al*, 2002).

Luciferase reporter assay The 5′ upstream regulatory region of the human *FOXE1* gene from −1934 to +539 relative to the transcriptional start site was amplified by PCR from genomic DNA

using FailSafe PCR system (Epicentre, Madison, WI). The following primers were used: Forward primer: 5′-GAATTAAGGAAGGAGA-GAACTGCGG-3′, reversed primer 5′-GAGAAGCTTCCGCGTGG-TGCTTCTCGAGGCGGGC-3′. PCR fragment was digested with *Hind*III and cloned into the *Hind*III site of pGL3basic reporter vector (Promega, Madison, Wisconsin) yielding the construct FOXE1-prom. For luciferase reporter assays, HaCaT cells were co-transfected in 12-well plates with FOXE1 (FOXE1prom) reporter plasmid and GLI2 expression plasmid (human full-length GLI2b (Acc. no. AB007296.1) (a gift from Dr David Markovitz) in pcDNA4/TO (Invitrogen)) or empty pcDNA4/TO. β-Galactosidase expression vector (pcDNA4/TO-LacZ; Invitrogen) was also co-transfected for normalization of transfection efficiency. Cells were harvested 48 h post-transfection and luciferase assays were carried out using Luciferase Substrate (Promega), according to the manufacturer's instructions. Luciferase activity was measured on a Lucy II luminometer (Anthos, Wals, Austria). To normalize results for lacZ activity, 20 μL of cell lysate was mixed with 240 μL Z-buffer (100 mM $NaPO_4$ (pH 7), 10 mM KCl_2, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol) and 50 μL *O*-nitro-α-D-phenyl-galactopyranoside (ONPG, SIGMA-Aldrich)(4 mg per mL in 100 mM $NaPO_4$) and incubated at 28°C until yellow color became visible. Reactions were stopped by adding 250 μL 1 M Na_2CO_3. β-Galactosidase activity was quantified by measuring absorbance at 405 nm on an SLT-Spectra plate reader (SLT (Tecan), Groedig, Austria).

***In situ* hybridization** DIG-labeled FOXE1 sense and antisense probes were generated from a 332 bp *Bam*HI/*Pst*I fragment of the 3′ UTR of *FOXE1* (corresponding to nucleotides 2148–2480, GenBank GI No. 2078532) subcloned into pBluescript II KS, using a DIG labeling kit (Roche) as described by the manufacturer's instructions. 8 μm tissue sections were pre-hybridized in pre-hyb-solution (4 × SSC, 1 × Denhardt's, 50% formamide, 500 μg per mL tRNA and 500 μg per mL salmon testes DNA (denatured at 100°C for 10 min and placed on ice before adding to the mix) and incubated at 42°C for 3–4 h. Hybridization was carried out using fresh pre-hyb-solution containing 80–100 ng labeled probe (denatured at 65°C for 5 min) at 42°C overnight. Next day, the sections were washed in 2 × SSC for 5 min (two times) and in 2 × SSC, 1 × SSC, 0.5 × SSC, each containing 50% formamide, at 45°C–55°C and in 0.1 × SSC 50% formamide at 50°C –60°C for 20 min. A final wash in 2 × SSC and rinsed in DIG buffer 1 (100 mM Tris-HCl, 150 mM NaCl pH 7.5). Sections were blocked with 10% normal sheep serum (NSS) in DIG buffer 1 and incubated with anti-digoxigenin-alkaline phosphatase-conjugated (Roche) diluted 1:400 in 1% NSS DIG buffer 1 for 2 h, followed by washing in DIG buffer 1 (× 2) and DIG buffer 2 (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 10 min. The hybrids were visualized by incubating the section with BCIP/NBT (SIGMA-Aldrich) liquid substrate in dark at 4°C overnight. The color reaction was stopped by immersing the sections in (10 mM Tris-HCl pH 8, 1 mM EDTA) for 30 min. The developed slides were mounted and examined under a light microscope.

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