

Activation of the BCL2 Promoter in Response to Hedgehog/GLI Signal Transduction Is Predominantly Mediated by GLI2

Gerhard Regl,¹ Maria Kasper,¹ Harald Schnidar,¹ Thomas Eichberger,¹ Graham W. Neill,⁴ Michael P. Philpott,⁴ Harald Esterbauer,² Cornelia Hauser-Kronberger,³ Anna-Maria Frischauf,¹ and Fritz Aberger¹

¹Department of Molecular Biology, Division of Genomics, University of Salzburg, Salzburg, Austria; ²Medical University Vienna, Institute of Medical and Chemical Laboratory Diagnostics, Vienna, Austria; ³Department of Pathology, St. Johann's Hospital, Paracelsus Medical School, Salzburg, Austria; and ⁴Center for Cutaneous Research, Barts and The London Queen Mary's School of Medicine & Dentistry, University of London, United Kingdom

ABSTRACT

Aberrant activation of the Hedgehog (HH)/GLI signaling pathway has been implicated in the development of basal cell carcinoma (BCC). The zinc finger transcription factors GLI1 and GLI2 are considered mediators of the HH signal in epidermal cells, although their tumorigenic nature and their relative contribution to tumorigenesis are only poorly understood. To shed light on the respective role of these transcription factors in epidermal neoplasia, we screened for genes preferentially regulated either by GLI1 or GLI2 in human epidermal cells. We show here that expression of the key antiapoptotic factor BCL2 is predominantly activated by GLI2 compared with GLI1. Detailed promoter analysis and gel shift assays identified three GLI binding sites in the human BCL2 *cis*-regulatory region. We found that one of these binding sites is critical for conferring GLI2-specific activation of the human BCL2 promoter and that the selective induction of BCL2 expression depends on the zinc finger DNA binding domain of GLI2. *In vivo*, GLI2 and BCL2 were coexpressed in the outer root sheath of hair follicles and BCC and in plasma cells that infiltrated BCC tumor islands. On the basis of the latter observation, we analyzed plasma cell–derived tumors and found strong expression of GLI2 and BCL2 in neoplastic cells of plasmacytoma patients, implicating HH/GLI signaling in the development of plasma cell–derived malignancies. The results reveal a central role for GLI2 in activating the pro-survival factor BCL2, which may represent an important mechanism in the development or maintenance of cancers associated with inappropriate HH signaling.

INTRODUCTION

The Hedgehog (HH)/GLI signaling pathway plays a critical role during vertebrate embryogenesis, during which it controls pattern formation, proliferation, and differentiation of a number of different cell types. Its pivotal role in development is underlined by the fact that mutations leading to aberrant HH/GLI signaling can result in severe developmental anomalies and tumor formation (1–4).

HH signal transduction is initiated by binding of secreted and post-translationally modified HH protein to the 12-pass transmembrane receptor Patched (PTCH), which in the absence of ligand represses signaling by inhibiting the 7-pass transmembrane protein Smoothed (SMOH). On ligand binding, the repressive activity of PTCH is abrogated, allowing SMOH to transduce the signal toward the nucleus by a complex mechanism that in vertebrates is not fully understood at present (1, 5).

Binding of HH to PTCH induces the transcriptional activation of

GLI1 and/or GLI2, both members of the GLI family of zinc finger transcription factors, which mediate the signal in the nucleus of responsive cells by regulating HH-target gene expression. GLI1 and GLI2 are expressed in overlapping yet distinct domains and have a highly conserved zinc finger DNA binding domain, each binding to the consensus GLI binding site GACCACCCA (6, 7).

Evidence indicates that aberrant HH signaling is involved in the development of a number of different types of malignancies, including medulloblastoma, rhabdomyosarcoma, and basal cell carcinoma (BCC; refs. 8–10). Deregulated HH/GLI signaling recently also has been implicated in common highly aggressive cancers of the lung, gastrointestinal tract, and pancreas, where it not only appears to be involved in tumor formation but also in tumor maintenance because the survival of transformed cells depends on active HH signaling (11–13).

In the case of BCC, a wealth of data suggest that ligand-independent activation of HH/GLI signaling resulting either from PTCH loss of function or from SMOH gain of function mutations represents the primary genetic lesion sufficient to initiate and propagate carcinogenesis (14–16). GLI1 and GLI2 are expressed at high levels in BCC, and transgenic expression of human GLI1 or rodent Gli2 in mouse epidermis showed that the oncogenic HH signal could be mediated by these transcription factors. Increased expression of GLI1 or Gli2 resulted in the formation of epidermal tumors including BCC, but the frequency with which certain tumor types were induced was different (17–20). Although these experiments convincingly showed that HH-induced tumorigenesis could be mediated by the increased activity of GLI1 and GLI2, the relative contribution of each transcription factor to tumorigenesis still remains to be addressed.

Distinct target gene specificities of Gli1 and Gli2 were revealed by heterologous expression of *Gli* genes in *Drosophila* (21) and were further supported by the finding that Gli2 acts in ventral mesodermal patterning in amphibians independent of HH signaling. In this context, Gli2 but not Gli1 was able to directly activate mesodermal patterning genes (22). Conversely, Gli1 can compensate for the lack of Gli2 function when expressed from the *Gli2* locus, indicating that Gli1 and Gli2 regulate a similar set of target genes during early mouse embryogenesis. Later in development, however, new gain-of-function defects emerge, suggesting context-dependent regulation of the biological activities of GLI factors (23).

Whether GLI1 or GLI2 or a combination of both factors is required for HH-induced tumorigenesis is still unclear. Recent evidence uncovered an essential role of Gli2 in transducing HH signaling during hair follicle development in mice, whereas Gli1 function appears to be dispensable for this process (24). By contrast, overexpression studies and the strong expression of GLI1 in the majority of HH-associated tumors argue that GLI1 plays a central role in mediating the oncogenic HH signal (20, 25–27).

To better understand the contribution of GLI1 and GLI2 in the neoplastic conversion of epidermal cells, we compared the mRNA expression profiles of GLI1- and GLI2-expressing human keratinocytes by cDNA array analysis and found that the key antiapoptotic

Received 3/29/04; revised 7/14/04; accepted 8/23/04.

Grant support: FWF projects P14227 and P16518-B14 (Austria Science Fund), the Austrian Genome Research Program GEN-AU (project Ultra-sensitive Proteomics and Genomics), and the University of Salzburg (research focus "Biosciences and Health").

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Notes: G. Regl and Maria Kasper contributed equally to the work; Supplementary data for this article can be found at Cancer Research Online (<http://www.cancerres.aacrjournals.org>).

Requests for reprints: Fritz Aberger, Department of Molecular Biology, Division of Genomics, University of Salzburg, Hellbrunnerstrasse 34, A-5020 Salzburg, Austria. Phone: 43-662-8044-5792; Fax: 43-662-8044-144; E-mail: fritz.aberger@sbg.ac.at.

©2004 American Association for Cancer Research.

washes, detection was performed using biotinylated goat antimouse or biotinylated rabbit antigoat and horseradish-streptavidin-biotin complex (DakoCytomation), followed by development with diaminobenzidine. Specimens were counterstained with hematoxylin.

For double-immunofluorescence staining, primary antibodies were applied simultaneously, followed by incubation with biotinylated rabbit antigoat antibody, Alexa Fluor 555 goat antimouse (1:50), and Streptavidin-Alexa Fluor 488 (1:100; Molecular Probes, Eugene, OR). Positive and negative control samples were included, and preabsorption controls using appropriate blocking peptides (Santa Cruz Biotechnology) were performed. Confocal microscopy analysis was done on a Zeiss LSM 510 laser scanning microscope (Zeiss, Oberkochen, Germany).

RESULTS

Predominant Activation of BCL2 mRNA Expression by GLI2 in Human Epidermal Cells. GLI1 and GLI2 have been implicated in mediating HH signal transduction in BCC development, although their specific contribution to epidermal neoplasia is unclear at present. To elucidate their respective roles in tumorigenesis and to address whether GLI1 and GLI2 display distinct target gene specificities, we compared by cDNA array technology the mRNA expression profile of the human keratinocyte cell line HaCaT either expressing GLI1 or GLI2 under the control of the tetracycline repressor (20, 30). In this screen, the key antiapoptotic factor BCL2 was identified as the target gene preferentially induced by GLI2 compared with GLI1. The array result then was analyzed in detail by quantitative real-time reverse transcription-PCR analysis.

RNA isolated from pools of four independent HaCaT lines expressing GLI1 or GLI2 under tetracycline control was used for the measurements to minimize interclone variability. In GLI2-expressing HaCaT cells, significantly elevated levels of BCL2 mRNA were already detected after 12 hours of tetracycline treatment (8.4-fold increase), whereas in GLI1-expressing cells, the level of BCL2 mRNA was only moderately higher at that time point compared with uninduced cells (1.6-fold increase). The predominant induction of BCL2 mRNA expression by GLI2 also was observed at later time points (Fig. 1A). The GLI1 and GLI2 HaCaT pools expressed comparable levels of transgene, showing that the differences in target gene activation are not caused by differences in GLI1 or GLI2 expression levels (Fig. 1; see Supplementary Data). The known direct GLI target gene *PTCH* also was induced to comparable levels by GLI2 and GLI1 (Fig. 1B), excluding the possibility that GLI1 protein was generally less active than GLI2. The results were validated with retrovirally transduced primary human keratinocytes expressing GLI1 or GLI2 for 72 hours. Like in HaCaT cells, only GLI2 induced high levels of BCL2 mRNA (7.1-fold induction by GLI2 *versus* 1.3-fold induction of BCL2 by GLI1), whereas GLI2 and GLI1 stimulated transcription of *PTCH* (Fig. 1C). Moderate activation of BCL2 mRNA expression in response to GLI1 was only observed at later time points (96 hours post-transduction; data not shown). Induction of BCL2 expression in response to GLI2 also was confirmed by Western blot analysis (Fig. 1D). Collectively, the data suggest that in human keratinocytes, BCL2 expression is predominantly activated by GLI2 in response to HH/GLI signaling.

GLI2 Directly Activates BCL2 Expression by Binding to a Short Region of the BCL2 Promoter Containing Three Closely Spaced GLI Binding Sites. Recent work by Bigelow *et al.* on the BCL2 promoter region has shown that GLI1 but not GLI3 can directly activate BCL2 expression (28). The rapid and strong activation of BCL2 expression in response to GLI2 prompted us to ask whether GLI2 also directly activates the BCL2 promoter and if so, which molecular mechanisms account for the selective activation of BCL2 expression by GLI2.

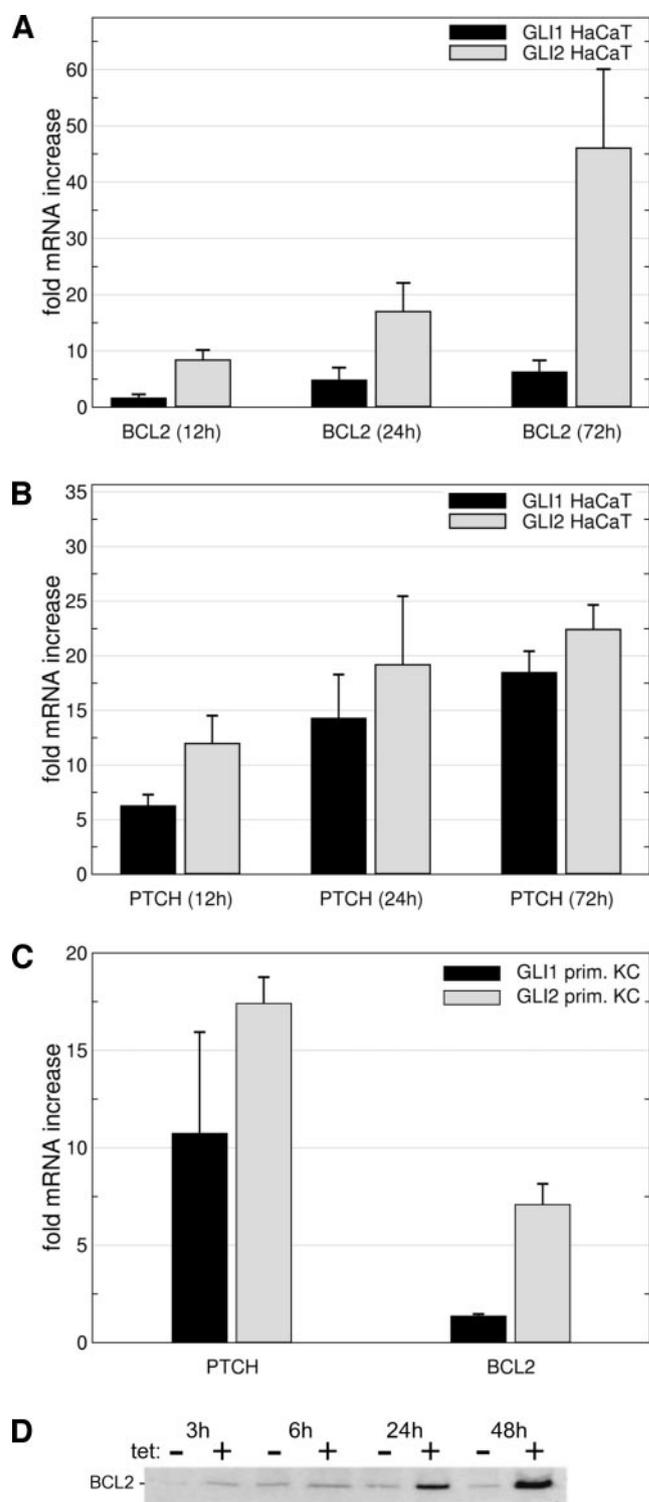


Fig. 1. Preferential induction of BCL2 expression by GLI2 in human keratinocytes. *A*, real-time PCR analysis of BCL2 mRNA induction in HaCaT keratinocytes expressing either GLI1 or GLI2 under tetracycline control. Transgene expression was induced by tetracycline for the time indicated. Fold mRNA values represent the ratio of mRNA levels in induced cells to mRNA levels in uninduced cells. *B*, analysis of *PTCH* mRNA expression in response to GLI1 or GLI2. The same cells as described in *A* were used for measurements. *C*, differential induction of BCL2 expression in primary human keratinocytes expressing either GLI1 or GLI2. Results shown in *A–C* represent the mean fold induction calculated from three independent measurements. *D*, Western blot analysis of GLI2-expressing HaCaT cells showing gradual increase in BCL2 protein levels following induction of GLI2 expression by tetracycline (*tet*). Cells were either treated with tetracycline for the times indicated or left untreated.

In silico analysis of the human BCL2 promoter using the ScanAce motif search program (35) revealed the presence of three closely spaced putative GLI binding sites (bs1, bs2, and bs3) with significant homology to the consensus GLI binding site GACCACCCA (ref. 6; Fig. 2A). To address whether GLI2 is able to bind to these sequences, we performed electrophoretic mobility shift assays (EMSA). As shown in Fig. 2B, all three sequences were specifically bound by the GLI2 zinc finger, suggesting that like GLI1, GLI2 regulates BCL2 expression by directly binding to the BCL2 promoter. To corroborate this, we isolated the putative BCL2 promoter region from position -1291 to +610 relative to the transcriptional start site (36) and cloned the fragment containing the three GLI binding sites into a luciferase reporter vector yielding BCL2prom (1.9 kb). As a control, we used a reporter construct in which the three putative GLI binding sites located within the region from -1291 to -1022 of the BCL2 promoter fragment were deleted [BCL2prom (cont); Fig. 2C, top]. The reporter constructs were tested in HaCaT keratinocytes for activation by GLI1, GLI2, or GLI3. To exclude that the weak induction of BCL2 expression by GLI1—as observed in our real-time PCR analysis—could be because of cytoplasmic rather than nuclear localization of GLI1 protein, we used a modified GLI1 construct that directs GLI1 protein to the nucleus by the presence of an NH₂-terminal NLS. Consistent with previous results (28), GLI1 induced BCL2 reporter activity (Fig. 2C). Activation of the BCL2 promoter by GLI2, however, was >10-fold stronger compared with GLI1-mediated reporter activation. By contrast, activation of a PTCH reporter by GLI2 was only moderately stronger (1.8-fold) than activation by GLI1 (Fig. 2C, inset), showing a similar activator potential of both transcription factors. GLI3 did not significantly induce activation of the BCL2 reporter, and neither GLI1 nor GLI2 was able to activate BCL2 reporter expression on deletion of the three GLI binding sites [BCL2prom (cont); Fig. 2C]. Together with our studies on endogenous BCL2 mRNA levels in GLI1- and GLI2-expressing keratinocytes, these results suggest that among the three human *GLI* genes, GLI2 is the predominant factor involved in the direct activation of BCL2 expression in response to HH/GLI signaling.

Activation of BCL2 Expression by GLI2 Is Independent of Endogenous GLI1. We have shown previously that GLI1 is strongly up-regulated by GLI2 in human keratinocytes and that this is caused by direct binding of GLI2 to the GLI1 promoter (20, 34). Therefore, the strong activation of BCL2 expression in response to GLI2 may be mediated by the increase of endogenous GLI1 protein. To determine the contribution of endogenous GLI1 to activation of the BCL2 promoter in response to GLI2, we knocked down endogenous GLI1 expression in GLI2-transfected cells by small-interfering RNA technology (Fig. 3A and B). The knockdown efficiency was tested in transient transfection assays, in which a cytomegalovirus promoter GLI1-EGFP expression construct was cotransfected with shRNA targeting GLI1. As shown in Fig. 3B, shRNA-GLI1C reduced the levels of GLI1-EGFP protein by >80%.

In reporter assays, coexpression of shRNA-GLI1C and GLI2 did not significantly reduce BCL2 promoter activation compared with cells expressing GLI2 only (Fig. 3A), suggesting that activation of BCL2 expression in human epidermal cells can be directly mediated by GLI2 independent of GLI1.

The Zinc Finger DNA Binding Domain of GLI2 and GLI Binding Site bs3 Is Required for GLI2-Specific Activation of the BCL2 Promoter. Vertebrate *Gli* genes have been shown to bind to the consensus Gli binding site (GACCACCCA; refs. 6, 37). This has led to the hypothesis that the distinct biological activities of GLI proteins rely on differential post-translational modifications, proteolytic processing, or specific interactions with transcriptional cofactors such as CBP rather than on selective binding site recognition (38–41).

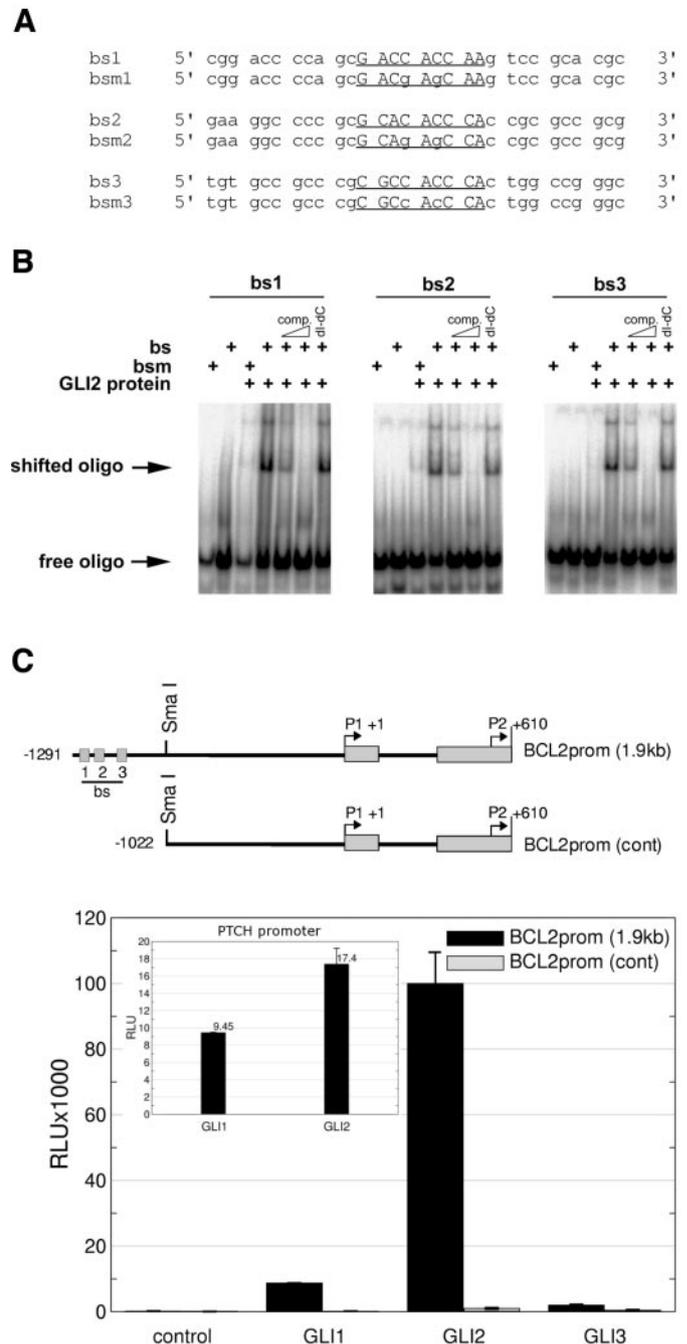


Fig. 2. GLI2-mediated activation of BCL2 expression involves three GLI binding sites in the BCL2 promoter. *A*, sequence of three closely spaced GLI binding sites (bs1, bs2, and bs3) identified in the BCL2 promoter. The same oligonucleotide sequences were used for EMSA analysis. For reasons of simplicity, the complementary sequences are not shown. The core binding sites with homology to the consensus GLI binding sequence are underlined and in uppercase; bsm1, bsm2, and bsm3 represent mutated oligonucleotides used for EMSA studies and competition experiments to show specific binding of GLI2 to the wild-type sequences. The bp exchanges are indicated by lowercase letters. *B*, EMSA analysis showing specific binding of recombinant GLI2 protein comprising the NH₂ terminus and zinc finger domain. Only the wild-type GLI binding sequences bs1, bs2, and bs3 were bound by GLI2. Point mutations at critical positions within the core binding site (bsm) abolish binding. Binding of GLI2 protein to bs1, bs2, and bs3 could be competed with excess wild-type binding sequences but not with poly(dI-dC) or bsm1, bsm2, or bsm3 oligonucleotides (data not shown), indicating that all three GLI binding sites are specifically bound by the GLI2 DNA binding domain *in vitro*. *C*, luciferase reporter assays showing preferential activation of the BCL2 promoter [BCL2prom (1.9 kb)] by GLI2 compared with GLI1 and GLI3. The control reporter construct lacking the three GLI binding sites [BCL2prom (cont)] was neither induced by GLI2 nor by GLI1. For comparison, activation of a PTCH reporter by GLI1 and GLI2 is shown as inset.

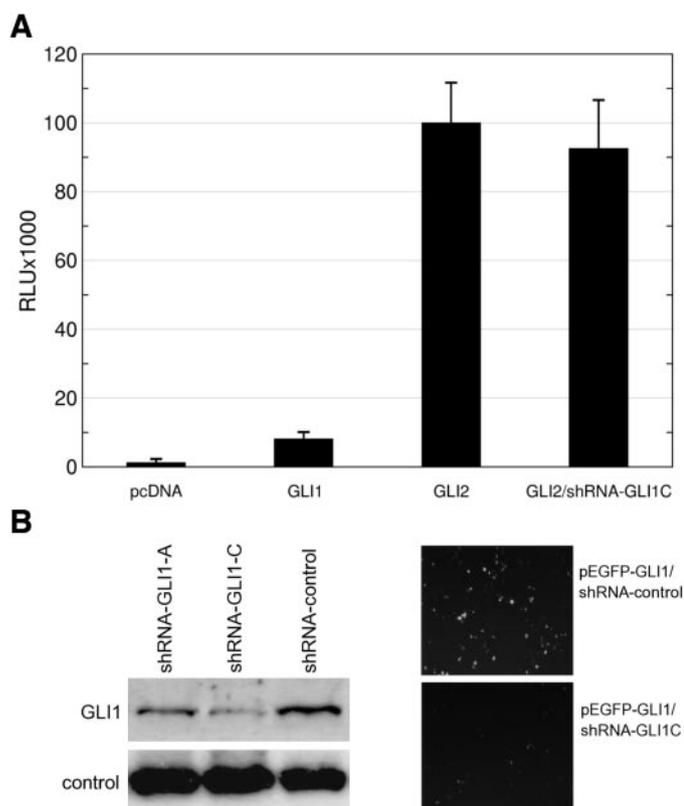


Fig. 3. GLI2-mediated activation of the BCL2 promoter is independent of endogenous GLI1 expression. *A*, luciferase reporter assays showing that expression of shRNA directed against GLI1 in GLI2-transfected cells (GLI2/shRNA-GLI1) has no significant impact on the selective activation of the BCL2 promoter by GLI2. *B*, Cotransfection of shRNA-GLI1C and EGFP-tagged GLI1 expression plasmid yields significantly lower GLI1 protein levels than cotransfection with control shRNA (shRNA-control), showing efficient silencing of GLI1 transgene expression by shRNA-GLI1-C. *Left*, Western blot analysis of cotransfected cells; *right*, fluorescence microscopy analysis of cells transfected with EGFP-tagged GLI1 and shRNA-GLI1-C plasmid (pEGFP-GLI1/shRNA-GLI1C) or with EGFP-GLI1 and control shRNA (pEGFP-GLI1/shRNA-control).

Conversely, the lack of quantitative data on the affinity of the DNA binding domains of different GLI proteins to variants of the consensus Gli binding site makes it difficult to assess the role of the zinc finger domain in mediating the distinct biological activities of GLI proteins.

We addressed the roles of the zinc finger DNA binding domain of GLI2 and of the three GLI binding sites in the induction of BCL2 expression. We reasoned that if the zinc finger domains of GLI2 were responsible for strong induction of BCL2 expression, replacing the zinc finger domain of GLI1 with that of GLI2 would convert GLI1 into a strong inducer of the BCL2 promoter. To test this, we constructed a chimeric protein termed GLI121, consisting of the NH₂-terminal region of GLI1, the zinc finger domain of GLI2, and the COOH-terminal region of GLI1 (Fig. 4*B*). The ability of wild-type GLI2, GLI121, and NLS-tagged GLI1 protein to activate the BCL2 promoter and the contribution of each of the three GLI binding sites to promoter activation were measured using the reporter constructs shown in Fig. 4*A*.

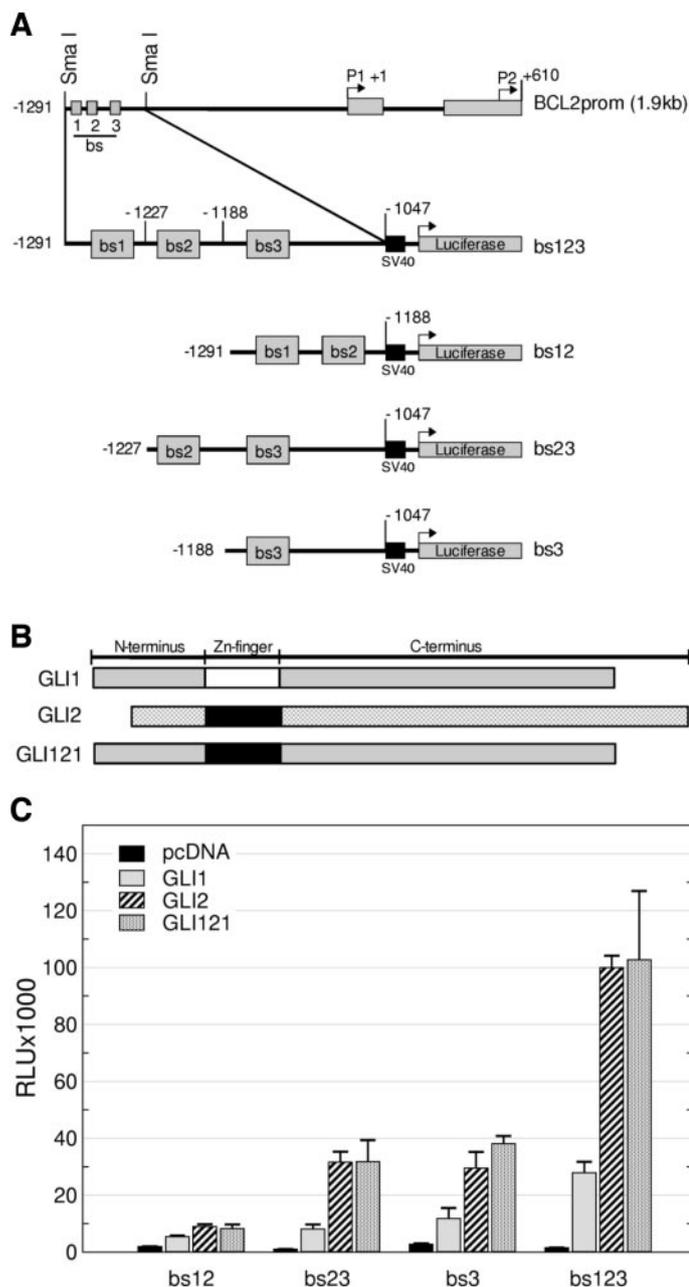
In line with our results on the 1.9-kb promoter fragment of BCL2, we found that the cassette comprising all three GLI binding sites [bs123 ranging from -1291 to -1047 of BCL2prom (1.9 kb)] was sufficient to recapitulate the GLI2-specific activation of the entire BCL2 promoter [BCL2prom (1.9 kb); see above]. This suggests that all of the *cis*-regulatory elements required for activation of BCL2 expression in response to *GLI* genes are located within the 244-bp fragment analyzed. Induction of luciferase activity by GLI2 was approximately four times higher compared with that of GLI1 (Fig. 4*C*;

bs123). Intriguingly, the chimeric GLI121 protein was as efficient in inducing reporter activity as wild-type GLI2, suggesting that the zinc finger domain accounts for the different abilities of GLI2 and GLI1 to activate the BCL2 promoter.

We next addressed the question of whether any one of the three GLI binding sites is required for selective promoter activation by GLI2 and GLI121, respectively. We found that construct bs12 containing binding sites bs1 and bs2 but lacking bs3 was only poorly activated by GLI1, GLI2, and GLI121. Although bs1 has been identified previously as a critical site for GLI1-mediated activation of the BCL2 promoter (28), these results suggest that bs1 is not responsible for the differential activation of the promoter by GLI1 and GLI2, respectively. By contrast, constructs bs23 and bs3 were strongly activated by GLI2 and GLI121 but only poorly by GLI1, suggesting that bs3 represents the *cis*-regulatory element involved in mediating GLI2-specific activation of the BCL2 promoter. Because no significant difference between constructs bs23 and bs3 was observed, we propose that bs2 only plays a minor role in mediating BCL2 activation in response to HH/GLI signaling. The critical role of bs3 in mediating selective BCL2 activation is further underlined by the fact that among the three binding sites found in the human BCL2 promoter, only bs3 has been fully conserved between human, mouse, and rat, as shown by sequence alignment of the respective genomic regions using Pip-Maker sequence comparison software (ref. 42; Fig. 4*D*). Notably, although bs1 was not required for preferential activation of the BCL2 promoter by GLI2 and GLI121, it was required to achieve maximum activation of luciferase activity, which may point to cooperative binding of GLI2 to the BCL2 promoter.

GLI2 and BCL2 Are Coexpressed in Outer Root Sheath Cells of Hair Follicles and BCC. To address the *in vivo* relevance of our findings, we performed coexpression analysis of BCL2 and GLI2 in normal skin and BCC. In line with previous reports on BCL2 and GLI2 expression (20, 34, 43), both proteins were coexpressed in outer root sheath cells of hair follicles (Fig. 5*A* and *B*) and BCC (Fig. 5*C* and *D*). Notably, virtually all of the cells expressing GLI2 also stained positive for BCL2, suggesting that GLI2 is likely to regulate BCL2 expression *in vivo*.

Coexpression of GLI2 and BCL2 in Infiltrating Plasma Cells and Plasmacytoma. When we analyzed the expression pattern of GLI2 and BCL2 in human BCC sections, we also detected strong staining for either protein in immune cells that infiltrated the tumor islands. The characteristic morphology of these BCL2/GLI2-positive cells (eccentric nucleus and a large cytoplasm) clearly identified them as plasma cells (Fig. 5*E* and *F*), which prompted us to analyze the expression of GLI2 in plasma cell-derived tumors, such as plasmacytoma of bone marrow, a malignancy characterized by the presence of aberrant neoplastic plasma cells. For the analysis of GLI2 expression, we used bone marrow biopsies that had been diagnosed for plasmacytoma by a histopathologist according to the World Health Organization guidelines. Bone marrow biopsies derived from plasmacytoma patients revealed strong GLI2 expression specifically in malignant plasma cells (11 of 11 biopsies stained strongly positive for GLI2; Fig. 6*A*). The specificity of the immunoreactivity was verified by peptide-blocking experiments (Fig. 2; see Supplementary Data). We next asked whether BCL2, which has been shown to play a critical role in the development of B-cell neoplasia and plasmacytoma (44, 45), also is expressed in neoplastic plasma cells. As for GLI2, we found strong expression of BCL2 in malignant cells of plasmacytoma bone marrow biopsies (Fig. 6*B*, *arrowheads*). Weaker expression of BCL2 also was detected in morphologically normal B cells (Fig. 6*B*, *small arrows*). Coexpression of GLI2 and high levels of BCL2 were confirmed by confocal microscopy analysis of double immunofluorescence staining of GLI2 (green) and BCL2 (red; Fig. 6*C* and *D*).



Intriguingly, GLI2-positive cells expressed high levels of BCL2, whereas GLI2-negative cells expressed significantly lower levels or were even negative for BCL2. In contrast, expression of GLI3, which might function as negative regulator of BCL2 transcription (28), was strikingly different from that of GLI2. GLI3 was mainly expressed in cells that stained only weakly for BCL2, whereas it was absent in cells with high levels of BCL2, implying that neoplastic plasma cells do not express significant levels of GLI3 (Fig. 6E and F). We also found GLI1 expression in the bone marrow of plasmacytoma patients, but unlike GLI2, which was exclusively detected in plasmacytoma cells, GLI1 protein was expressed in a variety of cells with different leukocytic differentiation (data not shown). These data suggest that like in epidermal cells, GLI2 is likely to be involved in the regulation of BCL2 expression in neoplastic cells of plasmacytoma patients, thereby implicating HH/GLI signaling in the development and/or maintenance of this highly aggressive B cell-derived malignancy.

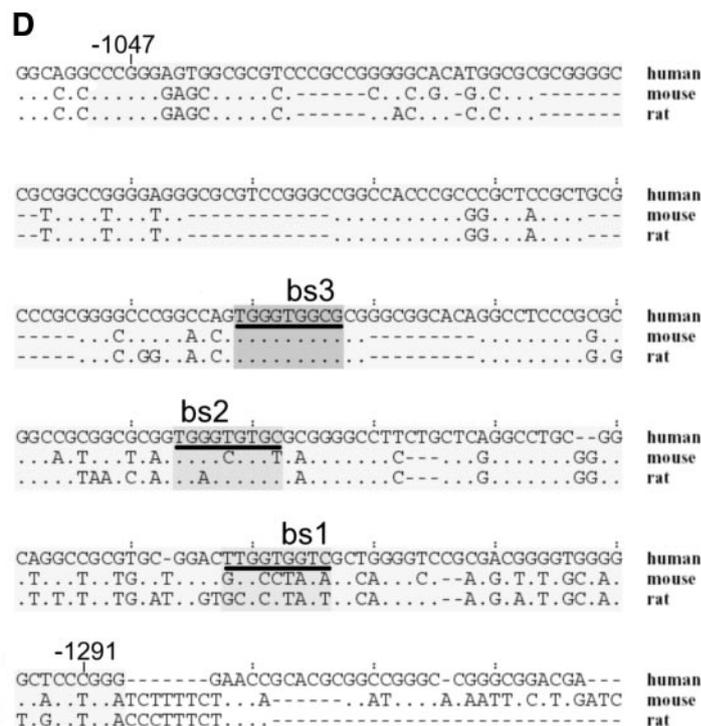


Fig. 4. The bs3 and the zinc finger domain of GLI2 are required for preferential activation of the BCL2 promoter by GLI2. *A*, schematic illustration of the BCL2 reporter constructs used to map critical binding sites. *B*, schematic illustration of the expression constructs GLI1 (with NH₂-terminal NLS), GLI2, and GLI121. The GLI2 zinc finger domain is highlighted by a black box, and the GLI1 zinc finger domain is indicated by a white box. *C*, luciferase reporter assays showing that preferential induction of the BCL2 reporter depends on the presence of bs3 and the GLI2 zinc finger domain. Cells were cotransfected with different reporter constructs (bs12, bs23, bs3, or bs123) and either empty control plasmid (pcDNA), NLS-tagged GLI1 (GLI1), wild-type GLI2, or GLI121 expression plasmids. Data show mean values of six independent transfection experiments. *D*, multiple sequence alignment of the human, mouse, and rat BCL2 promoter region containing the three GLI binding sites (-1047 to -1291). Dots represent conserved nucleotides, and dashes indicate nucleotides absent either in the human mouse and/or rat sequence. Respective human/mouse/rat promoter sequences were downloaded from the UCSC Bioinformatics Site (<http://genome.ucsc.edu/>) and aligned using Pip-Maker (<http://bio.cse.psu.edu/pipmaker>). Note that aligned sequences are shown in reverse orientation, according to the orientation of BCL2 on human chromosome 18q21.33.

DISCUSSION

GLI1 and GLI2 have been shown to act as positive mediators of HH signaling in early vertebrate development and in tumorigenesis (2, 46). Both factors, which have overlapping yet distinct biological activities, are highly expressed in BCC, but their relative contribution to skin carcinogenesis in response to aberrant HH signaling is unclear.

In this report, we provide evidence that GLI2 rather than GLI1 is able to induce high-level expression of BCL2, although either factor induces comparable levels of the known direct GLI target gene *PTCH*. Together with the observation that GLI3 is unable to activate the BCL2 promoter, this suggests that GLI2 represents the major GLI factor involved in activation of BCL2 expression in response to HH/GLI signaling. Given the pivotal role of BCL2 in promoting cell survival and tumorigenesis, our results uncover a potential mechanism

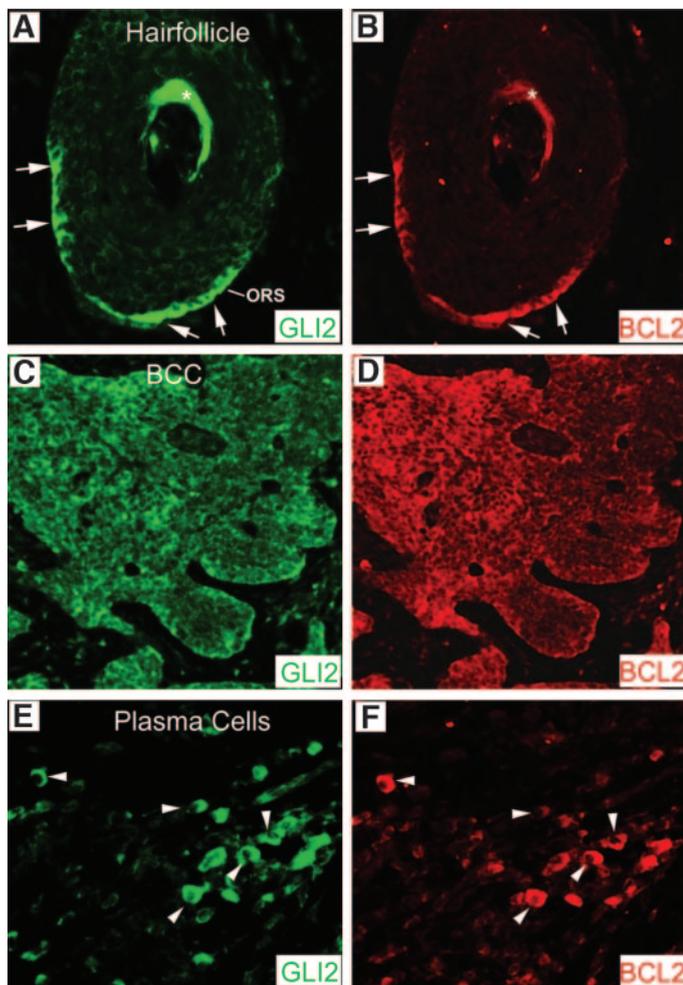


Fig. 5. Coexpression of GLI2 and BCL2 in the outer root sheath (ORS), BCC, and infiltrating plasma cells. *A* and *B*, coexpression of GLI2 (green) and BCL2 (red) in the ORS of hair follicles. Arrows depict regions with high levels of GLI2 and BCL2 protein. Asterisk highlights unspecific staining of GLI2 and BCL2 in the hair canal. *C* and *D*, coexpression of GLI2 and BCL2 in BCC tumor islands and (*E* and *F*) in plasma cells infiltrating the tumor islands. Arrowheads denote plasma cells with typical characteristics such as eccentric nucleus and large cytoplasm.

by which overexpression of the *GLI2* oncogene contributes to BCC development.

By replacing the DNA binding domain of GLI1 with that of GLI2, we showed that the zinc finger domain of GLI2 is responsible for GLI2-specific activation of the minimal BCL2 reporter construct comprising all three GLI binding sites. Notably, all of the amino acids of the GLI1 zinc finger domain that establish base contacts with the Gli consensus binding site GACCACCA are conserved in the GLI2 zinc finger domain (ref. 47; data not shown). Together with the overall high degree of sequence similarity between the GLI1 and GLI2 DNA binding domain, it appears that only minor differences in the amino acid sequence of the zinc finger domains of GLI transcription factors can have a dramatic effect on the selective activation of GLI target genes.

Detailed analysis of the *cis*-regulatory elements involved in the activation of the BCL2 promoter in response to GLI1 and GLI2, respectively, identified bs3 as the critical element involved in selective activation of the BCL2 promoter by GLI2. Unlike bs1 and bs2, bs3 is fully conserved between mouse, rat, and human, suggesting that preferential activation of BCL2 expression by GLI2 may represent a common regulatory mechanism in mammals.

Bigelow *et al.* (28) recently identified bs1 (corresponding to bind-

ing site 4 in Bigelow *et al.* (28)) as the major site required for activation of the BCL2 promoter in response to GLI1, whereas bs3 was not studied. In our experiments, bs1 had little effect on selective reporter activation by GLI2 when analyzed in the absence of bs3 but was able to increase reporter activity in combination with bs3. shRNA knockdown of GLI1 also showed that activation of endogenous GLI1 by GLI2 is not required for high-level activation of the BCL2 promoter. Thus, a possible scenario for the activation of the BCL2 promoter could be that GLI2 preferentially binds to bs3, which then would facilitate binding of another GLI2 molecule to bs1, a hypothesis that is consistent with our observation that full reporter activation is more than additive and depends on the presence of bs1 and bs3.

Our results on coexpression of GLI2 and BCL2 in tumors of BCC patients suggest that constitutive HH signaling induces BCL2 expression in BCC by enhancing the activity of GLI2. Aberrant HH signaling may thereby increase the viability of tumor cells. In a previous report, we have shown that GLI2 can stimulate epidermal proliferation by increasing the expression of genes required for G₁-S phase progression and that GLI2 is able to antagonize epidermal differentiation (30). Together with the findings presented in this study, the oncogenic

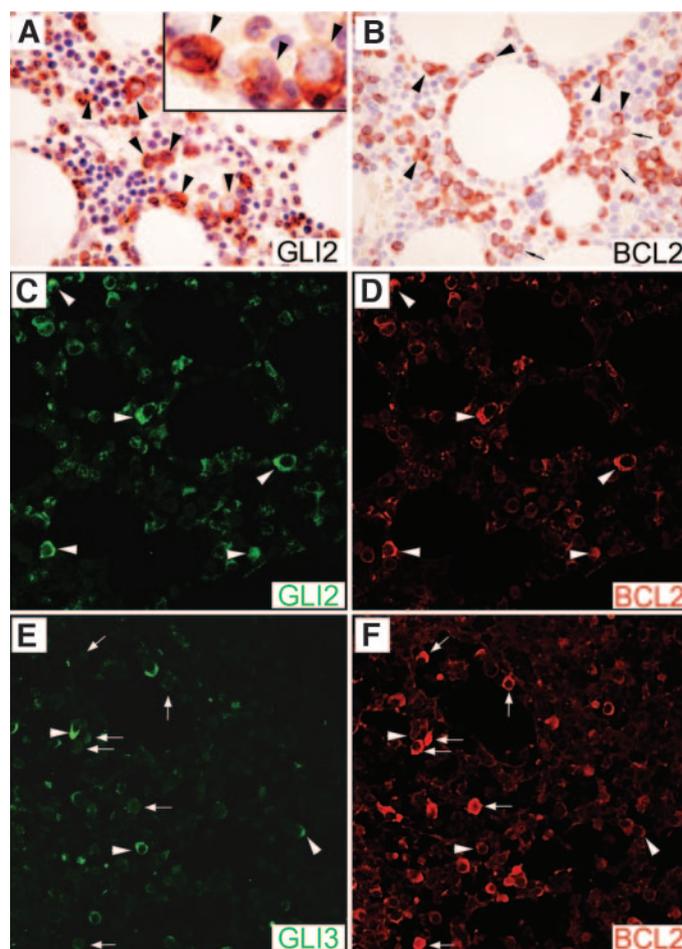


Fig. 6. Expression of GLI2, BCL2, and GLI3 in bone marrow biopsies of plasmacytoma patients. *A*, Strong GLI2 expression was detected in neoplastic plasma cells (black arrowheads) in the bone marrow. Inset shows aberrant malignant plasma cells (arrowheads) stained for GLI2 at higher magnification. *B*, strong BCL2 expression in neoplastic plasma cells (arrowheads). Normal plasma cells express only moderate levels of BCL2 (thin arrows). *C* and *D*, confocal microscopy analysis of GLI2-BCL2 double immunostainings of bone marrow biopsies of plasmacytoma patients. Cells expressing high levels of GLI2 (green, white arrowheads) also express increased levels of BCL2 (red). *E* and *F*, double immunostaining of plasmacytoma biopsies for GLI3 (*E*) and BCL2 (*F*). Cells expressing high levels of GLI3 protein (arrowheads) only express moderate levels of BCL2 (BCL2 (white arrows) are negative for GLI3).

activity of GLI2 in BCC therefore may rely on at least three distinct functions: (a) stimulation of cell proliferation; (b) repression of epidermal differentiation; and (c) induction of pro-survival signals such as BCL2.

When we observed GLI2/BCL2 coexpression in plasma cells infiltrating BCC tumor islands, we hypothesized a possible role of HH/GLI signaling also in plasma cell-derived malignancies. This was supported by a corresponding analysis of plasmacytoma samples. These results are consistent with experiments in which enforced expression of Bcl-2 significantly increased the incidence of plasmacytoma development in an appropriate mouse model, implicating BCL2 in neoplastic plasma cell development most likely by increasing the cells' resistance to apoptotic signals (45). Expression of GLI2 in malignant cells correlated well with high-level expression of BCL2. Conversely, atypical plasma cells expressing high levels of BCL2 were negative for GLI3, which mainly acts as a repressor of Hh/GLi target genes. In future experiments, it will be important to address the question of whether an increase in GLI2 activator function in plasma cells will result in increased proliferation and/or cell viability at the expense of terminal B-cell differentiation.

In summary, the observation that preferential activation of BCL2 expression by GLI2 depends on the GLI2 zinc finger domain and a single conserved GLI binding site in the BCL2 promoter uncovers a mechanism of differential target gene regulation by GLI proteins, which relies on selective binding site recognition rather than the requirement for additional coactivators. The predominant role of GLI2 in the activation of the key antiapoptotic factor BCL2 reveals an additional molecular pathway by which aberrant HH signaling may induce or maintain tumor development. Its ability to stimulate cell proliferation, to repress epidermal cell differentiation, and to activate pro-survival signals make GLI2 an attractive molecule to be targeted for therapeutic purposes in future experiments.

ACKNOWLEDGMENTS

We thank Dr. Shi for providing pBS/U6 plasmid, Mag. Carmen Schmid for providing the PTCH-reporter plasmid, Sabine Siller for excellent technical assistance, and Ing. Klappacher and Dr. Hermann for help with confocal microscopy.

REFERENCES

- Ingham PW, McMahon AP. Hedgehog signaling in animal development: paradigms and principles. *Genes Dev* 2001;15:3059–87.
- Ruiz i Altaba A, Sanchez P, Dahmane N. Gli and hedgehog in cancer: tumours, embryos and stem cells. *Nat Rev Cancer* 2002;2:361–72.
- Pasca di Magliano M, Hebrok M. Hedgehog signalling in cancer formation and maintenance. *Nat Rev Cancer* 2003;3:903–11.
- Mullor JL, Sanchez P, Altaba AR. Pathways and consequences: Hedgehog signaling in human disease. *Trends Cell Biol* 2002;12:562–9.
- Kalderon D. Hedgehog signaling: costal-2 bridges the transduction gap. *Curr Biol* 2004;14:R67–9.
- Kinzler KW, Vogelstein B. The GLI gene encodes a nuclear protein which binds specific sequences in the human genome. *Mol Cell Biol* 1990;10:634–42.
- Matisse MP, Joyner AL. Gli genes in development and cancer. *Oncogene* 1999;18:7852–9.
- Hahn H, Wojnowski L, Zimmer AM, et al. Rhabdomyosarcomas and radiation hypersensitivity in a mouse model of Gorlin syndrome [see comments]. *Nat Med* 1998;4:619–22.
- Goodrich LV, Milenkovic L, Higgins KM, Scott MP. Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science* 1997;277:1109–13.
- Ingham PW. The patched gene in development and cancer [published erratum appears in *Curr Opin Genet Dev* 1998;8:371]. *Curr Opin Genet Dev* 1998;8:88–94.
- Berman DM, Karhadkar SS, Maitra A, et al. Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. *Nature* 2003;425:846–51.
- Watkins DN, Berman DM, Burkholder SG, et al. Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer. *Nature* 2003;422:313–7.
- Thayer SP, di Magliano MP, Heiser PW, et al. Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. *Nature* 2003;425:851–6.
- Hahn H, Wicking C, Zaphiropoulos PG, et al. Mutations of the human homolog of *Drosophila* patched in the nevoid basal cell carcinoma syndrome. *Cell* 1996;85:841–51.
- Johnson RL, Rothman AL, Xie J, et al. Human homolog of patched, a candidate gene for the basal cell nevus syndrome. *Science* 1996;272:1668–71.
- Xie J, Murone M, Luoh SM, et al. Activating Smoothed mutations in sporadic basal-cell carcinoma. *Nature* 1998;391:90–2.
- Nilsson M, Uden AB, Krause D, et al. Induction of basal cell carcinomas and trichoepitheliomas in mice overexpressing Gli-1. *Proc Natl Acad Sci USA* 2000;97:3438–43.
- Sheng H, Goich S, Wang A, et al. Dissecting the oncogenic potential of Gli2: deletion of an NH(2)-terminal fragment alters skin tumor phenotype. *Cancer Res* 2002;62:5308–16.
- Grachtchouk M, Mo R, Yu S, et al. Basal cell carcinomas in mice overexpressing Gli2 in skin. *Nat Genet* 2000;24:216–7.
- Regl G, Neill GW, Eichberger T, et al. Human GLI2 and GLI1 are part of a positive feedback mechanism in basal cell carcinoma. *Oncogene* 2002;21:5529–39.
- Aza-Blanc P, Lin HY, Ruiz i Altaba A, Kornberg TB. Expression of the vertebrate Gli proteins in *Drosophila* reveals a distribution of activator and repressor activities. *Development* 2000;127:4293–301.
- Brewster R, Mullor JL, Ruiz i Altaba A. Gli2 functions in FGF signaling during antero-posterior patterning. *Development* 2000;127:4395–405.
- Bai CB, Joyner AL. Gli1 can rescue the in vivo function of Gli2. *Development* 2001;128:5161–72.
- Mill P, Mo R, Fu H, et al. Sonic hedgehog-dependent activation of Gli2 is essential for embryonic hair follicle development. *Genes Dev* 2003;17:282–94.
- Dahmane N, Lee J, Robins P, Heller P, Ruiz i Altaba A. Activation of the transcription factor Gli1 and the Sonic hedgehog signalling pathway in skin tumours [published erratum appears in *Nature* 1997;390:536]. *Nature* 1997;389:876–81.
- Ghali L, Wong ST, Green J, Tidman N, Quinn AG. Gli1 protein is expressed in basal cell carcinomas, outer root sheath keratinocytes and a subpopulation of mesenchymal cells in normal human skin. *J Invest Dermatol* 1999;113:595–9.
- Bonifas JM, Pennypacker S, Chuang PT, et al. Activation of expression of hedgehog target genes in basal cell carcinomas. *J Invest Dermatol* 2001;116:739–42.
- Bigelow RL, Chari NS, Uden AB, et al. Transcriptional regulation of bcl-2 mediated by the sonic hedgehog signaling pathway through gli-1. *J Biol Chem* 2004;279:1197–205.
- Boukamp P, Petrussevska RT, Breitkreutz D, et al. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J Cell Biol* 1988;106:761–71.
- Regl G, Kasper M, Schnidar H, et al. The zinc-finger transcription factor GLI2 antagonizes contact inhibition and differentiation of human epidermal cells. *Oncogene* 2004;23:1263–74.
- Sui G, Soohoo C, Affar el B, et al. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc Natl Acad Sci USA* 2002;99:5515–20.
- Martin KJ, Graner E, Li Y, et al. High-sensitivity array analysis of gene expression for the early detection of disseminated breast tumor cells in peripheral blood. *Proc Natl Acad Sci USA* 2001;98:2646–51.
- Sheffield P, Garrard S, Derewenda Z. Overcoming expression and purification problems of RhoGDI using a family of "parallel" expression vectors. *Protein Expr Purif* 1999;15:34–9.
- Ikram MS, Neill GW, Regl G, et al. GLI2 is expressed in normal human epidermis and BCC and induces GLI1 expression by binding to its promoter. *J Invest Dermatol* 2004;122:1503–9.
- Roth FP, Hughes JD, Estep PW, Church GM. Finding DNA regulatory motifs within unaligned noncoding sequences clustered by whole-genome mRNA quantitation. *Nat Biotechnol* 1998;16:939–45.
- Seto M, Jaeger U, Hockett RD, et al. Alternative promoters and exons, somatic mutation and deregulation of the Bcl-2-Ig fusion gene in lymphoma. *EMBO J* 1988;7:123–31.
- Ruppert JM, Vogelstein B, Arheden K, Kinzler KW. GLI3 encodes a 190-kilodalton protein with multiple regions of GLI similarity. *Mol Cell Biol* 1990;10:5408–15.
- Dai P, Akimaru H, Tanaka Y, et al. Sonic Hedgehog-induced activation of the Gli1 promoter is mediated by GLI3. *J Biol Chem* 1999;274:8143–52.
- Wang B, Fallon JF, Beachy PA. Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. *Cell* 2000;100:423–34.
- Sasaki H, Nishizaki Y, Hui C, Nakafuku M, Kondoh H. Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. *Development* 1999;126:3915–24.
- Price MA, Kalderon D. Proteolysis of the Hedgehog signaling effector Cubitus interruptus requires phosphorylation by Glycogen Synthase Kinase 3 and Casein Kinase 1. *Cell* 2002;108:823–35.
- Schwartz S, Zhang Z, Frazer KA, et al. PipMaker—a web server for aligning two genomic DNA sequences. *Genome Res* 2000;10:577–86.
- Rodriguez-Villanueva J, Colome MI, Brisbay S, McDonnell TJ. The expression and localization of bcl-2 protein in normal skin and in non-melanoma skin cancers. *Pathol Res Pract* 1995;191:391–8.
- Cory S, Vaux DL, Strasser A, Harris AW, Adams JM. Insights from Bcl-2 and Myc: malignancy involves abrogation of apoptosis as well as sustained proliferation. *Cancer Res* 1999;59:1685s–92s.
- Silva S, Kovalchuk AL, Kim JS, Klein G, Janz S. BCL2 accelerates inflammation-induced BALB/c plasmacytomas and promotes novel tumors with coexisting T(12;15) and T(6;15) translocations. *Cancer Res* 2003;63:8656–63.
- Ruiz i Altaba A. Gli proteins and Hedgehog signaling: development and cancer. *Trends Genet* 1999;15:418–25.
- Pavletich NP, Pabo CO. Crystal structure of a five-finger GLI-DNA complex: new perspectives on zinc fingers. *Science* 1993;261:1701–7.