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Characteristic overexpression of the forkhead box transcription factor *Foxf1* in *Patched*-associated tumors

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Abstract. Patients with nevoid basal cell carcinoma syndrome carry germline mutations in the tumor suppressor gene *Patched 1 (PTCH1)* and are predisposed to develop basal cell carcinoma (BCC), medulloblastoma (MB), and rhabdomyosarcoma (RMS). These tumors are also present in the murine model for *Ptch1* deficiency, the *Ptch1*$^{neo67/+}$ mouse. Previous studies, including those from our laboratory, have shown that the forkhead box transcription factor *Foxf1* is highly expressed in RMS of human and murine origin. We report on a more common role of *Foxf1* in *Ptch1*-associated tumorigenesis, since we found a striking up-regulation of *Foxf1* expression in *Ptch1*-associated BCC and MB compared with the respective non-neoplastic tissue. This overexpression was accompanied by increased levels of the Hedgehog target gene *Gli1* as well as the putative Foxf1 targets *Bmi1* and *Notch2* in these tumors. We also describe a striking *Foxf1* activation in *Ptch1* null embryos. In contrast, basal expression levels of *Foxf1*, *Gli1*, *Bmi1* and *Notch2* were detected in a variety of adult mouse tissues, such as liver, kidney, spleen, lung, heart and brain. In conclusion, our study suggests that *Foxf1* expression is characteristically up-regulated in tumors with a constitutively activated Hedgehog signaling pathway thereby defining a key role for Foxf1 in Hedgehog-associated tumorigenesis.

Introduction

The Hedgehog (Hh) signaling pathway plays a fundamental role in development and tumorigenesis and determines cell fate in a variety of tissues, including brain, muscle and skin (1,2). The core components of this signaling pathway are the morphogen Sonic Hedgehog (Shh) and its receptor Patched 1 (Ptch1) that inhibits the transmembrane protein Smoothened (Smo). Binding of Shh to Ptch1 suspends its inhibiting function and leads to the activation of the Gli-Krüppel zinc finger transcription factors (3). Constitutive activation of the Hh signaling pathway causes the formation of tumors including basal cell carcinoma (BCC), medulloblastoma (MB) and rhabdomyosarcoma (RMS) (4,5). Germline mutations in the *PTCH1* gene have been found in patients with nevoid basal cell carcinoma syndrome (NBCCS) (6,7) who are predisposed to BCC, MB, RMS and other tumors (8). Additionally, somatic PTCH1 mutations have been detected in sporadic cases of BCC and MB (9,10). Notably, *Ptch1*-deficient mouse strains develop tumors comparable to those in humans with NBCCS including BCC, MB and RMS. These tumors consistently overexpress downstream targets of the activated Hh signaling pathway such as *Gli1*, *Igf2* and *Ptch1* itself (4,5,11).

The forkhead box protein Foxf1 belongs to the superfamily of forkhead/winged-helix transcription factors that regulate cellular proliferation, differentiation and metabolic homeostasis (12). *Foxf1* expression begins at 6.5 days post coitum (13) and is essential for the development of gut derived organs, such as the esophagus, trachea, lung and intestine, as well as of the head and brain (13-16). As *Foxf1* plays an important role during early development, *Foxf1* knock out mice die *in utero* (17). However, this transcription factor is also expressed during adulthood in the lung, liver, intestine and brain (13,14,16,18,19). Notably, *Foxf1* haploinsufficiency in the mouse results in the same defects of the lung, foregut, trachea and oesophagus as seen in *Shh, Gli2* and *Gli3* mutants (20-23). Moreover, *Foxf1* heterozygous mice are described to exhibit diminished expression of *Gli3*, *Bmi1* (24), *Igf2* (17), *Hnf3β* and *Pdgfrα* (25), known target genes of Hh signaling (3). Our own group has reported on the significant *Foxf1* overexpression in RMS of *Ptch1*-haploinsufficient mice that show a constitutively activated Hh pathway (26). Altogether these studies suggest that *Foxf1* is a putative target gene of Hh signaling.

To substantiate this hypothesis we investigated whether *Foxf1* is overexpressed in *Ptch1*-associated tumors other than RMS and examined its expression levels in BCC and MB by means of semi-quantitative RT-PCR and *in situ* hybridization. Additionally, we tested whether the expression level of *Foxf1* is elevated in *Ptch1* null embryos, in which Hh

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signaling is aberrantly active. Since *Foxf1* haploinsufficiency has been demonstrated to diminish the expression of *Bmi1* and *Notch2* (27) and both genes have previously been found to be overexpressed in BCC and MB (28-32), we furthermore analyzed whether up-regulation of *Foxf1* in *Ptch1*-associated tumors consequently results in an increased expression of these genes. In the current study we demonstrated that *Foxf1* and *Gli1* are concomitantly overexpressed in *Ptch1*-associated tumors and *Ptch1* null embryos thus suggesting that *Foxf1* might be a target gene of the Hh signaling pathway. We furthermore showed for the first time that *Bmi1* and *Notch2* are up-regulated in RMS in comparison to normal skeletal muscle.

Materials and methods

Animals and tissue specimens. RMS and MB were excised from *Ptch1*$^{neo67/+}$ mice maintained on a CD1 and C57/BL6 background, respectively. Tumors were immediately frozen in liquid nitrogen and stored at -80˚C. Histological analysis of hematoxylin and eosin stained tumor sections was carried out by a pathologist. Only tumor tissues containing >95% tumor cells were used in this study. E9.5 *Ptch1*$^{+/+}$ and *Ptch1*$^{neo67/neo67}$ embryos from crosses between *Ptch1*$^{neo67/+}$ mice were isolated and genotyped as previously described (4). Two types of BCC of irradiated heterozygous *Ptch1*$^{neo67/+}$ mice that are known to be positive or negative for smooth muscle actin, respectively, were kindly provided by Dr M. Mancuso (33). Non-neoplastic tissue from skeletal muscle, cerebella and skin of the respective mouse strains as well as a variety of different tissue types (liver, kidney, spleen, lung, heart muscle and cerebral cortex) from wild-type mice, were harvested and immediately stored at -80˚C.

Isolation of RNA. Total RNA was extracted from tumors and normal tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was depleted from DNA and subsequently purified using the RNase free DNase set and RNeasy Mini Kit from Qiagen (Hilden, Germany) according to the manufacturer's protocol. The concentration of RNA was measured by photometry (BioPhotometer, Eppendorf, Hamburg, Germany) and RNA stored at -80˚C.

Semi-quantitative reverse transcription-PCR. Reverse transcription of total RNA was performed using random hexamers (Roche Diagnostics, Penzberg, Germany) and SuperScriptII reverse transcriptase (Invitrogen). PCR amplifications of the murine genes *ß-actin*, *Foxf1*, *Gli1*, *Bmi1* and *Notch2* were carried out with 50 ng of cDNA using the forward and reverse primers as given in Table I. PCR reactions were performed in a 20 μl final reaction mixture for 28-32 cycles consisting of 30 sec denaturation at 95˚C, hybridization of primers for 30 sec at 58˚C and extension for 1 min at 72˚C. The individual cycle number for each gene was defined by predetermining the linear range of the PCR. Amplification of *ß-actin* was used as a reference standard to control the amount of sample RNA.

In situ hybridization. Digoxigenin (DIG)-labeled RNA probes for *Gli1* and *Foxf1* were prepared from RT-PCR amplified products (primer sequences upon request) by standard RNA synthesis reactions with T7- or T3-RNA Polymerase (Promega, Mannheim, Germany) using the DIG-RNA labeling Mix (Roche Diagnostics). Labeling yield was validated by means of dot blot using Anti-Digoxigenin-AP Fab fragments (Roche Diagnostics). RNA expression analysis was performed on 5 μm thick paraffin-embedded sections of above mentioned RMS specimen that were rinsed in xylene, hydrated in a graded ethanol series and microwaved at 600 W for 30 min. Sections were treated with 0.3% Triton X-100 for 15 min and with proteinase K at 37˚C for 10 min followed by incubation with hybridization buffer (40% formamide, 10% dextran sulphate, 1X Denhardt's solution, 4X SSC, 250 μg/ml yeast total RNA) at 55˚C for 30 min. Then 10 μg salmon sperm DNA were denatured at 95˚C for 10 min, added to 1 μg labeled RNA probe in 100 μl hybridization buffer and heated for 2 min at 70˚C. Sections were incubated at 55˚C overnight in a humidified chamber and then rinsed with 2X SSC/1% SDS at room temperature for 5 min. This was followed by rinsing at 60˚C for 20 min with 0.2X SSC/1% SDS and at room temperature for 10 min with 100 mM Tris HCl and 150 mM NaCl at pH 7.5. Blocking with 4% BSA in 100 mM Tris HCl and 150 mM NaCl was performed at 37˚C for 30 min and sections were then incubated with anti-DIG-Fab-AP (1:500) at 37˚C in a humidified chamber for 2 h. Subsequently, sections were rinsed with 100 mM Tris HCl and 150 mM NaCl at pH 7.5. Sections were buffered at pH 9.5 with 100 mM Tris-HCl, 100 mM NaCl and 50 mM $MgCl_2$. For color detection nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate in 100 mM Tris-HCl, 100 mM NaCl and 50 mM $MgCl_2$ was used as recommended by the supplier (Roche Diagnostics). Staining was stopped by incubation with 10 mM Tris-HCl and 1 mM EDTA at pH 8.1 and sections were rinsed with distilled water and stained with hematoxylin for 1 min.

Immunohistochemistry. For immunohistochemical analyses 3 μm thick paraffin sections of RMS and MB were mounted on organo-silane coated slides (DakoCytomation, Hamburg, Germany). After deparaffination and rehydration antigen retrieval was accomplished by microwaving the tissue sections in 0.01 M citrate buffer at 700 W for 15 min. Primary antibodies for Myogenin (DakoCytomation) and Nestin (BD Biosciences, San Jose, CA, USA) were diluted 1:50 and 1:1000 in dilution buffer (DakoCytomation), respectively. For detection of primary antibodies the Animal Research Kit ARK™ (DakoCytomation) was used according to the manufacturer's protocol. Cryosections of BCC were fixed with ice-cold aceton prior to staining with an antibody for muscle actin (DakoCytomation) at a dilution of 1:1000 and detection with ARK™ (DakoCytomation).

Results

Previous studies have demonstrated that haploinsufficiency of *Foxf1* results in similar developmental defects of the lung, gut and brain as inactivation of Hh signaling components such as Shh, Gli2 and Gli3 (14,22), leading to the assumption that *Foxf1* is a putative target gene of this signaling cascade. In order to confirm this hypothesis, we first set out to study the expression pattern of *Foxf1* and *Gli1*, the latter being an

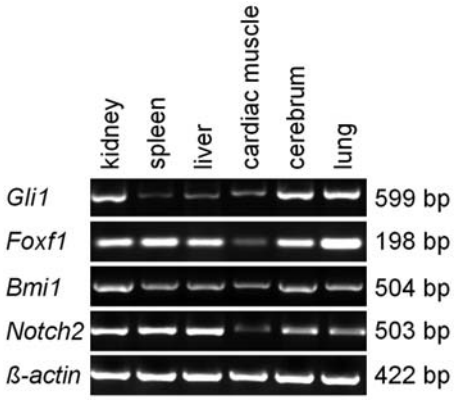

Figure 1. Differential expression of *Foxf1*, *Gli1*, *Bmi1* and *Notch2* in various non-neoplastic tissue types. Gene expression was analyzed in the kidney, spleen, liver, cardiac muscle, cerebrum, and lung using semi-quantitative RT-PCR. Expression of ß-actin was monitored to control the amount of sample RNA.

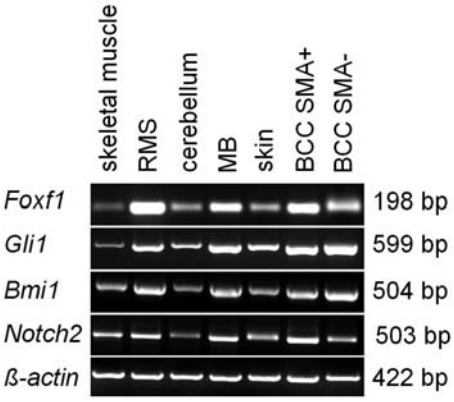

Figure 2. Overexpression of *Foxf1*, *Gli1*, *Bmi1* and *Notch2* in *Ptch1*-associated tumors. Gene expression was analyzed in RMS, MB and smooth muscle actin (SMA)-positive and negative BCC of $Ptch1^{neo67/+}$ mice and the respective non-neoplastic tissues (skeletal muscle, cerebellum and skin) using semi-quantitative RT-PCR, respectively. Expression of ß-actin was monitored to control the amount of sample RNA.

indicator of activated Hh signaling (9), in a variety of non-neoplastic adult tissue types of the mouse. Furthermore, two potential target genes of Foxf1, namely *Bmi1* and *Notch2*, were studied. We detected a high level of *Foxf1* and correspondingly of *Gli1* in lung and cerebral cortex tissue by means of semi-quantitative RT-PCR (Fig. 1). In kidney tissue we found an exceptionally high expression level of *Gli1*. Expression of these two genes as well as of the *Bmi1* and *Notch2* gene was also found in the other adult tissue types tested. However, no significant trend towards an especially high expression level of *Bmi1* or *Notch2* was found in any of the examined adult non-neoplastic tissue types (Fig. 1).

We and others have previously shown that *Foxf1* is highly expressed in RMS of human (34) and murine origin (26). To test whether *Foxf1* overexpression is rather associated with Hh signaling than with this specific tumor type, we aimed to determine the expression level of *Foxf1* and *Gli1* on RMS, MB and BCC of $Ptch1^{neo67/+}$ mice. To achieve this, we used tumors of $Ptch1^{neo67/+}$ mice maintained on an irradiated (BCC) and non-irradiated CD1 (RMS) or C57-BL6 (MB) background and validated their identity by immunohistochemical stainings. Myogenin, nestin and muscle actin, markers known to label RMS, MB and subtypes of BCC were analyzed, respectively (33,35-37). We detected a strong staining for the respective markers in the tumors under investigation thus substantiating their identity (data not shown). By analyzing these tumors using semi-quantitative RT-PCR, we confirmed an elevated transcription level of *Foxf1* in RMS in relation to the one in skeletal muscle, which is in line with our earlier data obtained by microarray and Northern blot analysis (26). Furthermore, we found an overexpression of *Foxf1* in BCC and MB in comparison to non-neoplastic skin and cerebellar tissue, respectively. In parallel to the high *Foxf1* expression, we detected an increased expression of *Gli1* in all three *Ptch1*-associated tumors compared to the respective non-neoplastic tissues (Fig. 2), which has already been described by others (4) and corresponds to its role as a marker of activated Hh signaling. Since *Foxf1* haploinsufficiency has been demonstrated to diminish the expression of *Bmi1* and *Notch2* on the transcriptional level (27), we additionally tested whether an up-regulation of *Foxf1* in *Ptch1*-associated tumors would consequently result in an increased expression of these two genes. Our semi-quantitative RT-PCR detected an up-regulation of *Bmi1* and *Notch2* in RMS, MB and BCC in comparison to the respective normal tissues (Fig. 2).

In order to ascertain whether *Foxf1* might be transcriptionally regulated by a constitutively activated Hh signaling pathway, we analyzed *Foxf1* expression in $Ptch1^{neo67/neo67}$ embryos by means of semi-quantitative RT-PCR. Homozygous loss of *Ptch1* alleles, which leads to aberrantly active Hh signaling, resulted in a strong increase in *Foxf1* and *Gli1* expression (Fig. 3A), the latter again was used as a positive control for the induction of the pathway. These results clearly show that the *Foxf1* gene is highly expressed in *Ptch1*-associated tumors and *Ptch1* null embryos and might be regulated by Hh signaling. Since our goal was to further explore the nature of *Foxf1* and *Gli1* overexpression and to localize the expressing cells within the tumor *in situ* hybridizations were performed exemplary with RMS. Here, we demonstrated that the enhanced expression takes place within the tumor cells thus making a pathologically activated Hh loop in RMS conceivable (Fig. 3B and 4).

Discussion

Forkhead box transcription factors play an important role during embryonic development. Amongst other transcription factors, Foxf1 has been linked to the Hh signaling pathway and is thought to be downstream of Shh (22). The expression pattern of *Foxf1* has been investigated previously mainly by means of *in situ* hybridization and ß-galactosidase staining in *Foxf1* haploinsufficient mice and has shown the involvement of this transcription factor in the development of the extra-embryonic mesoderm, allantois, lateral mesoderm and endoderm as well as its expression in adult lung, liver, intestine, brain, spleen, heart and urinary bladder (13,14,16,17,19,22,25,38). Gli1, however, is a transcriptional activator downstream of Shh that is up-regulated whenever the Hh pathway is activated (9). In our study we detected a basal expression level of *Foxf1*

Table I. Gene-specific primers used for semi-quantitative RT-PCR.

Primer	Forward (5'-3' orientation)	Reverse (5'-3' orientation)
Foxf1	CAGAACTGCAAGGCATCCCT	TGTCTTGGTAGGTGACCTGCTG
Gli1	AATACATGCTGGTGGTGCACAT	CCTTCATCCAAGCTGGACAAGT
Bmi1	AAAACCAGACCACTCCTGAACATAA	GCAAGTTGGCCGAACTCTGT
Notch2	CCTGCATCCACGGAAACTGT	CTGCCCTGAGTGTTGTGGC
ß-actin	TACCACAGGCATTGTGATGGA	CAACGTCACACTTCATGATGGA

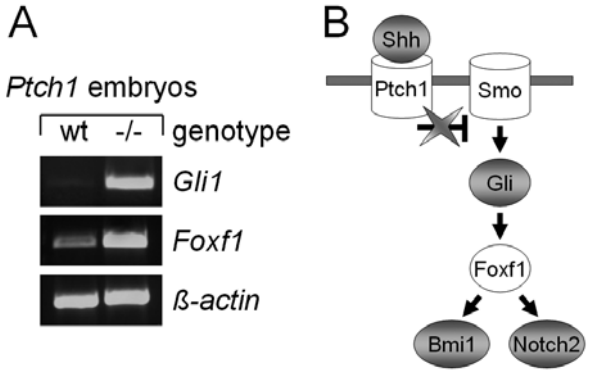

Figure 3. (A) Overexpression of *Foxf1* and *Gli1* in *Ptch1*$^{neo67/neo67}$ embryos. *Foxf1* and *Gli1* expression in Ptch1$^{neo67/neo67}$ embryos was analyzed by means of semi-quantitative RT-PCR. Expression of ß-actin was monitored to control for the amount of sample RNA. (B) Hedgehog signaling cascade integrating new target genes. Binding of the Sonic hedgehog (Shh) protein to its receptor Patched 1 (Ptch1) suspends its inhibiting function on the transmembrane protein Smoothened (Smo) and leads to the activation of Gli transcription factors. Integration of the new target genes *Foxf1*, *Bmi1* and *Notch2* into the Hedgehog signaling cascade distal to Gli.

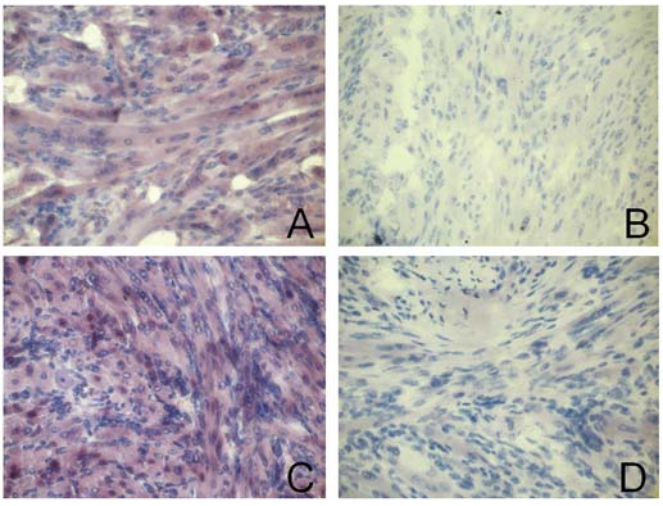

Figure 4. *In situ* hybridization confirming the overexpression of *Foxf1* and *Gli1* in RMS. To confirm the data produced by semi-quantitative RT-PCR analysis, the expression of *Foxf1* and *Gli1* was examined by *in situ* hybridization in RMS of *Ptch1*$^{neo67/+}$ mice. (A) Cytoplasmic expression of *Foxf1* in RMS as depicted by violet staining of the anti-sense RNA probe for *Foxf1* (x40). (B) Negative control using the sense RNA probe for *Foxf1* (x40). (C) Cytoplasmic expression of *Gli1* in RMS shown by violet staining of the anti-sense RNA probe for *Gli1* (x40). (D) Negative control demonstrating the absence of staining with the sense RNA probe for *Gli1* (x40).

and *Gli1* in the adult mouse tissue types tested by means of semi-quantitative RT-PCR confirming our hypothesis of *Foxf1* as a potential target gene of the Hh signaling pathway. Notably, we found an exceptionally high expression of *Foxf1* along with *Gli1* in the cerebrum, lung and kidney. This corresponds with the significant role of Hh signaling during lung and brain development as indicated by previous work (16,17) However, within the urinary tract the transcription factor Foxf1 had so far only been described in the urinary bladder and urethra of adult rats, but not in the kidney (14). Our finding that *Foxf1* is strongly expressed in the kidney is in line with a study of Hu and colleagues (39) that showed the importance of Gli1 during kidney development. Nevertheless, *Foxf1* showed relatively higher expression levels than *Gli1* in the spleen and liver. This inconsistency might be due to different levels of Hh signaling activity within the respective tissue types. Collectively, we detected an expression of *Foxf1* and *Gli1* in the examined adult tissue types indicating the importance of the Hh signaling pathway beyond development as well as *Foxf1* as part of this signaling cascade. In addition, we demonstrate ubiquitous expression of *Bmi1* and *Notch2* in non-neoplastic murine adult kidney, spleen, liver, cardiac muscle, cerebrum and lung as well as in skin, cerebellum and skeletal muscle. We found that the detected expression levels of *Bmi1* and *Notch2* are in relation to those of *Foxf1*. This suggests that *Bmi1* and *Notch2* might be targeted by Hh signaling via activation of Foxf1. It has been described that the Notch signaling pathway plays diverse roles during development and physiological tissue processes as well as in tumor formation (40,41), while the Polycomb protein BMI1 regulates stem cell maintenance and is associated with a variety of tumor types (29,42,43). The specific implication of these two genes in the different tissue types and its crosstalk with Hh signaling remains to be further investigated, but the corresponding expression levels of *Notch2*, *Bmi1*, *Gli1* and *Foxf1* point to a common regulatory circuitry (Fig. 3B).

In contrast, constitutive activation of the Hh signaling pathway, caused for example by mutations in the tumor suppressor gene *PTCH1*, is known to result in the formation of different tumors, amongst them RMS, BCC and MB (1,9). Accordingly, we detected increased expression levels of Gli1 as a marker of Hh signaling activation in these tumors in the *Ptch1*$^{neo67/+}$ mouse model. In a previous study we have already

up-regulation of *Foxf1* in RMS of these mice by DNA microarrays and Northern blot analysis (26). Here, we not only confirmed the overexpression of *Foxf1* in RMS by means of semi-quantitative RT-PCR, but also found increased levels of *Foxf1* in MB and BCC compared to the respective non-neoplastic tissues. High expression levels of GLI1 and FOXF1 have independently been described in human RMS (34,44). However, the concomitant expression of *Foxf1* and *Gli1* in our study rather suggests a common role of Foxf1 in Hh signaling than being a marker for RMS. Notably, a prominent role of forkhead box transcription factors in Hh signaling and associated tumors has already been suggested by others. Sasaki and colleagues were the first to describe that Hh signaling, being essential for the activation of the floor plate in vertebrates, is mediated by the forkhead box transcription factor Foxa2 (45). During somitogenesis and development of pharyngeal endoderm and head mesenchyme, Shh signals are transduced by Foxd2 and Foxc2 (46,47), respectively. On the other hand, MB are known to express high levels of *FOXM1* (48). Similarly, in human BCC, the most prominent tumor type associated with Hh signaling, overexpression of *FOXE1* and *FOXM1* has been reported to be caused by binding of GLI proteins (49,50). However, Teh and colleagues did not find an expression of *FOXF1* or *FOXA2* in human BCC by screening for the most abundantly expressed forkhead box transcription factors with degenerated PCR primers for the conserved DNA-binding protein motif of forkhead box transcription factors. This suggests that Foxf1 might not be the major forkhead box transcription factor in the transduction process of aberrantly activated Hh signaling during tumorigenesis. Nevertheless, by demonstrating the co-localization of *Foxf1* and *Gli1* within tumor cells of RMS by *in situ* hybridization and the concomitant expression of both genes in *Ptch1*$^{neo67/neo67}$ embryos by semi-quantitative RT-PCR our results strongly suggest an important role for *Foxf1* in the transduction of Hh signals during the formation of *Ptch1*-associated tumors.

If Foxf1 is transcriptionally activated through Hh signaling in *Ptch1*-associated tumors, known target genes of Foxf1 such as *Bmi1* and *Notch2* (24,27) should also be up-regulated in these tumors. Previous findings indicating diminished expression levels of *Bmi1* and *Notch2* as a consequence of *Foxf1*-haploinsufficiency further strengthen this notion (27). In proportion to this assumption, we detected a strong expression of *Bmi1* and *Notch2* in *Ptch1*-associated RMS, MB and BCC compared to their respective non-neoplastic tissues. This is in line with another study reporting that overexpression of *BMI1* was also found to be overexpressed in human BCC (29). However, the gene *NOTCH2* was demonstrated to be overexpressed in MB (31,32). Notably, Bmi1 was recently shown to enhance cell proliferation and play a role in tumor cell survival (42,43), whereas the Notch signaling pathway plays a significant role in tumorigenesis (41). In our study we demonstrated that the up-regulation of *Bmi1* as well as of *Notch2* corresponds to the overexpression patterns of *Foxf1* and *Gli1*, pointing to *Bmi1* and *Notch2* as target genes of *Foxf1* (Fig. 3B).

Taken together, our data and recent work by others strongly suggest that *Foxf1* functions as a target gene of the Hh signaling pathway in *Ptch1*-associated tumors thereby integrating downstream targets such as *Notch2* and *Bmi1* into the Hh pathway.

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