Zfx Facilitates Tumorigenesis Caused by Activation of the Hedgehog Pathway

Colin J. Palmer1, Jose M. Galan-Caridad1, Stuart P. Weisberg1, Liang Lei2, Jose M. Esquilin3, Gist F. Croft4, Brandon Wainwright5, Peter Canoll2, David M. Owens2,6, and Boris Reizis1

Abstract

The Hedgehog (Hh) signaling pathway regulates normal development and cell proliferation in metazoan organisms, but its aberrant activation can promote tumorigenesis. Hh-induced tumors arise from various tissues and may be indolent or aggressive, as is the case with skin basal cell carcinoma (BCC) or cerebellar medulloblastoma, respectively. Little is known about common cell-intrinsic factors that control the development of such diverse Hh-dependent tumors. Transcription factor Zfx is required for the self-renewal of hematopoietic and embryonic stem cells, as well as for the propagation of acute myeloid and T-lymphoblastic leukemias. We report here that Zfx facilitates the development of experimental BCC and medulloblastoma in mice initiated by deletion of the Hh inhibitory receptor Ptc1. Simultaneous deletion of Zfx along with Ptc1 prevented BCC formation and delayed medulloblastoma development. In contrast, Zfx was dispensable for tumorigenesis in a mouse model of glioblastoma. We used genome-wide expression and chromatin-binding analysis in a human medulloblastoma cell line to characterize direct, evolutionarily conserved targets of Zfx, identifying Dis3L and Ube2j1 as two targets required for the growth of the human medulloblastoma cells. Our results establish Zfx as a common cell-intrinsic regulator of diverse Hh-induced tumors, with implications for the definition of new therapeutic targets in these malignancies. Cancer Res; 74(20); 1–11. ©2014 AACR.

Introduction

The Hedgehog (Hh) pathway controls cell proliferation and differentiation in response to a gradient of secreted Hh ligands (1–4). Hh proteins such as Sonic Hedgehog (Shh) bind the inhibitory receptor Patched1 (Ptc1) on the cell surface, thereby releasing Ptc1-mediated inhibition of seven-pass transmembrane protein Smoothened (Smo). Smo is an essential, non-redundant transducer of Hh signals whose activation leads to activation of GLI-type transcription factors, which in turn induce the expression of Hh target genes. These target genes include downstream transcription factors such as Mycn (5), along with core Hh pathway components including Ptc1 and GlI1. The Hh pathway controls multiple processes in normal development, including specification of motor neuron and interneuron pools in the ventral spinal cord (6, 7), expansion of granule neuron precursors (GNP) and foliation in the cerebellum (8–10), and hair follicle morphogenesis (11, 12). On the other hand, aberrant activation of the Hh pathway may induce malignant transformation of these same Hh-dependent cell types (13).

In particular, inactivating mutations in Ptc1 are present in approximately 90% of human sporadic basal cell carcinoma (BCC), a predominantly indolent tumor of the adult epidermis (14). Furthermore, Hh pathway activation has been associated with a distinct GNP-derived subtype of medulloblastoma, a highly aggressive childhood cerebellar tumor (15–17). Both medulloblastoma and BCC can be induced in mice by Hh pathway overactivation due to Ptc1 mutation (18, 19). The resulting tumors arise from the stem/progenitor compartments of the corresponding tissues, including GNP or earlier neuronal progenitors (20, 21) and hair follicle stem cells (22). Chemical inhibitors of the Hh pathway component Smo show promising results for treatment of Hh-dependent cancers such as BCC and medulloblastoma (23, 24). However, the acquisition of Smo mutations by medulloblastoma to escape targeted molecular therapy has been demonstrated (25). Furthermore, targeting of the core Hh pathway in pediatric medulloblastoma should be done with caution, to avoid adverse effects on Hh-dependent normal cerebellar development and skeletal growth (13, 23, 26). Identification of novel cell-intrinsic regulators of...
the Hh pathway-initiated cancers could suggest novel therapeutic targets in contexts where Hh inhibitors induce resistance or cause serious developmental side effects.

Zfx is a transcription factor that is highly conserved in vertebrates and contains a large acidic transcriptional activation domain and a C-terminal zinc finger domain (27). Zfx is encoded on the mammalian X chromosome and is expressed ubiquitously, yet acts in a cell- and tissue-specific fashion. Zfx is required for the self-renewal of pluripotent embryonic stem cells (ESC) and adult hematopoietic stem cells (HSC; refs. 28, 29), as well as for B-lymphocyte development (30). On the other hand, Zfx is dispensable for ESC differentiation and for the growth of multiple cell types such as myeloid progenitors and embryonic fibroblasts. The role of Zfx in cancer development in vivo remains incompletely understood. A recent study using a transposon-based screen in a murine MYC-dependent liver cancer model reported Zfx as a potential tumor suppressor gene (31). In contrast, several studies have reported that Zfx knockdown in cancer cell lines in vitro impaired their growth (32–34). Our laboratory recently reported that Zfx is necessary for the initiation and maintenance of two disparate leukemias in vivo, acute myelogenous leukemia (AML), and T-cell acute lymphoblastic leukemia (T-ALL; ref. 35).

Our analysis of Zfx target genes in stem cells revealed that Zfx directly activates the expression of Smo (J.M. Galan-Caridad; unpublished observations). Together with the ubiquitous expression of Zfx and its cell-intrinsic requirement in multiple cancer types, these data raised the possibility that Zfx might facilitate Hh-driven malignancy. We therefore explored the role of Zfx in BCC and medulloblastoma, two tumors caused by Hh pathway activation in two different tissues. We now report that Zfx is required for the formation of BCC and optimal progression of medulloblastoma in vivo. The analysis of Zfx genomic targets revealed a conserved set of genes, some of which were required for growth of a human medulloblastoma cell line in vitro. Collectively, these results identify Zfx as a common cell-intrinsic regulator of distinct Hh-induced tumors such as BCC and medulloblastoma.

Materials and Methods

Supplementary Materials and Methods and associated references may be found in the Supplementary Data provided online.

Animals

Zfxlox (28), Ptenlox (36), and Pchlox (37) conditional mice have been previously described. The hGFP-Cre brain-wide deleter strain (38) and Rosa26-Smolox mice bearing a Cre-inducible, constitutively active allele of Hh signal transducer Smothened (39) were obtained from the Jackson Laboratory. Tamoxifen-inducible Rosa26-CreER mice were generously provided by Dr. Thomas Ludwig, Columbia University (New York, NY).

Pchlox and Zfxlox mice were crossed to generate doubly-conditional Pchlox Zfxlox mice. To model Hh pathway-dependent BCC formation in the skin, Pchlox and Pchlox Zfxlox mice were bred with Rosa26-CreER mice. To effect tamoxifen-induced deletion of Pch1 with or without concomitant Zfx deletion in the skin, 7- to 9-week-old mice were shaved across their lower dorsal skin (from above the tail to the posterior border of the ribcage) and treated topically for five consecutive days with 1 mg tamoxifen (Sigma) in 100 μL acetone. Rosa26-Smolox and Rosa26-Smolox Zfxlox mice were analogously generated, crossed with Rosa26-CreER deleter mice, and treated topically with tamoxifen to induce BCC.

Induction of Hh pathway-dependent medulloblastoma in mice via conditional deletion of Pch1 using hGFP-Cre has been previously described (21). Pchlox and Pchlox Zfxlox mice were bred to hGFP-Cre+ Pchlox mice to generate hGFP-Cre+ Pchlox Zfxlox and hGFP-Cre+ Pchlox Zfxlox mice that delete the inhibitory Hh receptor Pch1 with or without concomitant deletion of Zfx. Singly- and doubly-conditional pups, along with Cre– or Pch1 heterozygote controls, were followed for survival or were euthanized at postnatal time points for analysis by IHC.

Glioblastoma models were induced by stereotactic injection into brain of young Ptenlox and Ptenlox Zfxlox adult mice of retrovirus coding for PDGF-IRESCre, as previously described (40). All experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of Columbia University.

Cell culture

The DAOY human medulloblastoma cell line was obtained from the ATCC and cultured in accordance with ATCC recommended culture conditions.

Lentiviral shRNA knockdown

Lentiviral constructs containing Zfx-specific (H2, H3, H4) and nonspecific, scrambled (SCR) shRNAs in the pLKO.1 backbone were purchased from Open Biosystems. For each lentiviral construct, triplicate cultures of DAOY cells were transduced with ultracentrifuge-concentrated lentiviral supernatant for 16 to 24 hours. Puromycin-selected cells were collected for gene expression analysis and Western blot analysis 4 or 6 days after transduction.

Lentiviral particles encoding shRNAs targeting DIS3L and UBE2J1 mRNA or control shRNAs targeting non-mammalian sequence (NonM) were obtained from the MISSION shRNA library (Sigma-Aldrich). For each lentiviral construct (3–4 per gene), triplicate cultures of DAOY cells were transduced for 16 to 24 hours. Puromycin-selected cells were collected for gene expression analysis 5 days after transduction or were followed subsequently for growth curves.

Expression analysis, microarray, and chromatin immunoprecipitation sequencing

qPCR was performed as described (28). RT-PCR results were calculated by ΔΔCt method and normalized for a housekeeping gene (Actb or Gapdh).

RNA for microarray analysis of DAOY human medulloblastoma cells after ZFX knockdown was obtained 4 days after lentiviral transduction from one culture replicate for each of the ZFX-targeting shRNA constructs (H2, H3, H4) and non-targeting “scrambled” control (SCR). Labeled cDNA was
analyzed on Affymetrix Mouse Gene 1.0 ST arrays. Pattern-matching genes following the expression profile of Zfx were identified using NIA Array (41). Sonication-sheared chromatin from DAOY human medulloblastoma cells was generated and isolated using Covaris transChIP High Cell Chromatin Shearing Kit with SDS Buffer and S2 Sonicator. ZFX chromatin immunoprecipitation (ChIP) was performed using rabbit polyclonal antibody (28) and Protein A Dynabeads (invitrogen), and unprobed sheared chromatin was used as a control (Input). Library construction and sequencing were performed by the Yale Center for Genome Analysis. For comparison with murine ESCs, previously reported Zfx ChIP-seq data were downloaded from the NCBI Sequence Read Archive (SRX000552; ref. 42). Sequencing reads were aligned to the human genome (UCSC hg19) using the short read aligner, Bowtie (version 2.0.0; ref. 43). Significant ZFX-binding enrichment peaks were picked using MACS (1.4.0; ref. 44) and were visualized using DNA nexus. Peak distance to the nearest TSS was determined using PeakAnnotator (version 1.4; ref. 45). Unbiased motif analysis was performed on MACS-selected ChIP-seq peaks using the ChIPseeker FIRE module of ChIPseeker (2.0; ref. 46).

**IHC**

Paraffin sections (5 μm) from tamoxifen-treated skin were stained with hematoxylin and eosin (H&E), or were immunostained with anti-Zfx (1:500) or with anti-Sox9 (Santa Cruz Biotechnology, 1:500) antibodies, incubated with biotinylated anti-rabbit secondary antibody (Vector Laboratories), visualized with ABC and DAB Peroxidase Substrate Kits (Vector Laboratories), and counterstained with hematoxylin.

Sagittal paraffin sections (5 μm) from cerebella of P7 and P14 pups were cut at four levels equally spaced across half the cerebellum and subsequently stained with H&E, or immunostained with anti-Zfx antibody as described above.

Micrographs of histologic sections were recorded using an AxioImager.M2 microscope with attached AxioCam MRc camera (Zeiss). Measurements of cross-section area in cerebellar paraffin sections were made using Axiovision 4.8.2.

**Statistical analysis**

H&E-stained sections of BCC were scored by a pathologist who was blinded to the identity of the samples. Specimens were scored for the percentage of skin area containing BCC lesions (−, 0%; +, 1%–33%; ++, 34%–66%; ++++, 67%–100%), and the scores were analyzed using Fisher exact test extended to a 2 x 4 contingency table. Cerebellar cross-sectional areas were compared with unpaired, two-tailed Student t tests. Survival curves were analyzed with the log-rank test.

**Results**

The loss of Zfx prevents Hh-induced BCC formation

We first asked whether Zfx was required for BCC formation after the Hh pathway hyperactivation in vivo. To model Hh-induced BCC, mice with a conditional null allele of Pch1 (Pch1loxlox) with or without conditional null allele of Zfx (Zfxlox/lox) were crossed with the Rosa26-CreERT2 (R26-CreER) strain expressing tamoxifen-inducible Cre recombinase. Male mice carrying the Y chromosome and Zfxlox/lox allele on their single X chromosome (Zfxfl/+y) were targetted to Zfx. Topical application of tamoxifen enables deletion of Pch1 alone in R26-CreER Pch1loxlox (Pch1) mice or concomitant deletion of Pch1 and Zfx in R26-CreER Pch1loxlox Zfxlox/lox (Pch1-Zfx) mice.

To test the consequences of Zfx loss on Hh-induced BCC formation, tamoxifen was applied to the lower dorsal skin of Pch1, Pch1-Zfx, or control CreER-negative (Ctrl) mice for 5 consecutive days. Successful Cre-mediated deletion and the loss of Zfx protein from the basal layer of epidermis and hair follicle bulge of Pch1-Zfx skin were confirmed by immunostaining of skin sections isolated 3 days after tamoxifen treatment (Fig. 1). Within 8 to 9 weeks after treatment, Pch1 mice lost hair and developed gross induration in the treated skin patch, whereas Pch1-Zfx mice retained hair and intact skin (Fig. 2A). Histologic analysis of Pch1 skin at 8 week post-tamoxifen revealed frank BCC with massive skin infiltration and multiple dermal cysts in lieu of the intact hair follicles (Fig. 2B). These BCC lesions were positive for Zfx (Fig. 2B) and Sox9 (Supplementary Fig. S1), a marker of the hair follicle stem cell compartment that is also a marker of BCC (47). In contrast, Pch1-Zfx skin showed occasional aberrant hair follicles but no overt BCC. Importantly, the occasional aberrant follicles in Pch1-Zfx skin were composed of Zfx-expressing cells (Fig. 2B), suggesting that they arose exclusively from cells that escaped Zfx deletion. The analysis of dorsal skin sections confirmed that the extent of BCC outgrowth was significantly reduced in Pch1-Zfx mice, with most Pch1-Zfx sections (11/13) receiving the lowest score above the wild-type baseline (Fig. 2C). Thus, Zfx is required for BCC formation caused by Pch1 loss in the epidermis.

We also tested the consequences of Zfx loss in an alternative model of the Hh pathway-dependent BCC initiated by Cre-mediated induction of the Rosa26-SmoM2loxstoplox (R26-SmoM2) transgene, which expresses a constitutively active version of the Hh pathway signal transducer Smo (39). We treated shaved lower dorsal skin of R26-SmoM2+ Zfxlox/y R26-CreER+ (SmoM2-Zfx) and R26-SmoM2+ R26-CreER− (SmoM2) mice with topical tamoxifen for 5 consecutive days, to induce SmoM2 expression and BCC formation, with or without Zfx codelletion. Although less severe than the phenotype observed in Pch1 mice, SmoM2 mice exhibited hair loss and gross induration in the treated skin patch by 8 to 10 weeks post-tamoxifen, whereas treated SmoM2-Zfx dorsal skin patches retained hair follicles and largely intact skin (Supplementary Fig. S2A). Frank BCC observable in histologic sections from SmoM2 dorsal skin was absent in sections from SmoM2-Zfx dorsal skin (Supplementary Fig. S2B). As with Pch1-Zfx mice in Fig. 2B, the residual hair follicle cells in tamoxifen-treated SmoM2-Zfx mice expressed Zfx, revealing a strong selection for occasional non-deleter cells (Supplementary Fig. S2C). SmoM2-Zfx mice also showed a significant reduction in the extent of skin infiltration by BCC lesions (Supplementary Fig. S2D). Thus, Zfx is also required for formation of an alternative BCC model initiated by expression of constitutively active Smo.
The loss of Zfx delays Hh-induced medulloblastoma development

To test whether Zfx is also required for the development of Hh-induced medulloblastoma, we used brain-specific Ptch1 deletion mediated by the hGFAP-Cre deleter strain (38). The hGFAP-Cre+ Ptch1^{flox/flox} mice show early expansion of the GNP-containing external granular layer (EGL) of the cerebellum, develop medulloblastoma shortly after birth, and succumb to it by 4 weeks of age (21). To delete Ptch1 alone or concomitantly with Zfx, we generated hGFAP-Cre+ Ptch1^{flox/flox} (hGFAP-Ptch1) and hGFAP-Cre+ Ptch1^{flox/flox} Zfx^{lox/y} (hGFAP-Ptch1-Zfx) mice, respectively.

All hGFAP-Ptch1 pups at P7 and P14 showed disrupted cerebellar architecture with expanded EGL and minimal foliation (Fig. 3A). hGFAP-Ptch1-Zfx pups also showed EGL expansion composed of cells that lacked Zfx expression (Fig. 3B). Thus, in contrast with the inducible BCC model, Zfx deletion did not preclude transformation or favor the emergence of non-deleter cells. However, hGFAP-Ptch1-Zfx pups showed better preservation of cerebellar architecture and reduced EGL expansion (Fig. 3A), as confirmed by significantly smaller cross-sectional EGL areas in P7 cerebella (Fig. 3C). This was not due to developmental defects of Zfx deletion because hGFAP-Cre+ Zfx^{lox/y} mice exhibit normal cerebella throughout life (Supplementary Fig. S3). Nearly all hGFAP-Ptch1-Zfx mice died by 25 days, which corresponds to 4 to 6 days postweaning and suggests a failure to thrive independent of the dam (Fig. 3D). The majority of hGFAP-Ptch1-Zfx mice (~70%) also died around the same time, likely due to the similar inability to survive weaning (Fig. 3D). However, approximately 30% of hGFAP-Ptch1-Zfx mice survived past weaning and approximately 20% lived for >50 days, resulting in a significant (P < 0.01) improvement in survival. All hGFAP-Ptch1-Zfx mice developed and eventually succumbed to tumors, suggesting that the loss of Zfx does not prevent medulloblastoma initiation but impairs tumor propagation.

The observed effects of Zfx loss in medulloblastoma development could reflect a specific role of Zfx in Hh-driven medulloblastoma, or a more general role in brain tumorigenesis. Genome-wide expression data on murine medulloblastoma subtypes (48) show a prominent and specific overexpression of Zfx in Hh-driven compared with Myc-driven medulloblastoma or to normal neural stem cells (Supplementary Fig. S4A). To directly test the role of Zfx in other types of brain tumors, we used a model of glioblastoma in adult mice. We performed retroviral delivery of platelet-derived growth factor (PDGF) and Cre recombinase into the brains of mice with conditional null Pten allele (Pten^{flox/flox}), which results in aggressive Pten-deficient glioblastoma of the proneural subtype (40). In contrast with the medulloblastoma model,

Figure 1. Topical tamoxifen treatment successfully ablates Zfx in the skin. Ptch1^{flox/flox} Zfx^+/y R26-CreER^T (Ptch1) and Ptch1^{flox/flox} Zfx^{lox/y} R26-CreER^T (Ptch1-Zfx) mice, along with Ptch1^{flox/flox} R26-CreER^T controls (Ctrl), were treated topically with tamoxifen to induce deletion of Ptch1 alone or of both Ptch1 and Zfx in the skin. Shown is Zfx expression in the skin 3 days after tamoxifen treatment. Representative micrographs of IHC staining for Zfx in sections from treated dorsal skin are shown. Scale bars, 100 μm (top) and 50 μm (bottom). Epidermis (E), dermis (D), and hair follicle (F) are labeled, and the boundary between epidermis/follicles and dermis is marked (dotted line; top). Zoomed insets show decreased nuclear staining for Zfx in the hair follicle (bottom).
concomitant deletion of Zfx in Pten^floflo^Zfx^flofox/Zfx^flofoox^y mice significantly accelerated rather than delayed mortality (Fig. 4A). This was not due to inefficient deletion of Zfx, as the resulting tumors were completely Zfx negative (Fig. 4B). Thus, the role of Zfx in Hh-driven medulloblastoma formation does not reflect its general requirement for tumorigenesis in the brain.

**Zfx knockdown impairs growth in a human medulloblastoma cell line in vitro**

We investigated whether ZFX was also required in human medulloblastoma cells in vitro, using a system for ZFX knockdown with shRNAs recently validated in human ESC (29). The human medulloblastoma cell line DAOY was transduced with lentiviral vectors encoding ZFX-specific shRNAs (H2, H3, H4) or a control scrambled (SCR) shRNA. Two ZFX-specific shRNAs (H2, H3) strongly reduced Zfx protein (Fig. 5A) and transcript (Fig. 5B), whereas the third (H4) caused a more moderate reduction (Fig. 5B). These shRNAs impaired the growth of DAOY cells proportionately to the degree of ZFX knockdown (Fig. 5C), suggesting that ZFX is required for optimal growth of human medulloblastoma in vitro. Notably, genome-wide expression data on human medulloblastoma samples divided into molecular subtypes (49) reveal a significant enrichment of ZFX in the SHH subtype compared with two common Hh-independent subtypes (Supplementary Fig. S4B). Unlike murine Zfx, human ZFX escapes X inactivation, and its reduced dosage in male somatic cells is compensated by the Y-chromosomal gametolog ZFY. Within male medulloblastoma samples, ZFY was significantly enriched in the SHH subtype (Supplementary Fig. S4B). Collectively, these data suggest that the role of Zfx in Hh-driven medulloblastoma might be conserved between mice and humans.

**Identifying novel functional targets of ZFX in human medulloblastoma cells in vitro**

We hypothesized that an evolutionarily conserved set of target genes may contribute to the role of Zfx in Hh-induced tumors. To identify potential functional targets of Zfx, we first performed microarray-based gene expression analysis of the DAOY cell line at an early time point after transduction with lentiviral particles bearing ZFX-targeting shRNAs. We searched for genes whose RNA expression levels behaved similarly to that of ZFX, exhibiting strong reduction by qPCR with shRNAs H2 and H3 and more moderate reduction with shRNA H4. A set of 163 genes whose expression level patterns matched that of ZFX in transduced DAOY cells has been identified (Fig. 6A and Supplementary Dataset S1).

We then compared this gene set with direct binding targets of Zfx in mouse and human. Direct targets of Zfx in murine ESC have been previously identified via chromatin precipitation followed by massively parallel sequencing (ChIP-seq; ref. 42).
To identify direct targets of ZFX in human medulloblastoma, we performed anti-ZFX ChIP-seq on the human DAOY cell line. Then, we compared the list of 163 pattern-matching genes with overlapping ZFX direct binding targets that were within 1 kb of transcriptional start sites (TSS) of their nearest gene and were conserved ChIP-seq targets in both mouse ESC and human DAOY medulloblastoma cells (Supplementary Dataset S2). Of 163 genes identified by pattern-matching analysis of microarray gene expression data, 68 genes (42%) were bound by ZFX in DAOY cells, and 30 genes (18%) were bound in both DAOY and murine ESC (Fig. 6B and Supplementary Dataset S2).

Out of 30 conserved direct targets of ZFX, we have selected six genes (DIS3L, ETS2, FZD6, LRRC41, TNFAIP6, and UBE2J1) based on their likely involvement in cell growth control, and tested them by shRNA-mediated knockdown in DAOY cells (data not shown). The knockdown of four genes (ETS2, FZD6, LRRC41, and TNFAIP6) by multiple shRNAs yielded inconsistent effects on the growth of DAOY cells, whereas the knockdown of DIS3L and UBE2J1 consistently inhibited cell growth. DIS3L encodes a cytoplasmic RNA exonuclease with a possible role in mRNA degradation (50, 51). Ube2j1 encodes an E2 ubiquitin-conjugating enzyme that is thought to participate in the endoplasmic reticulum-associated degradation of misfolded proteins (52). ChIP-seq analysis of DAOY cells showed ZFX binding near the TSS of both genes in murine ESC and human DAOY cells (Fig. 6C and D). The expression of Dis3l (formerly known as AV340375) strongly depends on Zfx in all examined murine cells including HSCs and ESCs (Supplementary Fig. S5; ref. 28) and activated B lymphocytes (30). The expression of Ube2j1 is also Zfx dependent in HSCs (28) and B lymphocytes (30), although not in ESCs (Supplementary Fig. S5B; ref. 28). These data suggested DIS3L and UBE2J1 as candidate genes with potential functional relevance downstream of Zfx in both mouse and human.
Knockdown of ZFX targets Dis3L and Ube2j1 impairs human medulloblastoma growth in vitro

We tested whether the expression levels of Dis3L and Ube2j1 were affected in the context of BCC and medulloblastoma following Zfx loss. We confirmed that Dis3L and Ube2j1 expression levels were decreased after shRNA-mediated ZFX knockdown in DAOY cells (Fig. 7A). Moreover, both transcripts were reduced approximately 2-fold after tamoxifen-induced Zfx codeletion in epidermis and hair follicles of treated dorsal skin from the Ptch1-dependent BCC model in vivo (Fig. 7B).

Together with the ChIP-seq binding data, these data suggest that Zfx can directly regulate Dis3L and Ube2j1 expression in the contexts of BCC and medulloblastoma models in vivo and in vitro.

To determine whether Dis3L or Ube2j1 themselves are required for the growth of a human medulloblastoma cell line in vitro, we transduced DAOY cells with lentiviral vectors encoding several shRNAs targeting each gene. Compared with untreated cells and a control shRNA targeting non-mammalian sequence (NonM), all tested shRNAs against Dis3L (four shRNAs) or Ube2j1 (three shRNAs) impaired the growth of DAOY cells (Fig. 7C). Successful knockdown of the respective targeted genes by all shRNAs was confirmed by qPCR (Fig. 7D). Thus, Dis3L and Ube2j1 represent conserved direct targets of ZFX that are required for the optimal growth of DAOY human medulloblastoma cells in vitro.

Discussion

Our results demonstrate that Zfx expression is required for the development of two Hh pathway-dependent model tumors in vivo. In both BCC and medulloblastoma, Zfx seems to act in a cell-intrinsic manner to facilitate tumor propagation, although a role in the initiation of BCC could not be ruled out. This is in agreement with a recently reported cell-intrinsic role of Zfx in

Figure 5. Zfx knockdown impairs cell growth in a human medulloblastoma cell line in vitro. The human medulloblastoma cell line DAOY was transduced in vitro with lentiviruses encoding shRNAs targeting ZFX transcript (H2, H3, H4) or a scrambled control shRNA (SCR). Transduced cells were selected for by growth in puromycin-containing medium. A, Western blot analysis for ZFX and tubulin control in DAOY cells transduced with control (SCR) or ZFX-targeting (H2, H3, H4) lentivirus. B, expression of ZFX in DAOY cells after shRNA knockdown. Shown are ZFX expression levels 4 days after transduction, as determined by qPCR (mean ± SD of triplicate parallel cultures; representative of three independent experiments). C, growth curves for DAOY cells transduced with lentivirus expressing control (SCR) or ZFX-targeting (H2, H3, H4) shRNA (mean ± SD of triplicate parallel cultures, representative of three independent experiments).
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Figure 6. Identification of conserved direct targets of ZFX in human medulloblastoma cells. A, downregulated genes (n=163) identified in pattern matching analysis of Affymetrix Gene ST 1.0 array data from DAOY cells after shRNA knockdown. Full gene list is presented in Supplementary Dataset S1. B, conserved ZFX transcriptional targets. Shown are numbers of pattern-matching genes from ZFX KD microarray in A that had enrichment peaks <1kb from TSS in ZFX ChIP-seq from DAOY cells, from murine embryonic stem cells (mESC), from both cell types (Overlap), or from neither cell type (N/A). DAOY, mESC, and Overlap gene lists are presented in full in Supplementary Dataset S2. C, Zfx binding to Dis3L in murine and human ChIP-seq. Shown are sequencing-read enrichment peaks near TSS of Dis3L in anti-Zfx ChIP-seq from murine ESC (left; ref. 42), and in anti-ZFX ChIP-seq and sheared nuclear lysate control (Input) from DAOY human medulloblastoma cells (right). D, Zfx binding to UBE2J1 in murine and human ChIP-seq. Shown are sequencing-read enrichment peaks near TSS of Ube2j1 in anti-Zfx ChIP-seq from murine ESC (left; ref. 42), and in anti-ZFX ChIP-seq and sheared nuclear lysate control (Input) from DAOY human medulloblastoma cells (right).

the propagation of two molecularly distinct leukemias, AML and T-ALL (35). In contrast, Zfx appears dispensable for progression of a glioblastoma model in vivo, suggesting that a requirement for Zfx is not generalizable across all tumor models. ZFX expression is enriched in Hh-driven medulloblastoma compared with other medulloblastoma subtypes in both human patients and animal models (Supplementary Fig. S4), suggesting a preferential role for Zfx in this tumor subtype. It is possible that Zfx may act downstream of Hh signaling, for example, via transcriptional induction by or cooperation with the GLI proteins. A more likely possibility is that Zfx may activate the expression of key Hh pathway components such as Smo. Indeed, Zfx directly binds to the promoter of Smo in both murine ESCs (42) and in DAOY cells (Supplementary Dataset S2) and facilitates Smo expression in hematopoietic progenitors and differentiating ESCs (our unpublished data). However, the requirement for Zfx in SmoM2-induced BCC argues against Zfx-induced Smo expression as a sole mechanism. Most likely, Zfx exerts its role through a combination of targets specific for Hh-driven tumors as well as targets common for multiple tumor types, as discussed below.

Zfx is required in several cell types that undergo self-renewal, such as murine and human ESC and murine HSC (28, 29). At the same time, Zfx is dispensable in multiple cell types in which proliferation is followed by differentiation (e.g., myeloid progenitors). Thus, Zfx may control a specific genetic program that mediates prolonged self-renewal and therefore may become essential in malignant cells that acquire abnormal self-renewal capacity. In this context, Zfx would represent a clear example of the "nononcogene addiction" of tumors to an otherwise normal and largely dispensable endogenous gene (53).

We describe a broad Zfx-dependent genetic program that may maintain the self-renewal of multiple normal and malignant cells, including Hh-dependent tumors. We have focused on two functional components of this Zfx-dependent program, Dis3l and Ube2j1, identified as direct transcriptional targets of Zfx in both mouse and human. The knockdown of DIS3L and UBE2J1 demonstrated that each is required for optimal growth of human medulloblastoma cells in vitro (Fig. 7). These genes have not been previously implicated in cancer, although the DIS3L homolog DIS3L2 has been implicated in Perlman overgrowth syndrome and an associated kidney tumor (Wilms' tumor; ref. 54), supporting consideration of an RNA exonuclease for a possible role in cancer. The functions of DIS3L and UBE2J1 in cytoplasmic RNA exonuclease activity (50, 51) and ER-associated protein degradation (52), respectively, suggest that disregulation of either gene could potentially impact stress pathways, a hallmark of the phenomenon of nononcogene addiction in tumors (53). Although any requirements for DIS3L, UBE2J1, or other conserved direct targets of ZFX remain to be established in Hh-induced and other tumor models in vivo, they nevertheless highlight novel candidates for cell-intrinsic regulation and molecular targeting of Hh-dependent cancers.

We report here that the transcription factor Zfx is required for the development of Hh-induced BCC and medulloblastoma in vivo, two cancers that are highly distinct in their tissue of origin, growth rate, and clinical prognosis. Our results suggest that this effect may be partly due to direct regulation of a Zfx-dependent genetic program including conserved target genes like DIS3L and UBE2J1. A more complete understanding of the relevant functional targets of Zfx in the context of Hh-dependent medulloblastoma and BCC in vivo could facilitate molecular targeting of these malignancies. Identification of the precise downstream targets with functional relevance in a Zfx-defined cell-intrinsic program shared by these two cancers could yield potentially druggable molecules for treatment of both BCC and the Hh-dependent subtype of medulloblastoma.
Future targeting of such molecules, as alternatives to targeting core components of the Hh pathway like SMO, offers the promise of molecular therapies specific to Hh-dependent tumors that can help to bypass resistance to SMO inhibitors or spare normal Hh-dependent development in infants or children with Hh-dependent medulloblastoma.

Disclosure of Potential Conflicts of Interest
J.M. Galan-Caridad is engagement manager in IMS Consulting Group. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions
Conception and design: C.J. Palmer, J.M. Galan-Caridad, B. Reizis
Development of methodology: J.M. Galan-Caridad, L. Lei, G.F. Croft, B. Wainwright, D.M. Owens, B. Reizis
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.J. Palmer, S.P. Weisberg, L. Lei, J.M. Esquilin, B. Wainwright, P. Canoll
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.J. Palmer, J.M. Galan-Caridad, S.P. Weisberg, L. Lei, D.M. Owens, B. Reizis
Writing, review, and/or revision of the manuscript: C.J. Palmer, L. Lei, B. Wainwright, B. Reizis
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.P. Weisberg, L. Lei
Study supervision: D.M. Owens, B. Reizis

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