FBW7 suppression leads to SOX9 stabilization and increased malignancy in medulloblastoma

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Abstract

SOX9 is a master transcription factor that regulates development and stem cell programs. However, its potential oncogenic activity and regulatory mechanisms that control SOX9 protein stability are poorly understood. Here, we show that SOX9 is a substrate of FBW7, a tumor suppressor, and a SCF (SKP1/CUL1/F-box)-type ubiquitin ligase. FBW7 recognizes a conserved degron surrounding threonine 236 (T236) in SOX9 that is phosphorylated by GSK3 kinase and consequently degraded by SCF FBW7. Failure to degrade SOX9 promotes migration, metastasis, and treatment resistance in medulloblastoma, one of the most common childhood brain tumors. FBW7 is either mutated or downregulated in medulloblastoma, and in cases where FBW7 mRNA levels are low, SOX9 protein is significantly elevated and this phenotype is associated with metastasis at diagnosis and poor patient outcome. Transcriptional profiling of medulloblastoma cells expressing a degradation-resistant SOX9 mutant reveals activation of pro-metastatic genes and genes linked to cisplatin resistance. Finally, we show that pharmacological inhibition of PI3K/AKT/mTOR pathway activity destabilizes SOX9 in a GSK3/FBW7-dependent manner, rendering medulloblastoma cells sensitive to cytostatic treatment.

Keywords cisplatin; FBW7; medulloblastoma; metastasis; SOX9

Introduction

Medulloblastoma is one of the most common malignant childhood brain tumors with a survival rate of 70% following aggressive surgery, radiation, and chemotherapy (Gottardo & Gajjar, 2006). Survivors of the disease often demonstrate severe side effects from radiotherapy with neurological sequelae or psychological problems. New treatment options are needed not only to cure high-risk patients but also to alleviate therapy-induced side effects. Among the promising strategies employed to address these issues are the continuous refinement of molecular subtyping criteria and the development of genetically driven medulloblastoma models, which together are used as platforms to dissect the molecular biology and identify mechanisms of response and resistance based on pathway activity status. To date, medulloblastoma can be classified into four distinct molecular subgroups: WNT, SHH, Group 3 and Group 4 (Taylor et al, 2012). This categorization arguably provides a reliable foundation for predicting patient prognosis and outcome, as well as development of individualized targeted treatments. Nonetheless, it is also increasingly evident that molecular heterogeneity within a defined molecular subgroup of medulloblastoma accounts for variability in the efficacy of targeted treatments (as exemplified in Kool et al, 2014; Morrissy et al, 2016).

Our prior expression profiling analysis of human medulloblastoma specimens revealed that SOX9 (sex-determining region Y (SRY)-box 9) is commonly elevated in WNT and SHH medulloblastoma (Swartling et al, 2012). SOX9 is a member of the high-mobility group (HMG)-box class of transcription factors and controls establishment of broad transcriptional programs regulating stem cell properties, differentiation, proliferation, and survival.

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(Adam et al., 2015; Larsimont et al., 2015). In embryonal development, SOX9 transcriptional activity plays a crucial role in regulating the development of all germ layers (reviewed in Jo et al., 2014), including the maintenance and differentiation of neural stem cells in the developing brain (Stolt et al., 2003; Scott et al., 2010). Furthermore, both phosphorylation (by protein kinases A and G at S64 and S181) and SUMOylation of SOX9 protein have been reported to positively influence SOX9’s transactivation in chondrocytes (Huang et al., 2000) and in neural crest development (Taylor & Labonne, 2005). Deregulation of SOX9, including its overexpression and/or cooperation with other oncoproteins, contributes to evolution of central cancer hallmarks in various tumor types (Matheu et al., 2012). For instance, SOX9 has been reported to collaborate with the transcription factor SNAI2 (SLUG) to promote breast cancer metastasis through epithelial-to-mesenchymal (EMT) reprogramming (Guo et al., 2012). In another case, SOX9 enhances KRAS-driven pancreatic acinar malignant transformation (Kopp et al., 2012), possibly through SOX9-mediated protection of GLI1 from β-TrCP-mediated protein degradation (Deng et al., 2015). In sonic hedgehog (SHH)-driven basal cell carcinogenesis, SOX9 was further shown to be a critical downstream effector of WNT/β-catenin signaling for maintenance of the tumor-initiating cell population (Larsimont et al., 2015).

In medulloblastoma, our previous work demonstrated that SOX9 cooperates with the MYCN oncogene to promote tumor formation and penetrance (Swartling et al., 2012). While there is an abundant interest for SOX9 in initiating and driving oncogenicity of various malignancies, comparatively little is known regarding how SOX9 activity is regulated at the post-translational level. Work from de Crombrugghe’s laboratory provided evidence for proteasomal-dependent degradation of SOX9 (Akiyama et al., 2005) by the E6-AP/UBE3A HECT ubiquitin ligase in chondrocytes (Hattori et al., 2013). In cancer cells, we previously showed that activation of protein kinase G II (PKGII) phosphorylates and downregulates SOX9 protein leading to cell cycle arrest and increased neuronal differentiation of glioma cells (Swartling et al., 2009).

FBW7, a member of the WD40-repeat-containing F-box protein family, functions as a substrate recognition component of a multi-subunit ubiquitin ligase known as SCF (SKP1/CUL1/F-box) (Welcker & Clurman, 2008). Located on chromosome 4q32, the FBW7 gene is alternatively spliced into three isoforms, α (nucleus), β (cytoplasmic), and γ (enriched in the nucleolus) (Welcker & Clurman, 2008). FBW7 is best known for its function in regulating the stability of numerous oncoproteins including cyclin E, MYC, JUN, and NOTCH1, among others (Davis et al., 2014). FBW7 binds substrates through interaction of key residues located on the surface of its β-propeller, formed by its WD40 repeat domains, and a short motif in the substrate known as the Cdc4 phosphodegron (CPD), commonly phosphorylated by glycogen synthase kinase 3 (GSK3) or other kinases on serine (S) and/or threonine residues (T) (CPD consensus: S/TPPX/T/E/D) (Orlicky et al., 2003). In line with its role as a master oncoprotein suppressor, FBW7 inactivation in cancers by genetic deletion, loss of expression, or somatic mutations is thought to directly contribute to tumor development and progression (Tan et al., 2008; Wang et al., 2012b). Despite the indisputable function of FBW7 in carcinogenesis, the full set of target substrates remains to be discovered and knowledge of how FBW7 status influences and contributes to the progression of specific malignancies is limited. This has also hampered efforts to target the downstream oncogenic effectors in FBW7-inactivated tumors.

Here, we investigate the post-translational regulation of SOX9 in medulloblastoma and identify SOX9 as a novel FBW7 substrate for ubiquitylation and proteasomal degradation in response to phosphorylation by GSK3 kinase. Degradation of SOX9 through the GSK3/ FBW7 pathway likely represents an important mechanism for regulation of SOX9 activity in medulloblastoma as increased SOX9 protein level promotes migration, metastasis, and treatment resistance. FBW7 is frequently mutated in SHH medulloblastoma tumors and transcriptionally downregulated across all other patient subgroups. Accordingly, we observe a strong relationship between FBW7 loss-of-function and increased SOX9 protein levels in all medulloblastoma subgroups. Transcriptional profiling of medulloblastoma cells with stabilized SOX9 revealed differential expression of genes promoting metastasis through epithelial-to-mesenchymal (EMT) molecular reprogramming and genes directly associated with cisplatin resistance. Strikingly, our results provide evidence that targeting the PI3K/AKT/mTOR pathway, which correlates with poor prognosis in medulloblastoma (Kool et al., 2014), destabilizes SOX9 protein in a GSK3/FBW7-dependent manner thus further sensitizing medulloblastoma cells to cisplatin therapy.

Results

SOX9 interacts with FBW7α through a conserved degron motif phosphorylated by GSK3

To systematically identify new FBW7 substrates, we previously performed quantitative mass spectrometry using FBW7 knockout (KO) HCT116 cells combined with directed searches for proteins with FBW7 phosphodegron (CPD) motifs (Arabi et al., 2012). Enrichment of SOX9 protein in the nuclear fraction of the HCT116 FBW7-KO cells (Fig EV1A) and the presence of a canonical interaction motif (S/TPPX/T) at position threonine 236 (T236) (Fig 1A) indicated that SOX9 could be a substrate for FBW7, and most likely the alpha isoform that resides in the nucleus. Analysis of the SOX9 protein sequence and its evolutionary conservation across species revealed that the amino acid motif spanning T236 to T240 resembled the high-affinity consensus CPD found in other established FBW7α substrates (e.g., cyclin E, MYC, and JUN) (Figs 1A and EV1B).

To examine whether FBW7α interacts with SOX9 and to determine which amino acids may participate in the binding, we mutated T236, T240, or both T236/240 in SOX9 to alanine and assessed FBW7α binding. Whereas wild-type (WT) SOX9 immunoprecipitated with FBW7α, all three SOX9 mutants (T236A, T240A, and T236/240A) failed to bind FBW7α (Figs 1B and EV1C). As FBW7α interacts with phosphorylated substrates, we next analyzed whether phosphorylation of the SOX9 CPD sequence is required for the interaction. Synthetic peptides comprising the SOX9 motif surrounding residues T236 and T240 (amino acids 231–245) were immobilized on beads and tested for their binding to FBW7α. Only the phosphorylated peptide bound recombinant FBW7α in vitro (Fig 1C), suggesting that phosphorylation of the CPD motif triggers the interaction of SOX9 with FBW7α.
To further explore SOX9 CPD phosphorylation, we generated a phospho-antibody against the SOX9 231SQGPPtPPTpTPKTDV245 peptide. Importantly, using this tool, we were able to detect both SOX9-WT and SOX9-T240A, but not SOX9-T236A or T236/240A by immunoblot analysis (Fig EV1D), implying that the phospho-antibody primarily detects pT236-SOX9. Phosphatase treatment of immunoprecipitated exogenous and endogenous SOX9 demonstrated that the pT236-SOX9 antibody specifically detects SOX9-WT and SOX9-T240A.
phosphorylated SOX9 (Figs 1D and EV1E). The specificity of the pT236-SOX9 antibody was further validated by immunoblotting and immunofluorescence staining following RNAi-mediated SOX9 depletion in the medulloblastoma cell lines D324MED and Daoy (Fig EV1F and G). Given that GSK3 phosphorylates the central threonine position of the CPD in many FBW7a substrates, we next analyzed whether GSK3 also phosphorylates SOX9. Using purified recombinant GSK3a and GSK3b, we found that GSK3 kinase directly phosphorylates in vitro-translated (IVT) SOX9-WT but not the SOX9-T236/240A mutant (Figs 1E and EV1H). Finally, to investigate whether GSK3-mediated phosphorylation of SOX9 triggers its interaction with FBW7a, we incubated GSK3-phosphorylated SOX9 with purified recombinant SCF{FBW7}a complex. As shown in Figs 1F and EV1H, GSK3 indeed promoted the binding of SOX9 to SCF{FBW7}a in vitro. Altogether, these results suggest that GSK3-mediated phosphorylation of SOX9 on T236 is required for FBW7a to bind SOX9.

**FBW7a promotes SOX9 ubiquitylation and degradation in a GSK3-dependent manner**

In order to determine whether FBW7a regulates SOX9 protein stability, each FBW7 isoform was specifically depleted using siRNAs (as previously described in van Drogen et al, 2006) and endogenous SOX9 protein abundance was monitored. This experiment revealed that depletion of the nuclear isoform, FBW7a, increased the steady state and the half-life of SOX9 (Fig 2A). This was consistent with the reduced turnover and nuclear enrichment of SOX9 in the HCT116 FBW7-KO colon cancer cells as compared to HCT116 FBW7-WT cells (Figs EV1A and EV2A). No significant changes in SOX9 mRNA levels were observed upon FBW7a depletion, consistent with a post-translational mode of SOX9 regulation by FBW7 in these cells (Fig EV2B). Furthermore, FBW7a and SOX9 were observed to co-localize in the nucleus and ectopic expression of FBW7a reduced SOX9 protein levels in a dose-dependent manner (Fig 2B and C). Knockdown of FBW7a also increased both the steady state level and stability of SOX9 protein in different cancer cell lines derived from breast, brain, and colon (Fig EV2C and D). Consistent with the inability of the SOX9-T236/240A mutants to interact with FBW7a (Figs 1B and EV1C), we found that forced expression of FBW7a did not significantly change the level or stability of either SOX9-T236A or T236/240A as compared to SOX9-WT which was rapidly eliminated when co-expressed with FBW7a (Figs 2D and EV1D). In addition, the proteasome inhibitor MG132 prevented FBW7a-mediated depletion of SOX9-WT (Figs 2D and EV2E), strongly suggesting that FBW7a targets SOX9 for proteasomal degradation.

Given that GSK3 promotes FBW7–SOX9 interaction (Fig 1F), we next investigated whether inhibition of GSK3 activity interfered with FBW7a-mediated SOX9 degradation. As expected, treatment of cells with the GSK3 inhibitor (BIO) or depletion of GSK3 using siRNA significantly attenuated degradation of SOX9 by FBW7a (Fig 2E and F).

Finally, we assessed whether SCF{FBW7}a mediates the ubiquitylation of SOX9. Expression of FBW7a-WT promoted the formation of high molecular weight SOX9-ubiquitin conjugates, whereas expression of F-box-deleted (AF) FBW7 (which can bind protein substrates but not the SCF core ligase) or FBW7 with a WD40 domain mutant (R465A) (which binds the SCF core but lacks ability to interact with protein substrates) was unable to support SOX9 poly-ubiquitylation in vivo (Fig 2G). Supporting these results, depletion of FBW7a by siRNA significantly reduced SOX9 ubiquitylation in cells (Fig EV2F). Finally, we reconstituted SOX9 ubiquitylation in vitro using purified recombinant proteins. As shown in Figs 2H and EV2G, only when the SCF{FBW7}a ubiquitin ligase was present in the reaction, SOX9 was efficiently ubiquitylated. Taken together, these results show that SCF{FBW7}a ubiquitylates and targets pT236-SOX9 for proteasomal degradation in a GSK3-dependent manner.

**SOX9 protein stabilization correlates with low levels of FBW7a in medulloblastoma patients**

Using whole-exome sequencing and expression analysis, we observed that FBW7 missense and nonsense mutations occur in approximately 11% of adult SHH subtype cases (Kool et al, 2014). The majority of the missense mutations target distinct arginine residues that have been shown to be directly involved in substrate binding and degradation (Fig 3A) (Akhoondi et al, 2007; Thompson et al, 2007; Forbes et al, 2015). We confirmed that SOX9 is degraded in medulloblastoma cells by ectopic expression of FBW7a-WT in medulloblastoma-derived Daoy cells, whereas arginine mutant FBW7a (R465A or R479A) was deficient in SOX9 degradation (Fig 3B). We also demonstrated that only FBW7a-WT significantly interacted with SOX9, whereas the FBW7a arginine mutations identified in primary medulloblastoma specimens did not (Fig 3A and C). As mentioned above, depletion of FBW7a using siRNAs increased SOX9 protein levels in Daoy medulloblastoma cells (Fig EV2C).

To explore whether FBW7 may be transcriptionally downregulated in primary medulloblastoma specimens, we used a large combined set of gene expression profiles from primary medulloblastomas and analyzed overall FBW7 expression levels (using the R2 database: http://r2.amc.nl). As compared to both normal fetal (n = 5) and adult cerebellum (n = 13), FBW7 transcript levels were significantly lower in all medulloblastoma molecular subgroups (Fig 3D). In light of these findings, we further examined whether FBW7 expression could be linked to clinical outcome in medulloblastoma. Indeed, FBW7 significantly separated medulloblastoma tumors according to overall survival when the expression data were dichotomized into high vs. low FBW7 groups, as evaluated by log-rank analysis of Kaplan–Meier curves (Fig 3E). Using a best cutoff scanning approach, we also found a statistically significant correlation between low FBW7 groups and worse outcome in the SHH molecular subtype (Fig EV3A). Similar trends were found in the Group 3 and Group 4 medulloblastoma molecular subtypes (Fig EV3A), albeit not statistically significant upon correcting for multiple testing.

We next used the combined set of gene expression profiles to analyze SOX9 mRNA levels in 423 medulloblastoma samples. In accordance with our previous report (Swartling et al, 2012), SOX9 mRNA levels were found elevated in the SHH cases as compared to both fetal and normal adult cerebellum (Fig EV3B). Both the Group 3 and the Group 4 tumors have lower SOX9 mRNA levels relative to normal fetal and adult cerebellum tissues (Fig EV3B). Additionally, FBW7 mRNA expression did not correlate with SOX9 mRNA expression across the medulloblastoma subgroups (Fig EV3C). In order to test whether a correlation exists between...
Figure 2. FBW7α promotes SOX9 protein turnover in a GSK3-dependent manner through ubiquitin–proteasome system.

A. Endogenous SOX9 protein turnover in the presence of cycloheximide following RNAi depletion of FBW7α, FBW7β, or FBW7γ. HEK293 cells were transfected with 20 nM of scrambled siRNA or siRNAs specifically targeting FBW7α, FBW7β, or FBW7γ for 72 h prior to chase with the addition of 100 ng/ml cycloheximide. Immunoblot of cyclin E, an established SCF<sub>SOX9</sub> substrate, was used to assess the efficacy of RNAi-mediated depletion of FBW7 protein. GAPDH served as a protein loading control.

B. Immunofluorescence staining of D324MED medulloblastoma cells showing nuclear co-localization of HA-SOX9 WT (Alexa Fluor 568, red) and FLAG-FBW7α (Alexa Fluor 488, green). The cell nuclei were counterstained with DAPI (blue). Images are representative of multiple fields taken at 10× objective magnification. Scale bar indicates 20 µm.

C. Reaction mixture lacking the UbE<sub>α</sub> or FBW7α protein. GAPDH served as a protein loading control. The blots shown are representative of three independent experiments.

D. Expression of FLAG-FBW7α enhances HA-SOX9-WT protein turnover but not HA-SOX9-T236A or HA-SOX9-T236A/T240A protein turnover over a 4-h cycloheximide (100 ng/ml) chase in HEK293 cells. β-actin protein served as a loading control. The blots shown are representative of four independent experiments.

E. Treatment of HEK293 cells with increasing concentration of the GSK3α/β inhibitor BIO, increase HA-SOX9-WT protein level in a dose-dependent manner. HA-SOX9-WT and FLAG-FBW7α were co-expressed in HEK293 cells prior to treatment with different concentrations of BIO for 4 h. Whole-cell lysates were collected for Western blotting with anti-HA (SOX9) and anti-FLAG (FBW7) antibodies. GAPDH served as a protein loading control. The blots shown are representative of two independent experiments.

F. siRNA depletion of GSK3β attenuate FBW7α-induced HA-SOX9-WT turnover in HEK293 cells. HA-SOX9-WT protein turnover was examined following 48 h depletion of siGSK3β (20 nM) in the presence of cycloheximide (100 ng/ml). Immunoblots with GSK3β antibodies demonstrated depletion of GSK3β protein with the siRNA. Changes in SOX9 protein levels were analyzed relative to GAPDH levels using ImageJ. The blots shown are representative of three independent experiments.

G. Expression of different FLAG-FBW7α constructs and endogenous SOX9 protein were examined in the whole-cell lysates. GAPDH protein was used as a loading control. The blots shown are representative of four independent experiments.

H. Reconstitution of SOX9 poly-ubiquitylation by FLAG-FBW7α in vitro. Immunoblots with GSK3β antibodies demonstrated depletion of GSK3β protein with the siRNA. Changes in SOX9 protein levels were analyzed relative to GAPDH levels using ImageJ. The blots shown are representative of three independent experiments.

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FBW7 expression and SOX9 protein levels, we performed tissue microarray (TMA) analysis using an independent cohort of 142 medulloblastoma tumor specimens, encompassing all four molecular subgroups. Using a novel multiplex RNA in situ hybridization technique, the RNAscope technology (Wang et al., 2012a), we evaluated FBW7 and SOX9 mRNA levels and compared them to SOX9 protein levels as measured using immunohistochemistry (Fig EV3D).

Notably, when SOX9 expression was scored using the ratio of SOX9 protein/mRNA, a significant negative correlation with FBW7 expression was observed regardless of medulloblastoma subgroup (Fig 3F). Collectively, these data show that SOX9 is a substrate of FBW7 in medulloblastoma cells and its accumulation is associated with downregulation of FBW7 in primary medulloblastoma tumors, potentially contributing to medulloblastoma malignancy.

Figure 3. SOX9 protein stabilization correlates with low levels of FBW7a in medulloblastoma patients.

A FBW7 mutations across medulloblastoma molecular subgroup as identified by exome sequencing. In the SHH medulloblastoma subgroup, FBW7 mutations occurred approximately 11% in a cohort containing 133 SHH medulloblastoma cases (Kool et al., 2014) and can be categorized as stop-gain (n = 3) and non-synonymous (n = 7). One case of splicing error mutation is detected in the Group 3 medulloblastoma.

B Immunoblotting of HA-SOX9-WT protein following expression of FLG-FBW7a constructs including wild-type (WT), R465A or R479A mutation in Daoy cells. The cells were transfected with equal amounts of HA-SOX9-WT and FLAG-FBW7a, and the whole-cell lysates were collected 24 h following the transfection. GAPDH expression was used as a loading control. The blots shown are representative of two independent experiments.

C Co-immunoprecipitation of HA-SOX9 with either wild-type (WT) or R465A-FLAG-FBW7a. Unlike the wild-type protein, FLAG-FBW7a containing arginine 465 mutation did not significantly co-immunoprecipitate with HA-SOX9 (IP with anti-HA antibody) and vice versa (IP with anti-FLAG antibody). HA-SOX9 and FLAG-FBW7a-WT or R465A mutant were assessed in the whole-cell extract (WCE) of the transfected HEK293 cells with GAPDH protein used to indicate total protein loading.

D Comparison of FBW7 mRNA expression across the medulloblastoma subgroups (WNT, SHH, Group 3, and Group 4) in a cohort containing 423 medulloblastoma cases. Expression of FBW7 in both normal fetal (n = 5) and adult cerebellum (n = 13) was used for comparison against the medulloblastoma.

E Overall survival analysis of medulloblastoma patients based upon FBW7 expression level. Analysis was performed on 383 out of the 423 cases from which the survival data were available. Cohort was divided into high (blue line) and low-FBW7 (red line)-expressing subgroup using FBW7 median expression as group classifier. A log-rank test was used to show differences between groups.

F Correlation analysis between SOX9 protein/mRNA and FBW7 mRNA expression level from medulloblastoma tissue microarray consisting of 142 tissue samples. SOX9 protein level was scored following immunohistochemistry staining, while SOX9 and FBW7 mRNA expression was analyzed by the RNAscope as described in Materials and Methods. A negative correlation was determined by Kendall’s correlation test (τ = -0.223, P < 0.001) between SOX9 protein/mRNA and FBW7 mRNA expression. Affiliation of samples with medulloblastoma subgroups is indicated by different colored circle (blue = WNT, red = SHH, yellow = Group 3, green = Group 4, gray = undetermined).

Source data are available online for this figure.
FBW7\(\alpha\) suppresses the migration and metastasis of SOX9-driven medulloblastoma cells

To further examine the biological functions of FBW7\(\alpha\)-mediated repression of SOX9 in medulloblastoma, we transduced Daoy cells with SOX9 and doxycycline-inducible FBW7\(\alpha\) (i.e., Daoy-SOX9/T-FBW7\(\alpha\)). Doxycycline treatment resulted in a dose-dependent suppression of phospho-T236 and total SOX9 protein levels in these cells (Fig EV4A). To assess the consequence of FBW7\(\alpha\)-mediated control of exogenous SOX9 on proliferation, we measured cell-doubling time in parental versus SOX9-transduced Daoy with or without doxycycline-induced FBW7\(\alpha\) expression. Interestingly, constitutive expression of SOX9 in cultured Daoy cells prolonged cell-doubling time compared to parental cells, and FBW7\(\alpha\) induction reversed this effect (Fig EV4B). Induction of FBW7\(\alpha\) was not associated with changes in cell death as analyzed by PARP and caspase-3 cleavage (Fig EV4C and data not shown). Orthotopic engraftment of these cells into nude mice resulted in complete mortality within 45 days post-injection in the absence of doxycycline treatment. By comparison, feeding the mice with doxycycline-containing chow (and hence, inducing FBW7\(\alpha\)) significantly increased overall survival \((P = 0.0285;\) Fig 4A). Postmortem examination of the mouse brain from the Daoy-SOX9 group revealed not only aggressive tumor at the sites of injection in the cerebellum but also metastatic spread in the ventricles, brain stem, and parts of the forebrain. Mice treated with doxycycline (Daoy-SOX9/T-FBW7\(\alpha\)), on the other hand, presented a constrained tumor at the site of injection in the cerebellum (Fig 4B). Assessment of cell proliferation by immunohistochemistry showed a slight increase in Ki67-positive cells in the doxycycline-treated mice compared to untreated mice (Fig EV4D). No significant difference in the levels of SOX9 staining was evident in the Daoy tumors with constitutive expression of SOX9 from mice treated with or without doxycycline. Interestingly, SOX9 protein staining was found to be suppressed in the center part of FBW7\(\alpha\)-induced (+ doxycycline) tumors (Fig EV4E–H, black arrow). Migrating droplets of cells were only found in tumors without FBW7\(\alpha\)-induction (Fig EV4E1 and G2). We also performed immunohistological staining of tumors with or without FBW7\(\alpha\) expression to check for differences in cell death (cleaved caspase-3) or in differentiation markers of gliogenesis (GFAP) and neurogenesis (Syp and TuJ1), but no clear differences between the different groups of mice were found (not shown).

We reasoned that the observed tumor containment in response to induction of FBW7\(\alpha\) in vivo was due to suppression of SOX9-induced cell motility. To test this, we performed Transwell migration assays comparing SOX9-transduced cells with Daoy cells co-expressing SOX9 and FBW7\(\alpha\). As shown in Fig 4C, induction of FBW7\(\alpha\) (+ dox) potently suppressed cell motility \((> 70\%, P < 0.05)\) in SOX9-expressing Daoy cells (Fig 4C). In accordance, we recapitulated these findings using our previously developed medulloblastoma-initiating cells (MICs) derived from MYCN\(^{T248K}\)-transduced postnatal day 0 cerebellum mouse neural stem cells (Swartling et al, 2012). Orthotopic transplantation of the MICs led to intracerebral tumors (16 out of 16), while only three (18.75\%) of these disseminated and formed tumors in the frontal base of the forebrain. Strikingly, forced expression of SOX9 in these MICs (MIC-SOX9) increased the rate of metastasis to the forebrain to 100\% (8 out of 8) (Fig 4D), and only two (25\%) of the transplanted MIC-SOX9 cells formed intracerebellar tumors at the site of injection (Fig 4D).

In vitro Transwell assays again confirmed that SOX9 overexpression increased the motility of MICs (Fig 4E) and that expression of FBW7\(\alpha\), but not its dominant negative form ΔF-FBW7\(\alpha\), reduced the SOX9-induced cell motility (Fig 4F and G). Finally, to determine whether SOX9 protein correlates with metastasis, we went back to our TMA cohort of 142 medulloblastoma patients and correlated SOX9 protein levels with metastasis at diagnosis as determined by pathological grading of metastasis (M1–M4). No M4 cases were included in this cohort. We found that SOX9 protein levels
Figure 4.

A. Overall survival probability.

B. Daoy-SOX9/T-FBW7α
- Regular chow (no dox)
- Dox-containing chow

C. Daoy-SOX9/T-FBW7α
- No dox
- Dox (FBW7α)

D. Orthotopic xenograft
- Primary location
- Metastatic spread

E. MIC-MIC-SOX9

F. Migration relative to Control
- SOX9 / T-FBW7α
- SOX9 / T-ΔF FBW7α

G. MIC-SOX9/TRIPZ
- No Doxycycline
- 5 μg/mL Doxycycline

H. Number of tumors
- Low SOX9
- High SOX9
SOX9 stabilization promotes a metastasis-supportive program in medulloblastoma

To examine the biological function of SOX9 degradation for medulloblastoma progression in more detail, we generated doxycycline-inducible SOX9-WT or stable SOX9-T236/240A human primary MB002 cells (which have low levels of endogenous SOX9). Immediate transcriptional changes following an 8-h induction of SOX9-WT or SOX9-T236/240A were assessed relative to the MB002 cells transduced with an empty vector (EV) using RNA sequencing (RNA-Seq). Induction of SOX9-WT and SOX9-T236/240A generated similar transcriptional profiles ($R = 0.61$), with SOX9 itself being one of the genes most differentially expressed and significantly upregulated (Fig 5A). Hierarchical clustering of the most variable genes across these samples alone or in the context of human medulloblastoma signature genes (Northcott et al, 2011) showed that the different cell lines formed individual clusters, with SOX9-WT and SOX9-T236/240A being more similar to each other compared to the EV-transduced cells (Fig EV5A). Comparison of the RNA-Seq-derived transcription profiles to human medulloblastoma molecular subgroups (Northcott et al, 2011) revealed that all three MB002 cell lines were classified as Group 3 tumors.

Analysis of the FBW7 transcripts in the MB002 cells revealed that the alpha isoform is the most abundant transcript, while both the beta and the gamma isoforms are expressed at significantly lower levels (Fig 5B). Notably, neither expression of SOX9-WT nor that of SOX9-T236/240A significantly affected the endogenous level of the different FBW7 transcripts (Fig 5B).

Of the transcripts significantly upregulated with SOX9 expression when compared to EV, 38% of these transcripts (i.e., 329 out of 863) were shared between the WT and the FBW7-resistant SOX9 mutant (Fig 5C, red Venn diagram). Likewise, there were 377 out of 963 significantly downregulated transcripts (constituting 39%) that were shared between SOX9-WT and SOX9-T236/240A (Fig 5C, blue SOX9-T236/240A as compared to SOX9-WT Venn diagram).

Despite the similar transcriptional changes induced by SOX9-WT and T236/240A, detailed comparison of RNA-Seq profiling revealed subsets of differentially expressed transcripts among these cells (Fig 5C). Specifically, there were 207 among 228 transcripts that were exclusively upregulated and 209 out of 253 transcripts that were exclusively downregulated with the expression of SOX9-T236/240A in MB002 (Fig 5C, red Venn and blue Venn, respectively; Table EV1). Twenty-one transcripts (Fig 5D, upper panel; Table EV2) were significantly upregulated in SOX9-WT but also showed a further significant increase in SOX9-T236/240A as compared to EV (EV < SOX9-WT < SOX9-T236/240A). Conversely, 44 transcripts (Fig 5D, upper panel; Table EV2) were significantly downregulated more potently in SOX9-T236/240A as compared to SOX9-WT (SOX9-T236/240A < SOX9-WT < EV). To explore the functional significance of SOX9 accumulation in medulloblastoma, we next assessed the biological processes influenced by SOX9 in MB002 cells. First, we performed gene set overlap analysis of differentially expressed genes in response to induction of SOX9-WT or SOX9-T236/240A as well as between SOX9-WT and SOX9-T236/240A. Interestingly, among the HALLMARK gene sets, EMT genes were among the top 10 signatures upregulated in SOX9-WT or SOX9-T236/240A as compared to EV. The EMT functional category was also found to be the most significantly upregulated gene set in SOX9-T236/240A as compared to SOX9-WT (Table EV4). At the individual gene level, we identified several EMT hallmark factors (12 upregulated; four downregulated) including SNAI2 and VIM from our transcriptome profiles that were differentially upregulated or downregulated, respectively, in SOX9-T236/240A compared to SOX9-WT (Fig 5D). To confirm these results at the molecular level, SOX9 was induced for 8 and 24 h with doxycycline and analyzed for expression of SNAI2 and VIM. Quantitative real-time PCR (qRT-PCR) and immunoblot analysis demonstrated elevated mRNA and protein levels in the SOX9-T236/240A mutant cells as compared to EV and the SOX9-WT cells (Fig 5E and F). Thus, SOX9 and more potently stabilized SOX9 appear to promote expression of genes linked to EMT reprogramming in medulloblastoma. As EMT promotes migration/metastasis, these profiling data are well in line with the findings that SOX9 degradation controls migration and metastatic spread in medulloblastoma (Fig 4). To further characterize these aforementioned processes in more detail, we performed targeted GSEA on 19 preselected gene sets related to metastasis, migration, and EMT (Table EV5). Strikingly, this analysis revealed that in addition to EMT, metastasis signatures were found with significant enrichment among upregulated genes in SOX9-T236/240A and/or SOX9-WT (Fig EV5B).

To assess the change in expression of high-confidence SOX9 targets following induction of SOX9-WT or SOX9-T236/240A, we next compared differentially expressed genes to a list of genes putatively bound and regulated by SOX9 according to previous SOX9 ChIP studies (Kadaja et al, 2014; Oh et al, 2014; Larsimont et al, 2015). Importantly, we identified 24 genes that were documented as direct SOX9 target genes and overlapped with significantly upregulated or downregulated genes, respectively, in SOX9-T236/240A as compared to SOX9-WT (P = 0.0068) (Table EV3). This also included the recently described SOX9 target gene POU3F1 (OCT6) (Larsimont et al, 2015), which was found to be repressed at both the mRNA and protein level in SOX9-WT and more potently in SOX9-T236/240A mutant cells after 8 and 24 h of doxycycline treatment (Fig 5E and F). Interestingly, of these 24 genes, at least 14 have previously been recognized as pro-metastasis factors (e.g., PLD1, LAMA4, and NEDD9) again arguing for a significant role of SOX9 in promoting medulloblastoma tumor migration and metastasis (Nagato et al, 2005; Park & Min do, 2011; Speranza et al, 2012) (Fig 5G). Altogether, these results support a role for SOX9 stabilization as driver of medulloblastoma progression through orchestrating a metastasis-promoting transcriptional program.

SOX9 confers cisplatin resistance in medulloblastoma

Given that EMT reprogramming, stemness, and metastasis are linked to drug resistance in cancer cells, we next asked whether SOX9 abundance influences the response of medulloblastoma to cisplatin, a commonly used chemotherapeutic drug in standard medulloblastoma treatment. Using the doxycycline-inducible SOX9 model system, we found that SOX9 expression reduced the efficacy of cisplatin treatment not only in medulloblastoma MB002 and Daoy cells, but also in medulloblastoma-initiating cells (Fig 6A).
Quantitative comparison of the cisplatin IC_{50} values from these experiments showed between 2- and 15-fold increase in cisplatin resistance upon SOX9 overexpression (Fig 6A). Importantly, dox-induction of FBW7α in SOX9 expressing Daoy and MiCs improved sensitivity to cisplatin by approximately 2-fold (Fig 6B).

In order to test whether medulloblastoma cells with elevated levels of SOX9 are more resistant to cisplatin treatment in vivo, we transplanted mice with parental, SOX9-WT-transduced, or SOX9-T236/T240A samples. Heat map was generated using the GenePattern software (Reich et al., 2006).
SOX9-T236/240A-transduced Daoy cells. Importantly, cisplatin treatment for 14 days significantly prolonged survival in Daoy parental cells, whereas Daoy cells transduced with SOX9-WT or stable mutant SOX9 did not significantly respond to the cisplatin therapy (Figs 6C and EV6).

In an attempt to characterize the molecular mechanisms underlying SOX9-mediated cisplatin resistance, we re-assessed our MB002 RNA-Seq profiling data (Fig 5) for genes previously linked to cisplatin resistance (Galluzzi et al., 2012). Among the significantly differentially expressed genes between EV and SOX9-WT or SOX9-WT and SOX9-T236/240A (21 and 44 genes upregulated or downregulated, respectively) (Fig 5D), we identified 11 genes associated with cisplatin resistance (Fisher’s exact test P < 0.00001) (Fig 6D). Increased expression of the copper transporter ATP7A and decreased expression of DUSP2 and TTK were verified by quantitative RT–PCR and immunoblotting (Fig 6E and 6F).
PI3K/AKT/mTOR inhibitors sensitize medulloblastoma cells to cisplatin by targeting SOX9 through FBW7

Given the fact that PI3K/AKT signaling antagonizes GSK3 activity (Cross et al., 1995; Cheng et al., 2009), we reasoned that hyperactivation of the PI3K pathway could stabilize SOX9 protein. Strikingly, recurrent mutations affecting PI3K/AKT/mTOR signaling and phosphorylation of AKT and S6K are strongly associated with poor outcome in adult SHH medulloblastoma (Kool et al., 2014), and this pathway is commonly activated in clinically refractory and/or recurrent medulloblastoma (Buonamici et al., 2010). To explore the possibility that pharmacological inhibition of PI3K/AKT/mTOR signaling could potentially sensitize medulloblastoma to cisplatin treatment by targeting SOX9 for degradation through GSK3-FBW7, we utilized three pharmacological inhibitors (AZD8186, AZD5363, and AZD2014) to inhibit PI3K/AKT/mTOR activities in Daoy cells (Fig 7A). Specific inhibition of PI3K/AKT/mTOR signaling was confirmed by immunoblotting with a panel of pathway activity markers in medulloblastoma cells treated with each respective drug (Fig EV7A). Immunoblotting with GSK3α pSer21 and GSK3β pSer9 antibodies, established markers for the PI3K/AKT phospho-inactivated GSK3, revealed decreased levels upon inhibitor treatment (Fig EV7A), suggesting the presence of active GSK3. Notably, expression of FBW7α in the presence of these inhibitors resulted in more potent reduction of total and pT236 phosphorylated SOX9 protein (Fig 7A). Importantly, cycloheximide chase experiments demonstrated enhanced SOX9 degradation in response to PI3K/mTOR inhibition (AZD2014 and AZD8186), and depletion of FBW7α completely rescued inhibitor-induced turnover of SOX9 (Figs 7B and EV7B). Importantly, co-treatment of medulloblastoma cells (Daoy and MB002) with cisplatin and AZD2014 translated to a potent synergistic repression of cell viability (Fig 7C; CI (combination index) < 1.0). The drug synergism was also reproduced by drug combination (Fig EV7A) was associated with reduced expression of ATP7A and DUSP2 protein levels in SOX9-WT, SOX9-T236/240A, and DUSP2, whereas repression of endogenous SOX9 protein as compared to treatment with each drug alone (Fig 7D, lanes 6 and 12 vs. lanes 6 and 10, respectively). Altogether, these results suggest that SOX9 is targeted for degradation by FBW7 in response to cisplatin treatment and SOX9 degradation is further stimulated with therapeutic drugs inhibiting PI3K/AKT/mTOR signaling.

To characterize in more detail the role of SOX9 abundance and sensitivity to these drugs, we subjected MB002 cells expressing either SOX9-WT or the SOX9-T236/240A mutant to treatment with each individual drug or their combination. The FBW7-insensitive SOX9-T236/240A mutant remained stable in response to cisplatin treatment compared to wild-type SOX9 (Fig 7E, lane 10 vs. lane 6). As expected, the AZD2014 treatment stimulated degradation of SOX9 protein and the combination treatment (cisplatin+AZD2014) was more efficient in depleting SOX9-WT as compared to the SOX9-T236/240A mutant (Fig 7E). Interestingly, the AZD2014 inhibitor alone, as well as the combination treatment, also reduced SOX9-T236/240A protein to some extent, indicating that AZD2014 may repress SOX9 independently of FBW7 (Fig 7E). To this end, we examined whether SOX9 abundance affects drug response and cell viability in EV, SOX9-WT, or SOX9-T236/240A MB002 cells. As shown in Fig 7E, expression of either SOX9-WT or SOX9-T236/240A increased cisplatin resistance as measured by cleaved PARP and alamarBlue assay, respectively (Fig 7E and F). Nonetheless, SOX9-WT-expressing cells remained significantly sensitized, albeit to a lesser degree than EV-expressing MB002, to the cisplatin/AZD2014 combination treatment (Fig 7F), strongly arguing for FBW7-mediated degradation of SOX9 influences cisplatin sensitivity in these cells.

To further explore the underlying mechanism(s) of how SOX9 stabilization promotes resistance to cisplatin, we next analyzed ATP7A and DUSP2 protein levels in EV, SOX9-WT, and SOX9-T236/240A MB002 cells treated with single drugs or their combination. Indeed, we found that the combination treatment significantly attenuated upregulation of ATP7A expression in SOX9-WT cells compared to SOX9-T236/240A (Fig 7E, compare lane 8 vs. lane 5 and lane 12 vs. lane 9). Furthermore, the combination treatment also increased DUSP2 expression in MB002 cells (Fig 7E, compare lane 1 vs. lane 4), and in agreement with its role in repressing ERK1/2 activity, we observed reduced ERK1/2 phosphorylation in both SOX9-WT and SOX9-T236/240A cells treated with the drug combination as such the effect being less pronounced in the cells expressing FBW7-insensitive SOX9 (Fig 7E, compare lanes 5–8 vs. lanes 9–12). Supporting these results, we also found that the increased sensitivity to cisplatin upon induction of FBW7α in Daoy-expressing SOX9 (Fig 6B) was associated with reduced expression of ATP7A and increased DUSP2 protein levels (Fig 7G).

On the basis of these findings, we suggest that inactivation of PI3K/AKT/mTOR signaling sensitizes medulloblastoma cells to cisplatin treatment by stimulating GSK3/FBW7-mediated degradation of SOX9 proteasomal degradation (Fig 8).

Discussion

Stabilization of oncoproteins caused by dysregulation of SCF substrate adaptors, like F-box proteins, is an important driver of malignant...
brain tumors (Hede et al., 2014). To provide a more complete understanding of how the tumor suppressor FBW7 functions and controls tumorigenesis, we previously employed a proteomic screening approach designed to discover novel SCFFBW7 substrates (Arabi et al., 2012). One of the candidates identified in this screen was the transcription factor SOX9. Here, we show that the SCFFBW7 ubiquitin

**Figure 7.** Increased SOX9 turnover via PI3K/AKT/mTOR inhibition sensitizes medulloblastoma cells to cisplatin treatment.

A Western blotting of pT236-SOX9 and total SOX9 following treatment of Daoy cells with a panel of inhibitors targeting PI3K/AKT/mTOR pathway. The whole-cell lysates were collected for gel electrophoresis following 6-h treatment with 1 μM of AZD5363, AZD8186, or AZD2014. The blots shown are representative of three independent repeats.

B Western blotting of endogenous SOX9 protein turnover in the presence of PI3K/mTOR dual inhibitor, AZD2014. Daoy cells were transfected with either siScr or siFBW7 for 48 h prior to further treatment with 1 μM AZD2014. SOX9 protein turnover was examined 2 h following the inhibitor treatment by cycloheximide chase assay. Immunoblotting of cyclin E protein was used to indicate the efficacy of FBW7 knockdown. Changes in SOX9 protein level were quantified relative to GAPDH protein using ImageJ. The blots shown are representative of three independent experiments.

C Quantification of resazurin-based Daoy and MB002 cell viability following treatment with cisplatin (blue bars), AZD2014 (red bars), or their combination (green bars). Daoy cells were treated for 72 h, while MB002 cells were subjected to 5-day course of treatment. The cell viability was calculated relative to the vehicle-treated cells. The synthetic lethality combination index (CI) for each treatment was calculated from the mean of three independent experiments using the Compusyn software. Error bars indicate standard deviation.

D Time-course Western blotting of endogenous SOX9 and apoptotic markers including cleaved PARP and caspase-3 following MB002 treatment with cisplatin, AZD2014, or their combination. β-Actin was used as a loading control. The blots shown are representative of three independent experiments.

E Western blotting profiling of MB002 cells expressing EV, SOX9-WT, or SOX9-T236/240A following 48-h treatment with cisplatin, AZD2014, and their combination. The set of blots shown is representative of three independent experiments.

F Quantification of cell viability of MB002 cells expressing EV, SOX9-WT, or SOX9-T236/240A following 5-day treatment with either 10 μM cisplatin (black bars), 0.5 μM AZD2014 (blue bars), or their combination (red bars). Data are mean ± standard deviation from three independent experiments, from which statistical significance was analyzed by two-way ANOVA multiple comparisons with Bonferroni’s post-test.

G Immunoblots of ATP7A and DUSP2 protein following doxycycline treatment of Daoy-SOX9/T-FBW7α cells. The cells were either untreated or incubated with 5 μg/ml doxycycline for 72 h to induce FBW7α prior to being harvested for gel electrophoresis. The blots shown are representative of two independent experiments.
ligase targets SOX9 for proteasomal degradation and provide experimental evidence of a role for FBW7 in modulating medulloblastoma malignancy and cisplatin resistance by controlling SOX9 proteolysis.

As phosphorylation is the dominant mechanism controlling the interaction and subsequent ubiquitylation of substrates by F-box proteins (Skaar et al., 2013), we first investigated how FBW7-mediated degradation of SOX9 is regulated. Using a phospho-specific SOX9 antibody, we showed that GSK3α/β phosphorylates T236 in SOX9 which resides within a conserved SOX9 CPD motif. The phosphorylation of SOX9 by GSK3 is a prerequisite for the FBW7 to interact, ubiquitylate, and degrade SOX9 in vivo and in vitro. Knockdown of individual FBW7 transcripts showed that SOX9 specifically accumulates upon depletion of the FBW7α splice form which is the primary isoform responsible for SOX9 degradation in cells.

In the brain, SOX9 is required for development and lineage commitment and it is essential for gliogenesis while blocking neural differentiation (Scott et al., 2010; Martini et al., 2013). Consistent with its function in maintaining neural stem cells downstream of SHH, we previously reported that SOX9 expression is high in SHH/WNT medulloblastoma subtypes (Swartling et al., 2012). Recent large-scale sequencing efforts providing a comprehensive map of mutations in medulloblastoma defined this brain tumor as a heterogenous malignancy (Northcott et al., 2012). Here, we examined the relationship between FBW7 and SOX9 in medulloblastoma in detail. We identify recurrent FBW7 gene mutations in the adult SHH medulloblastoma subgroup and demonstrate that FBW7 expression is frequently downregulated in the other medulloblastoma patient subgroups. Importantly, we find that when FBW7 is functionally inactivated by mutation or reduced expression, SOX9 protein is upregulated demonstrating their inverse association in clinical samples and tumor-derived cell lines. In line with the notion that inactivation of FBW7 contributes to medulloblastoma malignancy through deregulation of SOX9, we demonstrate the requirement of SOX9 for tumor migration and metastasis and, further, that forced expression of FBW7α attenuates these SOX9-driven processes. Metastasis at diagnosis correlates with tumors with adverse outcome and characterizes medulloblastoma into a high-risk category (Zeltzer et al., 1999). The findings that FBW7 is transcriptionally downregulated and mutated in medulloblastoma and that elevated SOX9 levels correlate with poor outcome, tumor cell dissemination, and metastasis in clinical samples and brain tumor xenograft models suggest that SOX9 stabilization through loss of FBW7α could predict metastasis and may be used as a biomarker for medulloblastoma aggressiveness. Interestingly, SOX9 is frequently upregulated in many other malignancies, and recent reports reveal that deregulation of SOX9 promotes critical cancer processes by modulating the malignant transcriptome (Jo et al., 2014). Thus, it is likely that SOX9 stabilization through loss of FBW7 is a common feature also in other cancer types. Indeed, we found that FBW7 depletion stabilizes SOX9 in several different tumor-derived cell lines of different origins. Noteworthy, Pine and coworkers also identified SOX9 as a target of FBW7 and found that SOX9 is actively

Figure 8. Schematic model of FBW7α-mediated regulation of SOX9 in medulloblastoma cells. 
SCF^FBW7α^ together with GSK3 regulate SOX9 protein levels through the ubiquitin–proteasome system. FBW7α mutations, or transcriptional downregulation, lead to stabilization of SOX9 protein and SOX9-driven EMT-like reprogramming, migration and drug resistance in medulloblastoma. Small-molecule inhibitors of the PI3K/mTOR pathway can be used to stimulate GSK3/FBW7α-mediated SOX9 turnover and may provide a novel strategy to target SOX9-driven medulloblastoma.
degraded by FBW7 in response to DNA damaging agents in various cancer types (Hong et al., 2016).

Although not fully understood, SOX9 acts in concert with other oncogenic transcription factors and co-factors involved in normal brain or brain cancer development, several of which directly or indirectly promote SOX9 expression, including Gli (Bagheri-Fam et al., 2006). SOX9 is a downstream target of Gli1 and a positive-feedback loop between Gli1 and SOX9 was shown in pancreatic ductal carcinoma (Deng et al., 2015), involving SOX9-mediated degradation of β-TrCP, another SCF ubiquitin ligase that targets Gli1 for proteasomal degradation (Deng et al., 2015). Thus, it is possible that functional inactivation of FBW7 and consecutive SOX9 stabilization may further promote Gli1 protein stability and SOX9/Gli1-driven processes involving stemness and self-renewal properties. This is in agreement with our previous findings that SOX9 increases self-renewal capacity of medulloblastoma-initiating cells (Mics) (Swartling et al., 2012). Consistently, we have observed that forced expression of FBW7 suppresses the formation of SOX9-induced MIC spheres in culture (unpublished data). Moreover, SOX9 is a direct target of Notch1 and mediates Notch-induced mesenchymal features in lung adenocarcinoma (Capaccione et al., 2014) and neural lineage determination (Martini et al., 2013). Although our data suggest that defective SOX9 degradation directly promotes medulloblastoma pathogenesis, at this stage we cannot exclude the possibility that other FBW7 target substrates such as Notch1 and c-Jun may also contribute to medulloblastoma progression. For instance, deletion of FBW7 in the mouse brain has been shown to impair neural stem cell differentiation and increase progenitor cell death, phenotypes attributed to deficient degradation of the FBW7 target substrates Notch1 and c-Jun, respectively (Hoeck et al., 2010). Activated Notch1 can induce SHH-driven medulloblastoma when overexpressed in p53-deficient mice (Natarajan et al., 2013); however, Notch2 seems to maintain these tumors and is also the Notch member elevated in primary human medulloblastoma (Fan et al., 2004).

To assess the physiological significance of altered SOX9 stability in medulloblastoma, we studied how SOX9-WT or the FBW7-insensitive mutant influenced the transcriptional profiles in medulloblastoma MB002 cells. Strikingly, we found that the genes differentially expressed and upregulated in Sox9-expressing cells and between the Sox9-WT and the T236/240A mutant overlap significantly with previously known hallmark EMT factors. Furthermore, gene set enrichment analyses demonstrated that the transcriptional changes correlated significantly with gene signatures found to be upregulated in cancer metastasis and EMT processes. One of the genes, palladin, is further known to promote the expression of vimentin (Brentnall et al., 2012) that was also found to be elevated in medulloblastoma cells expressing the stable SOX9 mutant. Interestingly, AKT drives cell migration and invasion in part by phosphorylating both palladin and vimentin (Xue & Hemmings, 2013). Whether SOX9 is actively involved in regulating this process is currently unknown. Importantly, among the genes differentially regulated between the Sox9-WT and stable Sox9 mutant, a significant number of previously reported Sox9 targets genes were found, including a majority of which have previously been reported as pro-metastatic genes, such as SNAI2/SLUG. Sox9 has been reported to work in concert with SNAI2/SLUG and trigger an auto-regulatory gene expression program involved in inducing and sustaining the mammary stem cell state (Guo et al., 2012). A more direct relationship between SNAI2/SLUG and SOX9 was recently found in human lung carcinoma (Luanpitpong et al., 2015), where SNAI2 and SOX9 were shown to interact in the cytoplasm, and upon SNAI2/SLUG depletion, SOX9 was degraded. However, the ubiquitin ligase responsible for SOX9 degradation was not identified in this study. In our profiling studies, SNAI2/SLUG was found to be rapidly upregulated in response to SOX9 induction.

One of the major findings in this study is that the GSK3-SOX9-FBW7 regulatory axis is controlled by the PI3K/AKT/mTOR signaling cascade. The PI3K/AKT/mTOR pathway bridges many signaling pathways downstream of receptor tyrosine kinases (RTKs) and controls various critical processes in cancer progression (Fruman & Rommel, 2014). Inclusion of drugs targeting this pathway was recently suggested to be an option for combination therapies in several malignancies including medulloblastoma (Kool et al., 2014). Importantly, our data show that targeting the PI3K/AKT/mTOR pathway destabilizes SOX9 in medulloblastoma cells with functional FBW7, and thus further sensitizes tumor cells to the chemotherapeutic drug cisplatin. A correlation between loss or reduced FBW7 expression and cisplatin resistance through EMT has previously been reported in non-small-cell lung cancer cell lines (Yu et al., 2013). Here, we found that increased or stabilized SOX9 protein reduced cisplatin treatment efficacy in medulloblastoma, both in vitro and in vivo. Related to this process, we identified multiple genes associated with cisplatin resistance that may be directly controlled by SOX9, including ATP7A and DUSP2. Importantly, SOX9-induced changes in expression of ATP7A and DUSP2 were found to be reversed following destabilization of SOX9 by targeting the PI3K/AKT/mTOR signaling pathway with pharmacological compounds. In the future, it will be important to explore whether SOX9 can be stabilized in vivo by drugs targeting PI3K/AKT/mTOR activity and whether medulloblastoma patients with functional FBW7 are more sensitive to drugs targeting this pathway.

In summary, as illustrated in Fig 8, our study of SOX9 regulation by FBW7 provides insights into how loss of FBW7 might contribute to medulloblastoma malignancy by promoting SOX9-driven migration, metastasis, and drug resistance. These findings uncover a new molecular mechanism for the post-translational regulation of SOX9 stability through the GSK3-SOX9-FBW7 regulatory axis that opens up new strategies for targeting SOX9 in malignant brain tumors. For example, the use of selective inhibitors targeting PI3K/AKT/mTOR signaling could be a potential point of intervention for patients with recurrent, chemoresistant medulloblastoma with functional FBW7. In addition, development of specific SOX9 inhibitors, or compounds attenuating SOX9 expression, may also potentially be used for selective killing of FBW7-deficient, metastasis-prone, and drug-resistant medulloblastoma cells.

**Materials and Methods**

**Cell culture**

Daoy medulloblastoma cell lines were obtained from the American Type Culture Collection. Cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% FBS (Gibco) and 1% L-glutamine (Gibco).
MB002 cells have been described previously as derivative of primary medulloblastoma tumor with large-cell histology and gene expression markers consistent with Group 3 medulloblastoma (Bandopadhayay et al., 2014). MB002 cells were maintained in culture media made up of 1:1 (v/v) DMEM-F-12 (Gibco) and Neurobasal-A media (Gibco, #10888-022) supplemented with HEPES, sodium pyruvate, non-essential amino acid, L-glutamine, B27 (Gibco, #12587-10), 20 ng/ml EGF (Peprotech; #AF-100-15), 20 ng/ml basic fibroblast growth factor (Peprotech; #AF-100-18b), heparin (07980; Stem Cell), and 10 ng/ml leukemia inhibitory factor (LIF; #L1170-10; Millipore). Medulloblastoma-initiating cells (MICs) derived from postnatal, day 0, mouse cerebellar neural stem cells—transduced with MYCN\textsuperscript{T34A} alone or those which were further transduced from postnatal, day 0, mouse cerebellar neural stem cells—were cultured in Neurobasal-A media (Gibco, #10888-022) supplemented with L-glutamine, B27 (Gibco, #12587-10), 20 ng/ml EGF (Peprotech; #AF-100-15), and 20 ng/ml basic fibroblast growth factor (Peprotech; #AF-100-18b).

**Lentiviruses and generation of stable cell lines**

To generate stable doxycycline-inducible FBW7\textsuperscript{a}, SOX9\textsuperscript{wt}, and SOX9-T236/240A cell lines, lentiviruses were first generated in HEK293T cells by packaging the specified pTRIPZ lentivector with the packaging plasmids, psPAX2 and pMD2.G. Supernatants containing viral particles (48–96 h post-transfection) were pooled and used to infect the target cells in the presence of freshly added hexadimethrine bromide (8 mg/ml). Seventy-two hours post-infection, the transduced cells were subsequently selected and maintained in their appropriate culture medium in the presence of puromycin (0.125–0.5 \mu M).

**Drug treatment and cell viability assay**

cis-diammineplatinum (II) chloride (cisplatin) was obtained from Sigma-Aldrich, while small-molecule inhibitors of PI3K and/or mTOR including AZD5363, AZD8186, AZD2014, KU-0063794, and BEZ-235 were purchased from Selleckchem. For assessment of cell viability, the cells were treated with doxycycline when appropriate, counted, and seeded into 96-well plate for 24 h prior to drug treatment. Following 72–120 h of drug treatment, cell viabilities were measured by adding alamarBlue reagent (Life Technologies) into each well of 96-well plate.

**Immunoprecipitation, SDS–PAGE, and Western blotting**

For biochemical analyses of protein interactions and levels, the cells were lysed in NP-40 lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% NP-40) supplemented with both protease (Complete Mini; Roche) and phosphatase (PhosSTOP; Roche) inhibitors. Immunoprecipitation of protein complexes from cell lysate was performed using 1 \mu g of antibodies (rabbit anti-FLAG for exogenous FLAG-FBW7\textsuperscript{a}, or rabbit anti-SOX9 or mouse anti-HA for endogenous and exogenous SOX9, respectively), which subsequently was immobilized with Sepharose G beads (GE Life Science) and eluted under reducing–denaturing condition in SDS sample buffer at 95°C for 5 min. Proteins were separated by SDS–PAGE, blotted onto PVDF membrane, and detected by immunoblotting using the appropriate antibodies. The list of antibodies and their sources can be found in the Appendix.

**Generation of antibodies against SOX9 pT236&240**

Rabbit polyclonal antibody specific against SOX9 pT236&240 was produced by Innovagen AB (Lund, Sweden; Project # 92528). Synthetic phospho-peptide (CQSQGPP[T/P]PTPTPKTDV-NH\textsubscript{2}) identical to the phosphorylated SOX9 at Thr236&240 was used as an antigen to immunize New Zealand white rabbit. Following four rounds of immunization, sera were collected and subjected to peptide affinity purification against the non-phosphorylated peptide (CQSQGPP[T/P]PTPTPKTDV-NH\textsubscript{2}) to acquire the SOX9 pT236&240-specific antibody.

**In vitro kinase and binding assay**

Full length SOX9-WT and SOX9-T236/240A was synthesized in vitro using TnT T7 Quick Coupled Transcription/Translation kit from Promega (L1170) according to the manufacturer’s instructions. Following the synthesis, the proteins were immunoprecipitated and immobilized on Sepharose beads prior to being used for either in vitro kinase or binding assay. For the kinase assay, the immobilized SOX9 proteins were incubated with a complete GSK3 reaction buffer for 90 min at 37°C. Complete GSK3 reaction buffer consists of 50 mM Tris–HCl pH 7.4, 1 mM EGTA, 150 mM NaCl, 0.1% β-mercaptoethanol, 2 mM ATP, and 1 U of recombinant, active human GSK3β (Abcam, AB60863). For in vitro binding assay, Sepharose bead-immobilized full-length SOX9 protein or synthetic peptide was resuspended in 50 mM Tris–HCl pH 7.4 and 150 mM NaCl buffer in the presence of recombinant, active SCF\textsuperscript{FBW7} complex (Millipore, 23-030) under constant gentle agitation at 37°C for 1 h. Following the incubation, the supernatant was aspirated and the beads were washed three times with NP-40 lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% NP-40) prior to elution under reducing–denaturing condition in SDS sample buffer at 95°C for 5 min for SDS–PAGE.

**Ubiquitylation assay**

For in vivo ubiquitylation assay, cells were transfected with the indicated plasmids and/or siRNA (siFBW7 for knockdown experiment) and treated with MG132 (20 \mu M) for 4 h prior to harvest and extraction in 1% SDS and 10 mM N-ethylmaleimide (Sigma) at 95°C for 10 min. Protein extracts were subsequently sonicated and diluted ten times in NP-40 lysis buffer followed by immunoprecipitation of total SOX9 protein and gel electrophoresis. For in vitro ubiquitylation assay, Sepharose bead-immobilized full-length SOX9 protein was resuspended in ubiquitylation reaction buffer (25 mM Tris–HCl pH 7.6; 5 mM MgCl\textsubscript{2}; 100 mM NaCl) containing 2 mM ATP, 1 mM DTT, 200 mM Ube1 (Boston Biochem, E-305), 500 mM His\textsubscript{6}-UbcH3 (Boston Biochem, E2-610), 0.5 \mu g recombinant, active SCF\textsuperscript{FBW7} complex (Millipore, 23-030), and 5 \mu g ubiquitin (Boston Biochem, E2-100H). In vitro reactions were performed under gentle agitation at 37°C for 2 h prior to termination of reaction with the addition of 10 mM N-ethylmaleimide. The beads were subsequently washed three times with 50 mM HEPES pH 7.5/150 mM NaCl containing 10% glycerol, 1% Triton X-100, 1 mM EDTA, and...
1 mM EGTA prior to being eluted in SDS sample buffer for SDS-PAGE.

**In vitro cell migration assay**

Cell migration was performed using Transwell inserts (5-µm pores, Millipore, ECM220) according to the manufacturer's instructions. Briefly, 200,000 cells suspended in serum-free medium were added into Transwell insert and were let to migrate through the pores of the Transwell membrane toward the external laminin or fibronectin coating for 4 h. Cells that migrated were fixed and stained with 0.5% crystal violet from which the crystal violet absorbance was measured at 590 nm. For each independent experiment, two replicates per condition were used.

**Immunohistochemistry and RNAscope assay on tissue sections and TMAs**

Tissue microarrays (TMAs) were generated as previously described (Korshunov et al, 2010). Brain tumors were isolated, dehydrated, embedded in paraffin, and immunostained together with the TMAs as previously reported (Swartling et al, 2012) using ABC kits from Vector Labs. Antibodies against SOX9 (Millipore), Ki67 (Abcam) and STEM121 (TaKaRa) were used in 1:500, 1:2,000 and 1:1,000 dilution, respectively. RNascope experiments were performed on TMA sections according to the manufacturer’s instructions (Advanced Cell Diagnostics) using probes against human SOX9 (ACD-300031-C2) and FBW7 (ACD-300031-C). During the sectioning of the TMAs, some of the tumor samples were lost and some of the samples had less than 10% of the tissue left. These were not scored and are marked as N/A (not available) in the Source Data for Fig 3. The samples in the TMAs were judged one by one and for each channel separately to avoid selection bias.

Under the microscope, specific RNA signals appear as discrete, bright fluorescent spots in or around the DAPI-stained nuclei (Fig EV3D). To score “RNA intensity” in each tumor specimen, we quantified the number of RNA spots in each positive cell (indicating the relative number of RNA molecules per cell). The total number of RNA fluorescent spots was manually counted from at least three different image fields by two independent, blinded individuals, followed by adjustment to the number of DAPI-stained and RNA fluorescent signal-positive nuclei. RNA intensity was scored 0 for negative, 1 for low (≤3 spots/cell) to 3 (more than 10 spots/cell) accordingly. Similarly, the “percentage of tumor cells expressing the RNA” was analyzed by estimating the proportion of DAPI-stained nuclei with a positive RNA fluorescent signal in a given field (i.e., 0–100%). The average percentage (or RNA positivity) was calculated and likewise converted to scores: 0 for negative, 1 for low (≤ 20%), two for intermediate (20–80%), and three for high (more than 80%). To grade the overall RNA expression in a given tumor specimen, the “RNA intensity” score was multiplied with the score of the proportion of RNA-positive cells, generating the possible expression scores of 0, 1, 2, 3, 4, 6, or 9. In order to ensure linear distribution, the resulting multiplication scores were converted to “ranks” from 1 to 7, respectively. This assessment is illustrated in detail in sample images in Fig EV3D.

| SCORE | 0 | 1 | 2 | 3 | 4 | 6 | 9 |
|-------|---|---|---|---|---|---|---|
| RANK  | 1 | 2 | 3 | 4 | 5 | 6 | 7 |

Similar to RNA, an overall SOX9 protein score was calculated and converted to ranks (Fig EV3D). Finally, to analyze the correlation between SOX9 and FBW7, “SOX9 protein/RNA” ratio was used to account for variability in the levels of SOX9 transcript across different medulloblastoma molecular subgroups. It is known that the WNT and the SHH subgroups have elevated levels of SOX9 mRNA transcript (Swartling et al, 2012), probably as a consequence of activation of the WNT or SHH pathway. Therefore, the calculated SOX9 protein and RNA scores were first translated to rank (1–7; see Table 1), and subsequently used to obtain SOX9 protein/RNA. Details on scoring and ranking for SOX9 and FBW7 are exemplified in Fig EV3D with circles showing RNA spots and arrows pointing to SOX9 protein-positive cells.

The correlation between ranked FBW7 mRNA expression and SOX9 protein or the SOX9 protein/RNA ratio, respectively, was investigated using Kendall’s rank correlation coefficient $\tau$. The values of $\tau$ and $p$ depicted in Fig 3F indicate the correlation coefficient and $P$-value of the comparison, respectively.

**RNA-Seq preprocessing**

MB002 empty vector, MB002 SOX9-WT, and MB002 SOX9-T236/240A cells were treated with 100 ng/ml doxycycline in triplicate for 8 h. Cells were lysed in TRIzol (Life Technologies) and RNA was prepared using Qiagen RNeasy Mini Kit (Qiagen) according to the manufacturer’s recommendations. Sequencing of the RNA extracted from MB002 empty vector, MB002 SOX9-WT, and MB002 SOX9-T236/240A cells was conducted using the Ion Proton™ System for Next-Generation Sequencing and performed at NGI, Science for Life Laboratory, Uppsala Biomedicinska Centrum (BMC), Sweden. All RNA sequence reads were mapped against the human genome assembly GRCh37/hg19 (Lander et al, 2001) using STAR v2.4.0 (Dobin et al, 2013). The alignment was performed with a two-pass approach, where splice junctions discovered during a first alignment guided the forming of a second, final alignment (Veeneman et al, 2016). Unmapped reads were realigned using Bowtie2 v2.2.3 (Langmead & Salzberg, 2012) before duplicate reads were removed with Piccard v1.127 (Guo et al, 2012). The transcriptomes were then assembled and expression profiles quantified with Cufflinks v2.2.1 (Trapnell et al, 2010). Comparisons of transcriptomes and their expression levels were performed using Cuffcompare and Cuffdiff (Trapnell et al, 2013). Both the alignment and the quantification utilized RefSeq data downloaded from UCSC (Karolchik et al, 2004).

**Differential expression analysis**

The differential expression results produced by Cuffdiff were subsequently processed using the R package CummeRbund (Goff et al, 2013). Fold changes (FCs) were recalculated for each transcript after setting all expression values equal to FPKM+1 to avoid infinity results. Transcripts were considered significantly differentially expressed, if the recalculated FC > 1.5 and the adjusted $P$-value $p < 0.05$. 

| Table 1. Conversion of the final scores of protein or mRNA expression into RANK. |
|---|---|---|---|---|---|---|---|
| SCORE | 0 | 1 | 2 | 3 | 4 | 6 | 9 |
| RANK  | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
Gene set overlap and gene set enrichment analysis

Gene set enrichment analyses (GSEAs) were performed using the respective tool on the Gene Set Enrichment Analysis webpage (http://software.broadinstitute.org/gsea/index.jsp) using the predefined gene set databases (Subramanian et al., 2005). Gene set enrichment analyses (GSEAs) were performed on the GenePattern server (Reich et al., 2006) using the respective module and using either precompiled gene set databases or gene sets manually downloaded from the GSEA webpage above. Statistical significance in the GSEAs was performed using permutations of the gene sets rather than samples.

Medulloblastoma subgroup classification

Classification of RNA-Seq-derived transcription profiles into the four MB molecular subgroups (WNT, SHH, G3, G4) was performed using the metagene code for cross-platform, cross-species projection of transcription profiles (Tamayo et al., 2007). Raw CEL files for a total of 103 MB samples with known subgroup affiliation (eight WNT cases, 33 SHH cases, 27 Group 3 cases, and 35 Group 4 cases) (Northcott et al., 2011) were downloaded from ArrayExpress (E-GEOD-21140). CEL files were processed in the Affymetrix Expression Console using the Robust Multichip Average (RMA) method for background correction, quantile normalization, and probe summarization (Bolstad et al., 2003). To facilitate comparability between RNA-Seq and microarray identifiers, all gene names were translated using the HUGO Gene Nomenclature Committee (Gray et al., 2015). The expression profiles of the 103 MB samples were used to establish metagene classification signatures based on genes, which exhibited a FC > 5 and an absolute difference in expression of at least 600 between the means of any two subgroups. Subgroup affiliation of MB002 empty vector, MB002 SOX9-WT, and MB002 SOX9-T236/240R cells was then determined by a hierarchical clustering of the MB002 cell lines together with MB samples using the expression of metagenes. The result of the clustering was additionally verified by a Support Vector Machine (SVM) classification (data not shown).

Comparison to putative SOX9 target genes

Putative SOX9 target genes identified from three murine SOX9 transcription factor ChIP studies were downloaded (Kadaja et al., 2014; Oh et al., 2014; Larsimont et al., 2015) and matched to human orthologs using the BioMart software tool (http://www.ensembl.org/biomart/martview) and human gene names translated using the HUGO Gene Nomenclature Committee (Gray et al., 2015). All three studies investigated expression of putative target genes in the presence as well as conditional ablation/deletion of SOX9, producing a list of target genes potentially activated or repressed by SOX9. After mapping of orthologs and gene name translation, the study by Larsimont et al resulted in a list of 259 genes putatively activated by SOX9 and 201 genes putatively repressed by SOX9. From the study by Kadaja et al, 60 genes activated by and 12 genes repressed by SOX9 were extracted, while the study by Oh et al proposed 180 genes activated by and six genes repressed by SOX9. Putative target genes activated by SOX9 were mapped to genes significantly upregulated in SOX9-T236/240A as compared to WT, while target genes putatively repressed by SOX9 were mapped to genes significantly downregulated in SOX9-T236/240A as compared to SOX9-WT.

Quantitative real-time PCR (qRT–PCR)

To perform the qRT–PCR, RNA was first isolated using TRIzol reagent (Invitrogen, Life Technologies), followed by RNeasy Mini Kit (Qiagen). A total of 50 ng of the purified RNA was then used for cDNA synthesis (SuperScript VILO cDNA Synthesis Kit; Invitrogen, Life Technologies). Before preparing the PCR, the cDNA was diluted ten times and 4 µl was used for each PCR. Quantitative PCRs were carried out using SYBR Green or TaqMan probes as described in the Appendix. The sequence for each primer and probe can be found in the Appendix. The data were analyzed with ΔΔCT method and presented as relative expression.

Mice xenograft and cisplatin treatment

Tumor formation was monitored following stereotactic injection of 10³ medulloblastoma cells. Tumor cells were injected into the cerebellum of 6- to 9-week-old athymic nude Foxn1nu mice (obtained from Harlan Laboratories) as previously described (Swartling et al., 2012). All animal experiments were performed in accordance with local animal ethics committees. One week after transplantation, mice were treated intraperitoneally with cisplatin (2 mg/kg in 10% β-HP-cyclodextrin; Sigma-Aldrich) or vehicle (10% β-HP-cyclodextrin), for a total of six doses (administered every other day). Experimental endpoint for the treatment study was 80 days.

Statistical analysis

Data are presented as mean ± SD from two or more independent experiments. Statistical analyses were performed using the statistical package GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA; http://www.graphpad.com) using either log-rank (Mantel–Cox) analysis, one-way analysis of variance (ANOVA), or two-way analysis of variance (ANOVA) with Bonferroni’s post hoc test when appropriate. Correlation analyses were performed in MATLAB (MATLAB and Statistics Toolbox Release 2015b, The MathWorks, Inc., Natick, Massachusetts, USA) using either the Spearman rank correlation coefficient or Kendall rank correlation coefficient. Significance of overlap analyses between gene sets was determined in MATLAB using Fisher’s exact test. Survival analyses were performed in the R2: Genomics analysis and visualization platform using the implemented LogRank test.

Data availability

Primary data
Kool et al (2014); for gene expression values, GSE49243.

The MB002 RNA-Seq profiling data are available from NCBI with an access record of GSE84320.

Referenced data
Information about putative SOX9 target genes was obtained directly from the cited articles and/or the respective supplementary information (Kadaja et al., 2014; Oh et al., 2014; Larsimont et al., 2015). Raw CEL files with MB expression data from Northcott et al (2011) were downloaded from ArrayExpress (E-GEOD-21140).

Expanded View for this article is available online.
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Author contributions

The overall study was conceived and designed by ASR, FJS, and OS, with important contributions from VS. ASR and AB performed the majority of the in vitro experiments. SB prepared tumor samples for RNA-Seq and planned and performed in vivo studies and experimental treatments. SB and GR performed immunohistochemistry and subsequent analysis. GR and VS performed and analyzed RNAseq assays on TMAs. HW analyzed the RNA-seq data and performed the majority of bioinformatic analysis including clustering and GSEA of gene with important help from AS. HW, DTWJ, and MK performed and analyzed genes and protein expressions in the clinical cohorts. Y-JC, SMP, AK and MDT provided human medulloblastoma cohorts. Y-JC, SMP, AK and MDT performed and analyzed genes and protein expressions in the clinical cohorts. Y-JC, SMP, AK and MDT provided human medulloblastoma cohorts and tissue microarrays. AM designed primers and performed qPCR experiments. ASR, AM, MC, DK, SH and CS generated various plasmid constructs and retroviruses in this study. SH and AS provided technical support. ASR, VS, and HW analyzed the data and performed statistical analyses. ASR, VS, FJS, and OS wrote the manuscript with significant contributions from CS, SMP, and MK.

Conflict of interest

O Sangfelt and C Spruck report receiving a research grant from Astra Zeneca for a separate project. The other authors declare that they have no conflict of interest.

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Stabilization of SOX9 in medulloblastoma  Aldwin Suryo Rahmanto et al

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