Expanded View Figures

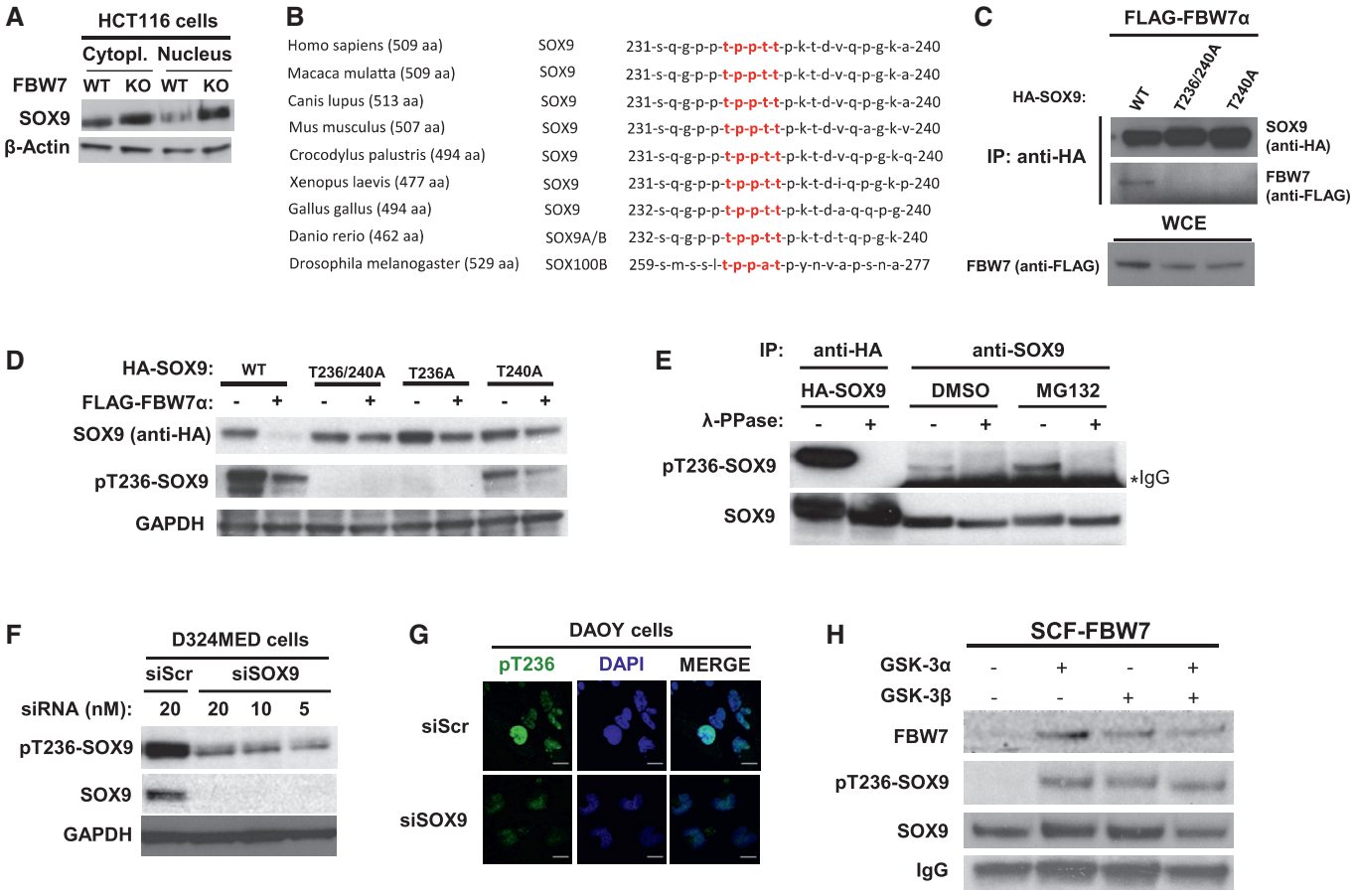


Figure EV1. SOX9 interacts with FBW7α through its conserved degron motif phosphorylated by GSK3.

A Western blotting of total SOX9 protein levels in the cytoplasmic and nuclear fractions of HCT116-FBW7 WT versus KO cells. The β-actin was used as a loading control.

B General evolutionary conservation for SOX9 amino acid sequence surrounding the human CPD motif (highlighted in red) of threonine 236–240 across species.

C Western blotting of FLAG-FBW7α eluted from the immunoprecipitated HA-SOX9 wild-type (WT) or CPD mutants (-T236/240A and -T240A). The HA-SOX9-WT and the CPD mutant constructs were transiently co-expressed for 24 h with FLAG-FBW7α in HEK293 prior to immunoprecipitation with anti-HA antibody. Equal protein expression of FBW7α across the HEK293 cells transfected with different SOX9 constructs was assessed by immunoblotting of the whole-cell extract.

D Co-expression of FBW7α with HA-SOX9 WT or various other CPD mutant constructs (-T236/240A, -T236A, or -T240A) in HEK293 cells. Whole-cell lysates were collected 24 h following transfection for Western blotting of the total exogenous and the phosphorylated SOX9 proteins using anti-HA and our pT236-SOX9 antibody, respectively. Immunoblot of GAPDH protein was used to indicate protein loading in each lane.

E Detection of both exogenous and endogenous phosphorylated SOX9 protein from SOX9 immunoprecipitates. HA-SOX9-transfected or non-transfected HEK293 cells were used as sources for exogenous and endogenous SOX9 protein, respectively. Following SOX9 immunoprecipitation with either anti-HA (for exogenous) or anti-SOX9 (for endogenous) antibody, the resulting immunoprecipitates were divided and either treated with λ-phosphatase or left untreated prior to gel electrophoresis and immunoblotting with pT236-SOX9 antibody. The SOX9 protein blot shows the total protein level present in each sample. Treatment of HEK293 with proteasome inhibitor MG132 (10 μM) increased the level of phosphorylated SOX9.

F Immunoblots of endogenous pT236 and total SOX9 protein 24 h following transfection of D324MED medulloblastoma cell line with either non-targeting scramble RNA (siScr) or increasing concentrations of siRNA against SOX9. GAPDH protein was used to indicated protein loading for each sample

G Representative immunofluorescence staining depicting high intensity of pT236-SOX9 (Alexa Fluor 488; green) staining in the nucleus (counterstained with DAPI; blue) in Daoy medulloblastoma cells. Transfection of Daoy cells with 20 nM siSOX9 depleted the nuclear staining of pT236-SOX9. Images were taken using a 40× objective. Scale bar: 20 μm.

H Bead-immobilized IVT HA-SOX9 WT were subjected to *in vitro* kinase reaction with 1 unit of recombinant active GSK3α, GSK3β, or their combination (i.e., 0.5 unit for each isoform) for 90 min at 37°C prior to elution and gel electrophoresis. The SOX9 blot shows total SOX9 protein eluted from the beads from each *in vitro* kinase reaction.

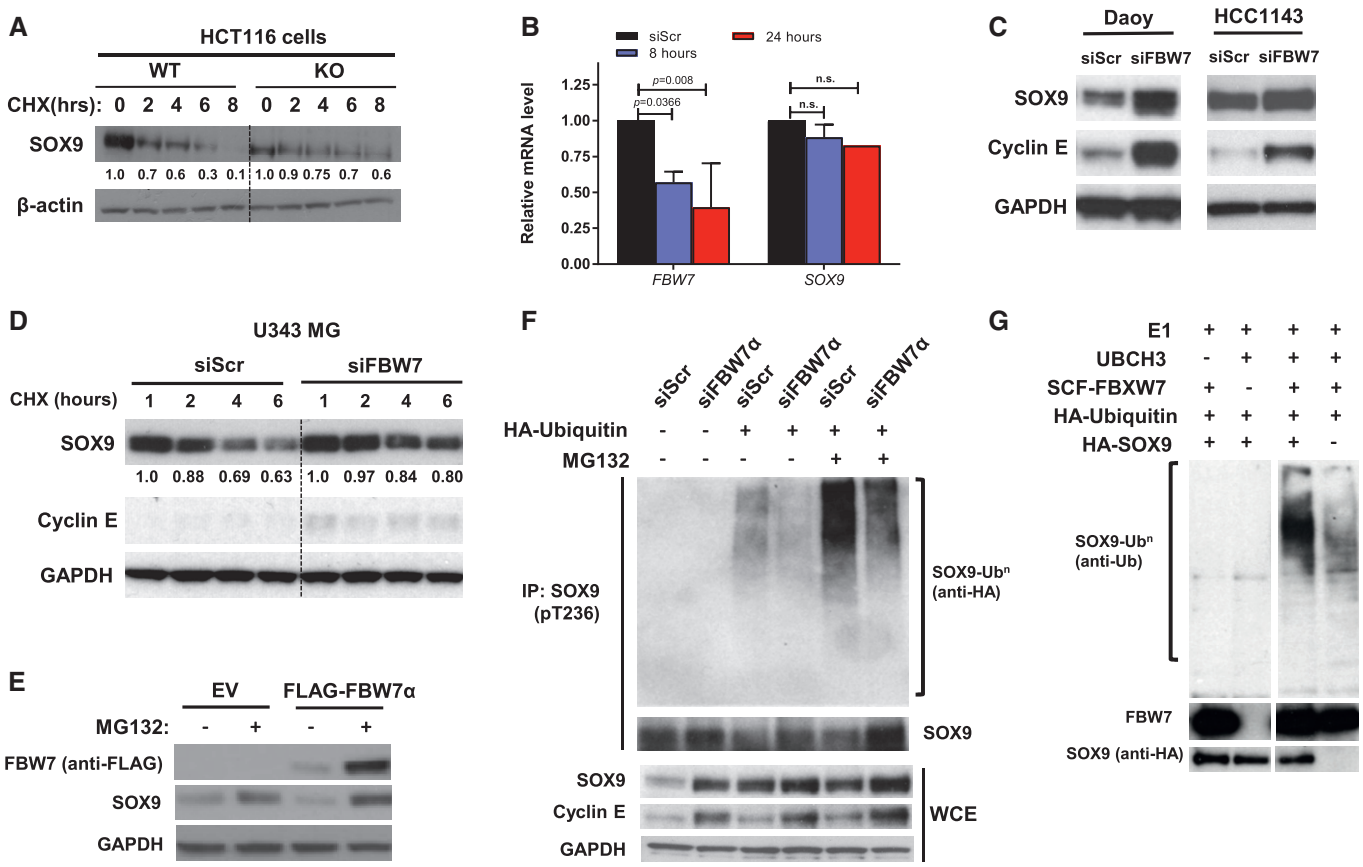


Figure EV2. FBW7α promotes SOX9 protein turnover in a GSK3-dependent manner through the ubiquitin–proteasome system.

A Endogenous SOX9 protein turnover in HCT116-FBW7 WT and KO cells over the course of 8 h following the addition of 100 ng/ml cycloheximide. β-Actin indicates total protein loading for each sample.

B Quantitative PCR analysis of FBW7 and SOX9 mRNA transcripts in Daoy at 8 and 24 h following transfection with 20 nM siFBW7. The FBW7 and SOX9 mRNAs were adjusted to the B2M mRNA prior to being expressed relative to the siScr control. Data are expressed as mean + standard deviation from two independent experiments, with statistical significance determined by multiple-comparison two-way ANOVA with Bonferroni's post-test.

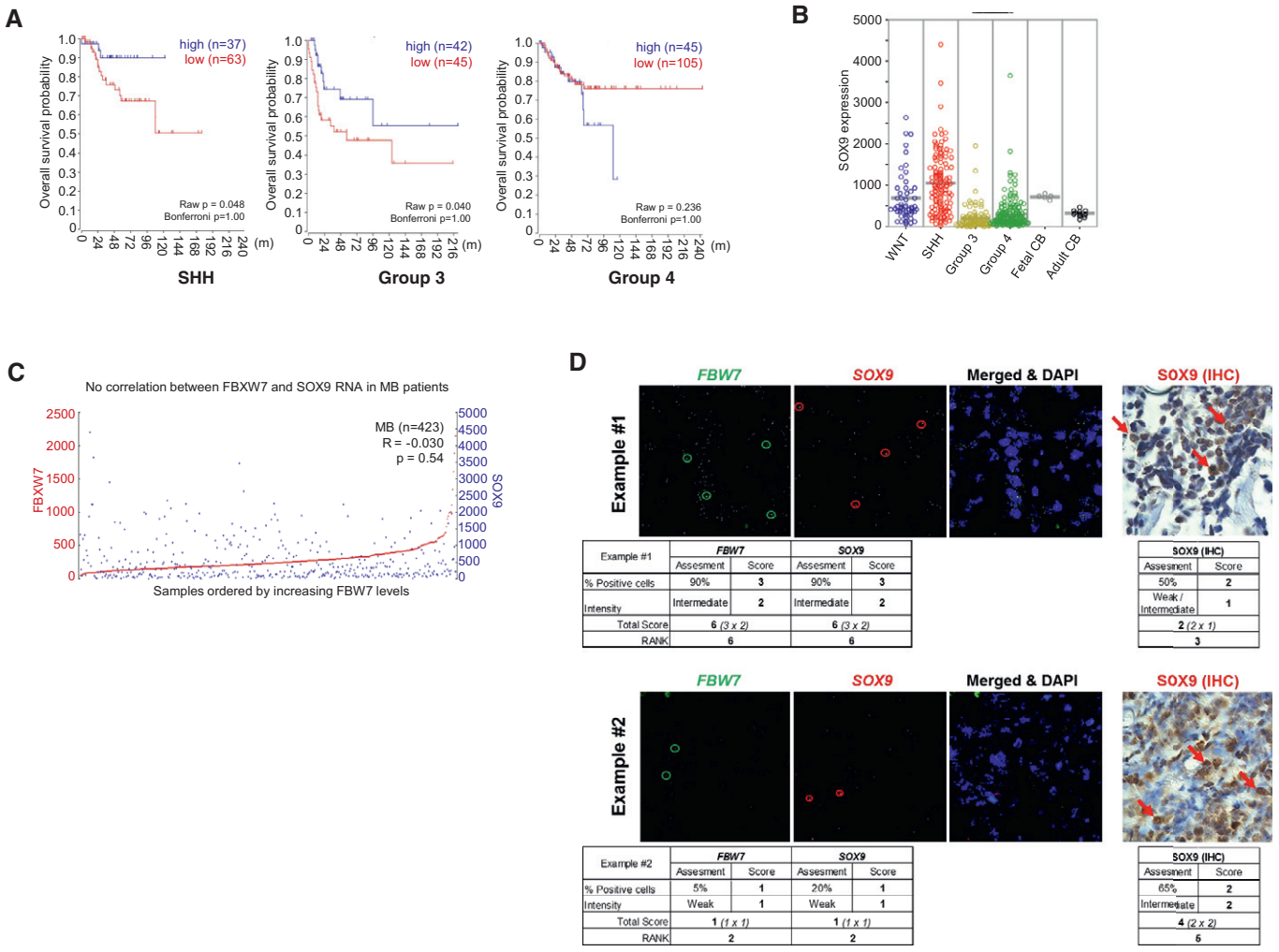
C Immunoblots of endogenous SOX9 protein in medulloblastoma cell line Daoy and breast cancer cell line HCC1143 following depletion of FBW7 by RNAi (20 nM) for 48 h. Accumulation of cyclin E is used to assess the efficiency of FBW7 knockdown. GAPDH immunoblot is shown as a loading control.

D Cycloheximide chase of endogenous SOX9 protein over the course of 6 h in glioma cell line U343MG. The cells were transfected with either non-targeting (siScr) or FBW7-specific siRNA for 72 h prior to experiments. Immunoblots of cyclin E, established SCF^{FBW7} substrate, indicated the efficacy of siFBW, while GAPDH protein was used as total protein loading control for each sample.

E Western blotting of endogenous SOX9 protein level upon treatment with 10 μM MG132. HEK293 cells were transfected with 1 μg FLAG-FBW7α for 24 h prior to treatment with the proteasome inhibitor. Whole-cell lysates were collected 4 h following MG132 treatment for gel electrophoresis and immunoblotting. GAPDH protein was immunoblotted to indicate total protein loading for each sample.

F RNAi depletion of FBW7α decreased endogenous SOX9 ubiquitylation in HEK293. The cells were transfected with either scramble (siScr) or siFBW7α for 72 h prior to assessment of endogenous SOX9 ubiquitylation in the absence or presence of MG132. Endogenous SOX9 protein was immunoprecipitated under denaturing condition (1% SDS) from the whole-cell lysate using the pT236-SOX9 antibody and eluted as described in Materials and Methods. Total SOX9, cyclin E, and GAPDH proteins in the whole-cell lysate were immunoblotted.

G Reconstitution of ubiquitylation reaction *in vitro* using bead-immobilized IVT HA-SOX9-WT and recombinant, active human $SCF^{FBW7α}$. Reaction mixture lacking the UbcH3, $SCF^{FBW7α}$, and IVT HA-SOX9-WT served as control for the experiments. Ubiquitylation was assessed following elution of IVT HA-SOX9-WT from the bead. Immunoblots of SOX9 and FBW7 proteins present in the eluted fraction are shown.



Example #1	FBW7		SOX9	
	Assesment	Score	Assesment	Score
% Positive cells	90%	3	90%	3
Intensity	Intermediate	2	Intermediate	2
Total Score	6 *(3 x 2)*		6 *(3 x 2)*	
RANK	6		6	

SOX9 (IHC)	
Assesment	Score
50%	2
Weak / Intermediate	1
2 *(2 x 1)*	
3	

Example #2	FBW7		SOX9	
	Assesment	Score	Assesment	Score
% Positive cells	5%	1	20%	1
Intensity	Weak	1	Weak	1
Total Score	1 *(1 x 1)*		1 *(1 x 1)*	
RANK	2		2	

SOX9 (IHC)	
Assesment	Score
65%	2
Intermediate	2
4 *(2 x 2)*	
5	

Figure EV3. SOX9 protein stabilization correlates with low levels of FBW7α in medulloblastoma patients.

A Overall survival in the different MB molecular subgroups of patients with low vs. high FBW7 expression. After Bonferroni correction, the results were not significant in any of the MB subgroups (Bonferroni P = 1.000).

B SOX9 expression in the four molecular subgroups of MB (423 tumors). Adult (n = 13) and fetal (n = 5) cerebellums were used as controls. ANOVA: P = 2.7e-05.

C FBW7 and SOX9 mRNA levels do not correlate with each other in a cohort of 423 SHH patients (R = −0.030; P = 0.54).

D Examples of FBW7 (green circles) and SOX9 RNAscope (red circles) images and corresponding SOX9 IHC protein staining (red arrows). Overall tumor expression analyses were performed based upon the "percentage of positive cells" and the staining "intensity" from at least three different image fields by two independent, blinded subjects for each tumor as described in detail in Materials and Methods. The overall score was subsequently converted to "Rank" for correlation analysis of SOX9 protein:RNA ratios against FBW7 expression.

Figure EV4. FBW7α circumvents SOX9-induced cell migration and metastasis in medulloblastoma.

A Immunoblots of WCE from SOX9-expressing Daoy cells with dox-inducible FBW7α expression. FBW7α expression was induced with different levels of dox for 24 h prior to cell harvesting for Western blotting. GAPDH immunoblot is shown as a loading control.

B Assessment of changes in Daoy cell viability upon constitutive expression of SOX9 and doxycycline induction of FBW7α. Changes in cell viability are expressed relative to day 0, at the time of seeding. Cell-doubling time compared to parental cells (Daoy T_d = 31.47 h vs. SOX9 T_d = 35.67 h), and FBW7α induction reversed this effect (SOX9 + FBW7α T_d = 30.20 h).

C Time-course Western blotting profiling of Daoy cells constitutively expressing SOX9 upon doxycycline induction of FBW7α.

D Quantification of Ki67-positive cells in SOX9-expressing Daoy cells with dox-inducible FBW7α tumors (+ and − dox). Representative IHC stainings used for quantification are shown. Scale bars: 50 μm. Box-and-whisker plots displaying the quantification of Ki67-positive cells. The horizontal line inside each box represents the median value, lower and upper box borders display the 25th and 75th percentiles, respectively, and the interval between the two whiskers include non-outlier values (min to max).

E IHC staining targeting human-specific epitope (STEM121) identifying transplanted human medulloblastoma cells in SOX9-expressing Daoy cells without FBW7α expression. Between the primary tumor and the metastatic compartment human tumor cell "droplets" are evident, as seen in the marked region 1.

F IHC targeting human-specific epitope (STEM121), identifying transplanted human medulloblastoma cells in SOX9-expressing Daoy cells with dox-induced FBW7α expression. Black arrow indicates the center part of the tumor (similar to the region indicated by the black arrow in H).

G Antibody targeting HA epitope identifying HA-SOX9 levels in SOX9-expressing Daoy cells without FBW7α expression. Between the primary tumor and the metastatic compartment human SOX9-positive tumor cell "droplets" are evident, as seen in the marked region 2.

H Antibody targeting HA epitope identifying HA-SOX9 levels in SOX9-expressing Daoy cells with dox-induced FBW7α expression. Black arrow indicates the center part of the tumor (similar to F) with reduced SOX9 expression to be compared to center regions of the tumor in (G).

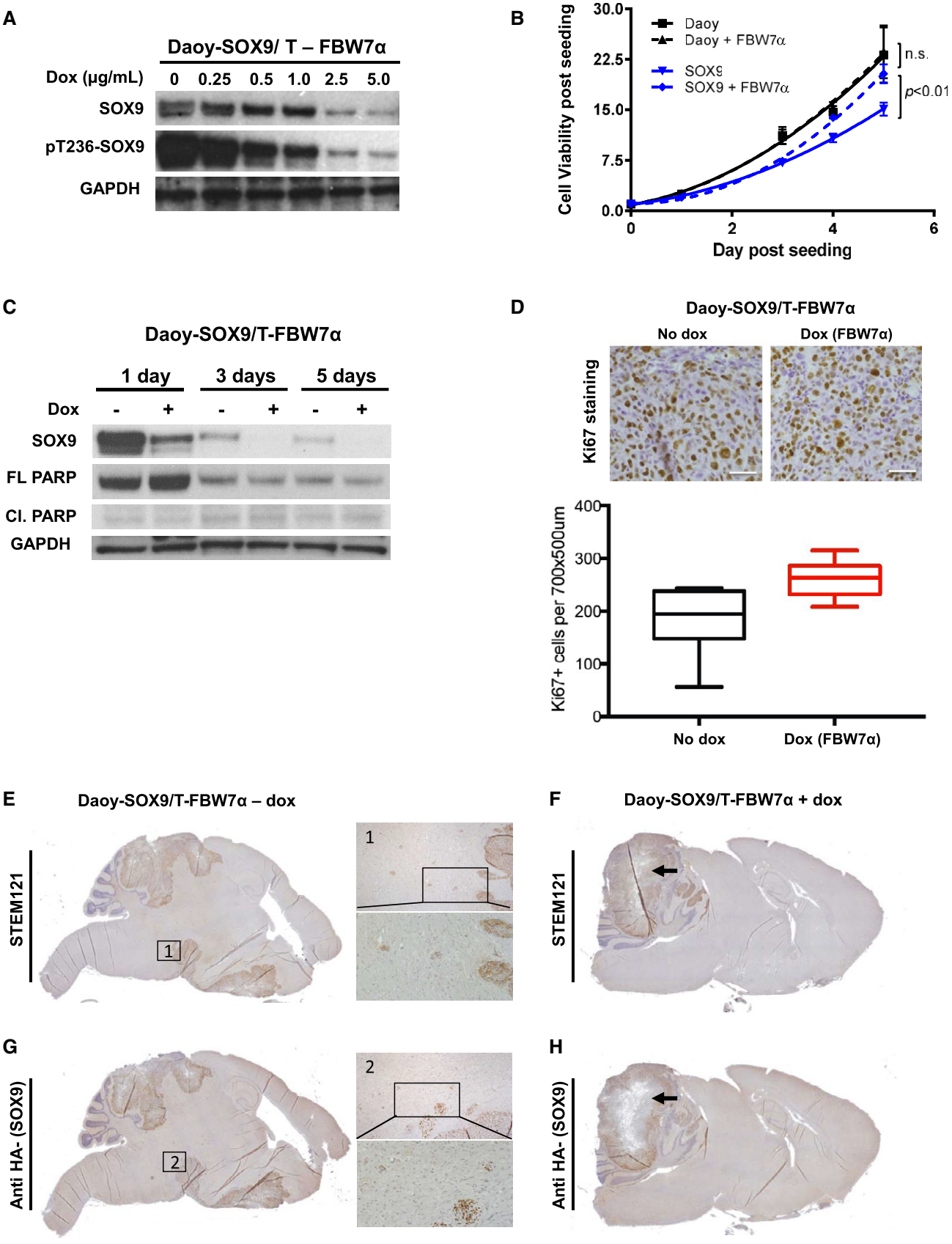


Figure EV4.

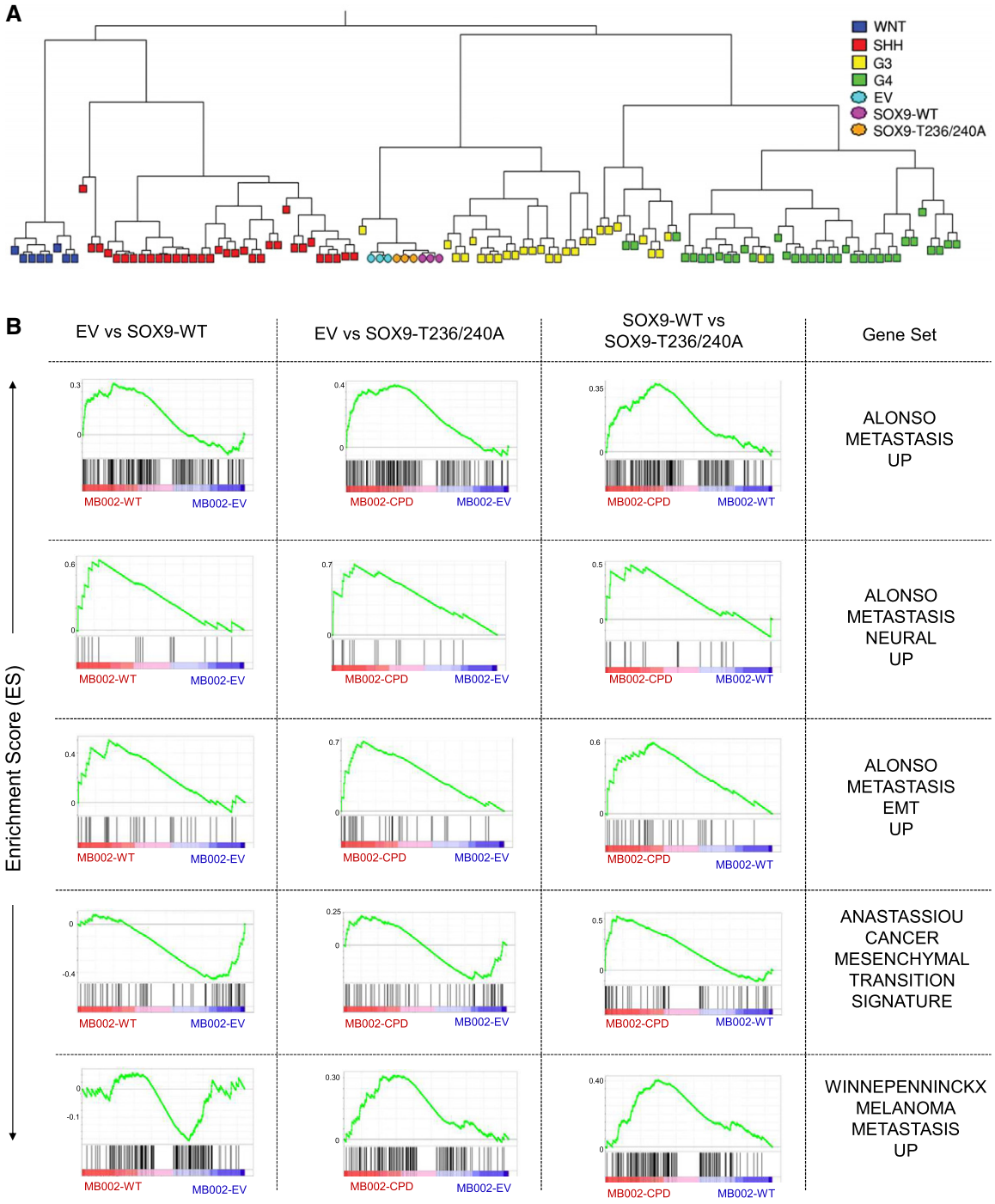


Figure EV5. Hierarchical clustering, MB subgroup classification, and GSEA of transcriptional profiles in MB002 cells overexpressing SOX9-WT or SOX9-T236/240A.

A Hierarchical clustering of 103 human medulloblastoma samples with known molecular subgroups (Northcott *et al*, 2011) together with triplicates each of MB002 cells transduced with EV (blue circles), SOX9-WT (orange circles), or SOX9-T236/240A (purple circles). The clustering was performed on the 3,167 signature genes most variably expressed between the four MB subgroups. Extraction of signature genes, hierarchical clustering, and plotting of the dendrogram were performed using the metagene code for cross-platform, cross-species projection of transcription profiles (Tamayo *et al*, 2007).

B Gene sets with significant enrichment in SOX9-expressing MB002 cells. A GSEA on a list of 19 gene sets related to metastasis, migration, or EMT (Table EV5) was performed for each pairwise comparison of MB002 EV, SOX9-WT, and SOX9-T236/240A. The figure shows the five gene sets (rows) with significant positive enrichment in either SOX9-WT against EV (left column), SOX9-T236/240A against EV (middle column), or SOX9-T236/240A against SOX9-WT (right column).

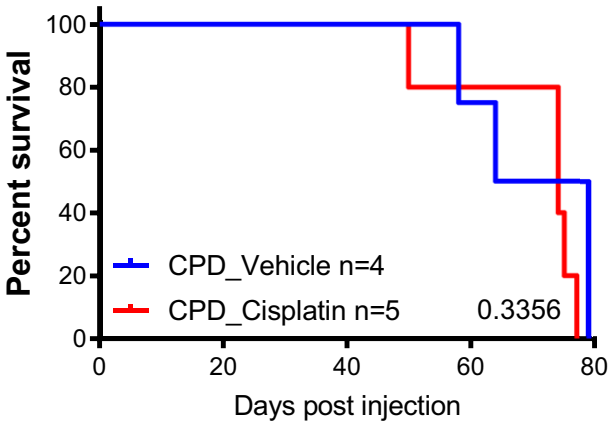


Figure EV6. SOX9 confers cisplatin resistance in medulloblastoma.
Overall survival of nude *Foxn1*^{nu} mice orthotopically engrafted with Daoy cells expressing SOX9-T236/240A treated with cisplatin (red curve) or a vehicle control (blue curve). The experiment was carried out as described in Fig 6C.

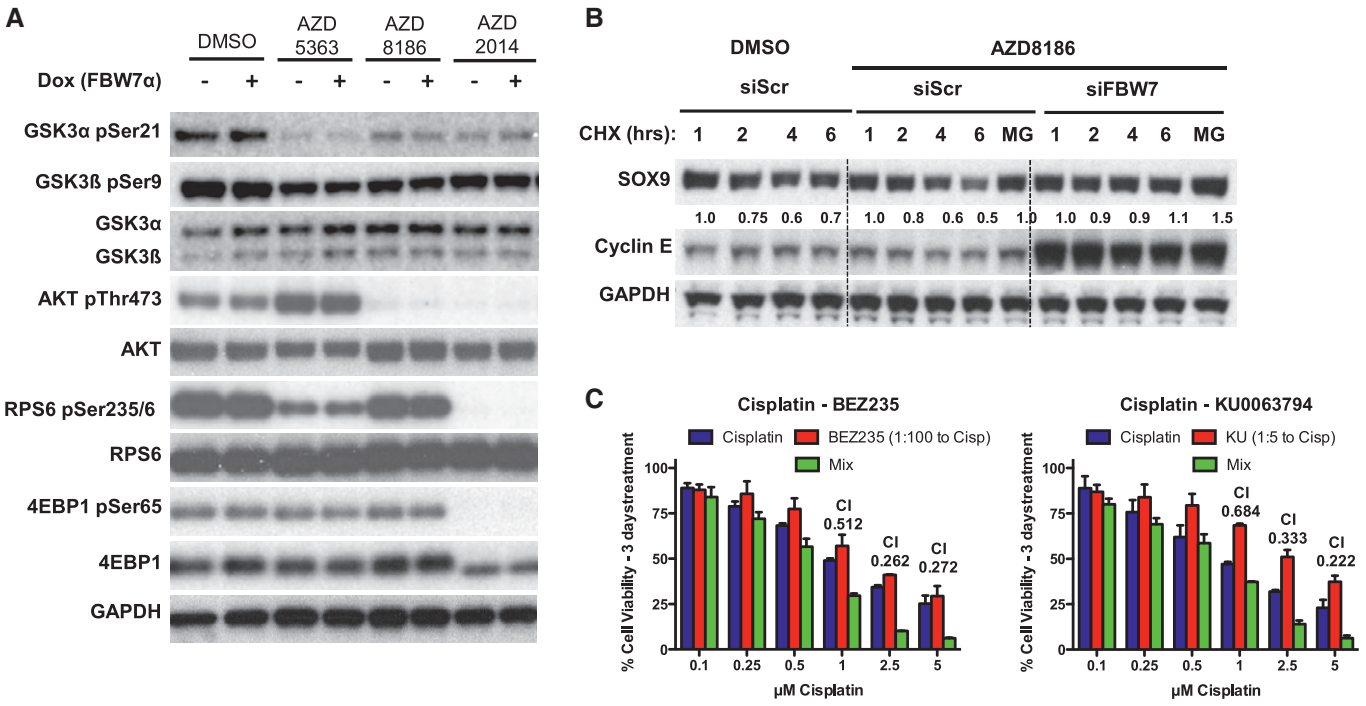


Figure EV7. SOX9 confers cisplatin resistance and targeting of SOX9 through PI3K/AKT/mTOR inhibition increases sensitivity to cisplatin treatment.

A Immunoblots of PI3K/AKT/mTOR pathway activity to assess inhibition upon treatment of Daoy cells with 1 µM AZD5363, AZD8186, or AZD2014.

B Western blotting of cycloheximide chase from Daoy cells treated with 1 µM PI3K inhibitor AZD8186. Cells were transfected with either non-targeting or FBW7-specific siRNA for 48 h prior to cycloheximide chase. siRNA depletion of FBW7 leads to stabilization of SOX9 protein and was calculated using Compusyn software.

C Cytotoxicity assay for Daoy medulloblastoma cells treated with a combination of cisplatin with (i) BEZ235 or (ii) KU0063794. The synthetic lethality combination index (CI) for each treatment was calculated using Compusyn software. Data are expressed as mean + standard deviation from two independent experiments.

D Quantitative PCR analysis of endogenous SOX9 mRNAs in MB002 following 24 and 72 h treatment with cisplatin, AZD2014, or their combination. The mRNAs levels were normalized relative to GAPDH and expressed relative to the DMSO-treated samples. Data are expressed as mean + standard deviation from two independent experiments, with statistical significance determined by multiple-comparison two-way ANOVA with Bonferroni's post-test.