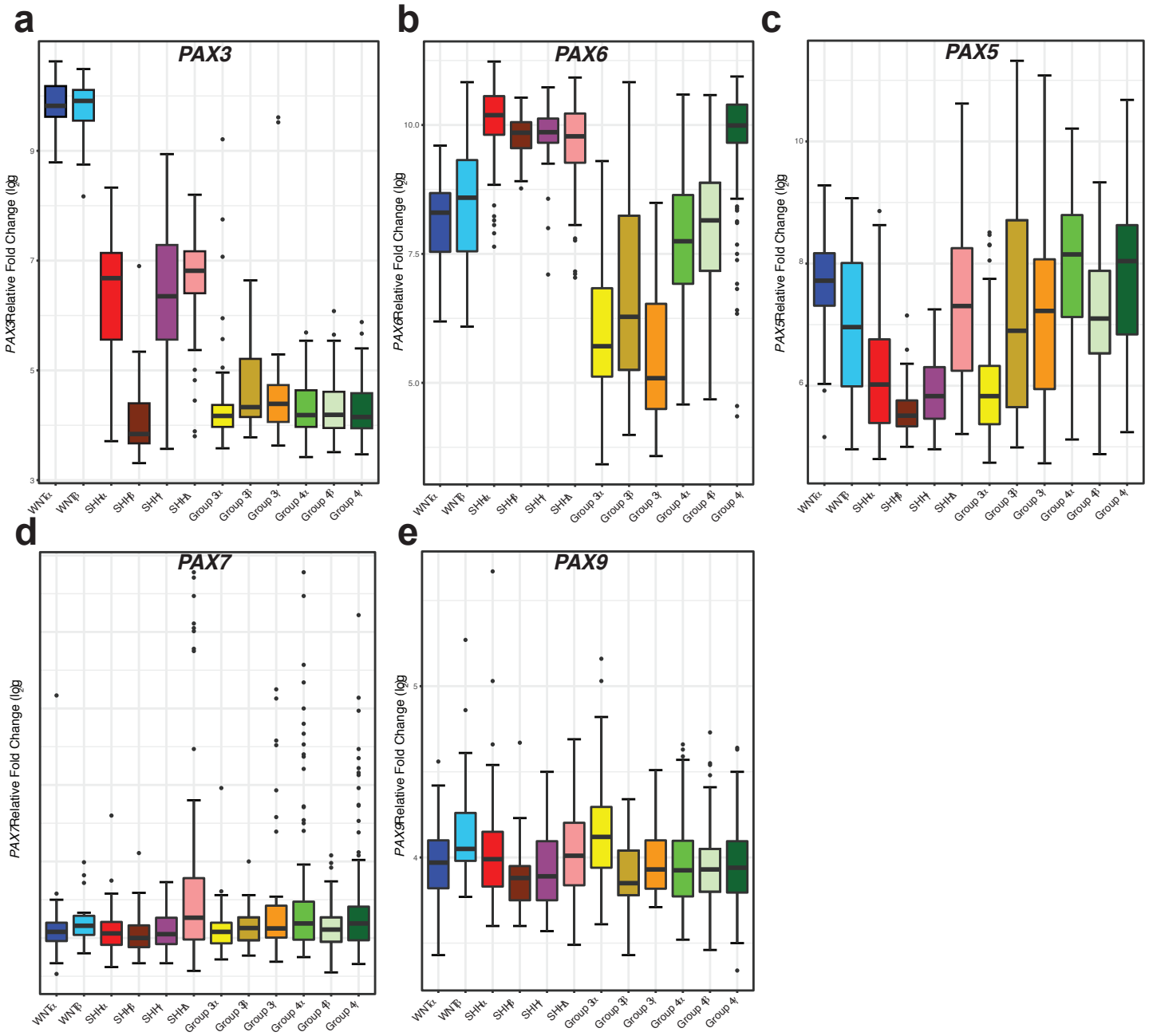
An OTX2-PAX3 signaling axis regulates Group 3 medulloblastoma cell fate

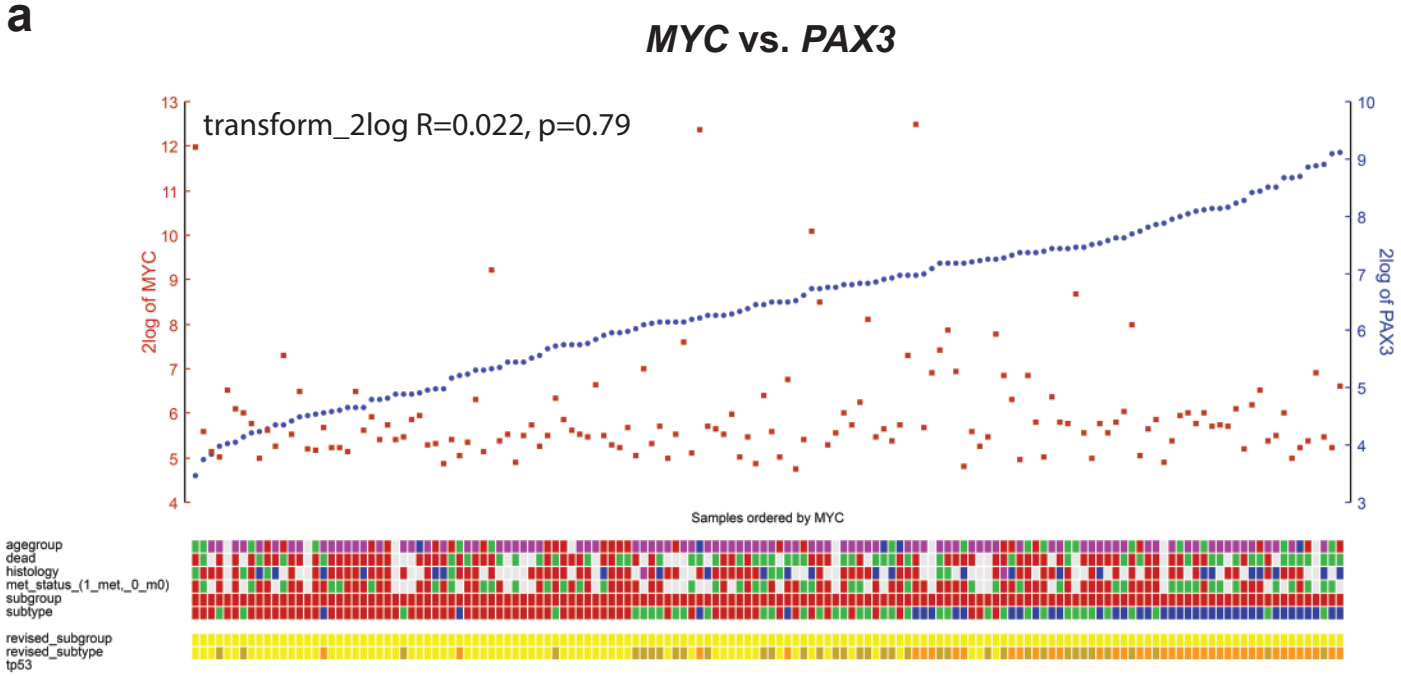
Zagozewski et al.,

Supplementary Information

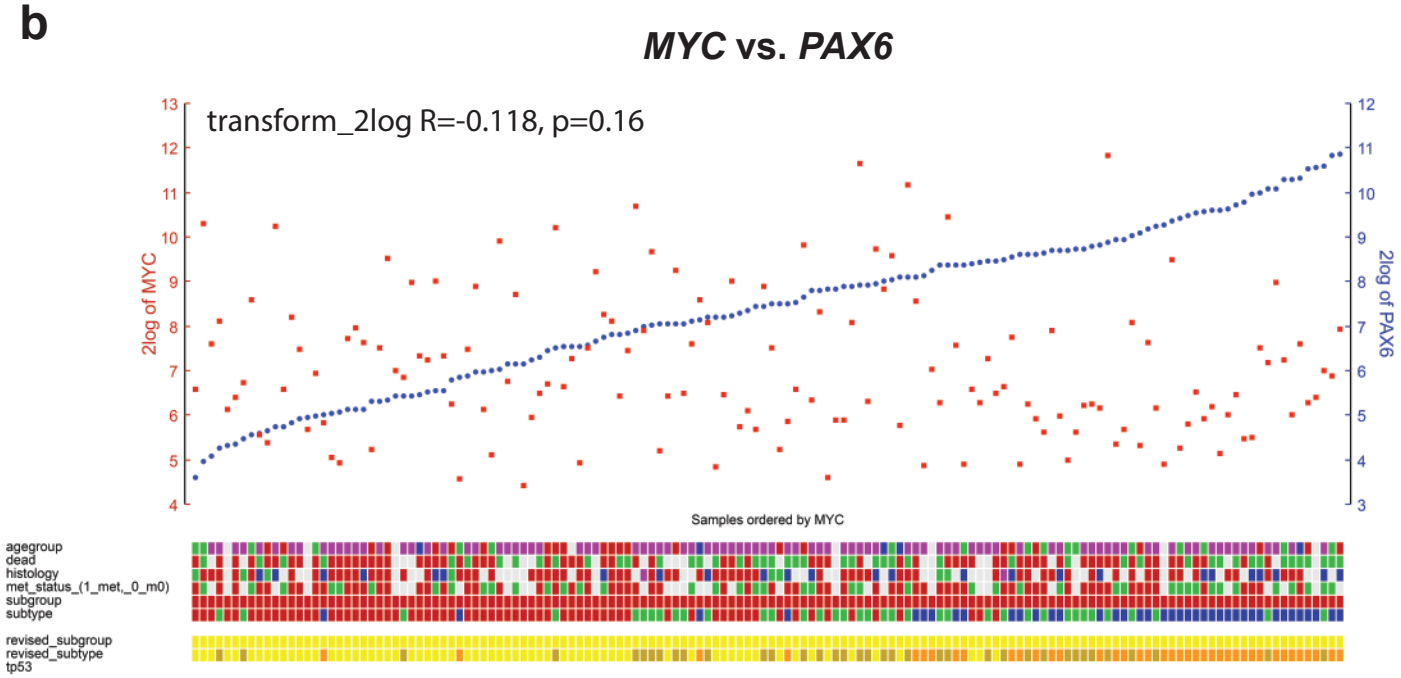

a
PAX3
PAX3 Relative Fold Change (log2)
b
PAX6
PAX6 Relative Fold Change (log2)
c
PAX5
PAX5 Relative Fold Change (log2)
d
PAX7
PAX7 Relative Fold Change (log2)
e
PAX9
PAX9 Relative Fold Change (log2)
WNTα
WNTβ
SHHα
SHHβ
SHH
SHHA
Group 3α
Group 3β
Group 3γ
Group 4α
Group 4β
Group 4γ

Supplementary Figure 1: PAX family gene expression across the 12 medulloblastoma subtypes. *PAX3* (a), *PAX6* (b), *PAX5* (c), *PAX7* (d) and *PAX9* (e) gene expression across the 12 medulloblastoma subtypes from 763 medulloblastoma patient samples (MAGIC cohort). The centre line represents the median, the box represents the interquartile range, and the whiskers are the 95% confidence intervals. Data are presented as log2-transformed signal intensity.

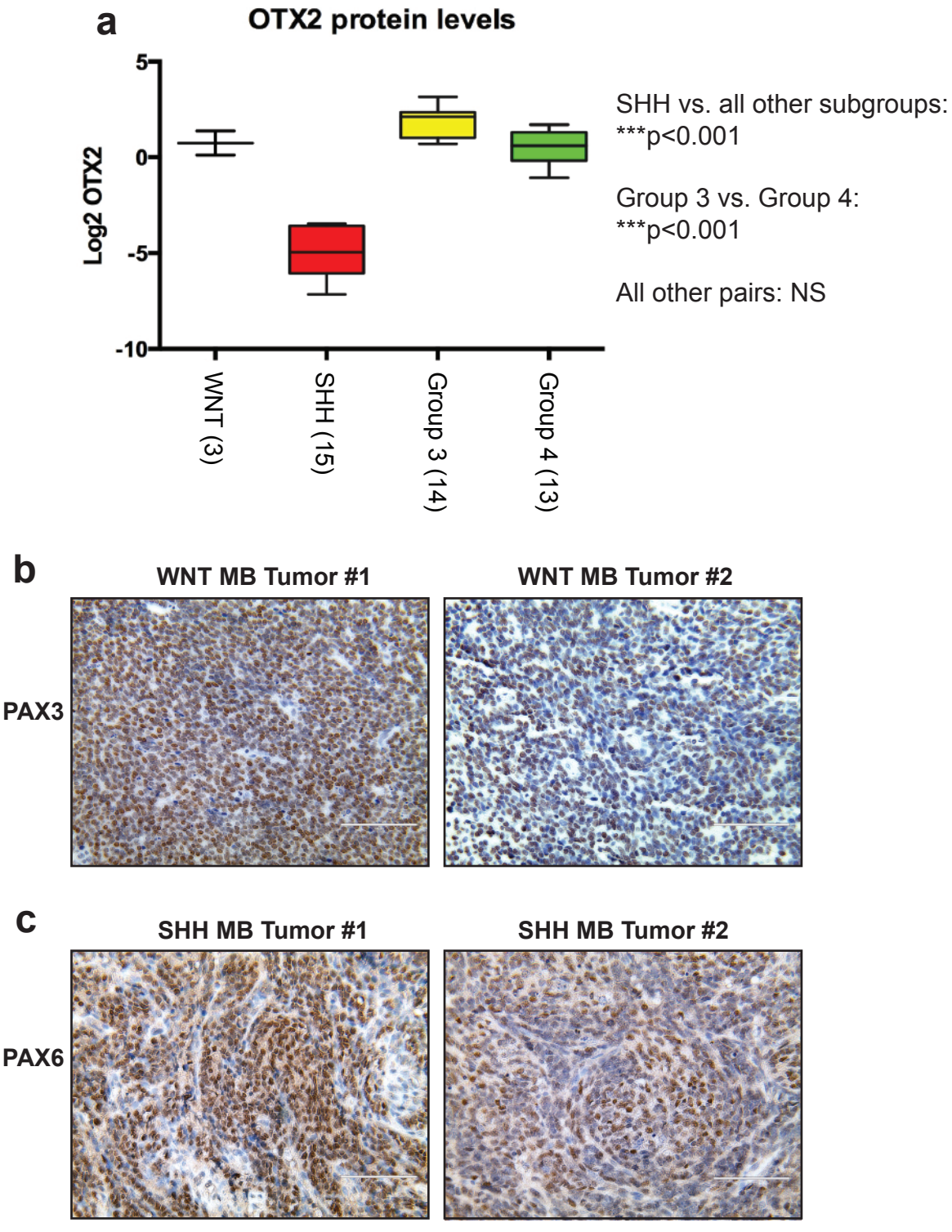
a


MYC vs. PAX3
transform_2log R=0.022, p=0.79
2log of MYC
2log of PAX3
Samples ordered by MYC
agegroup
dead
histology
met_status_(1_met,_0_m0)
subgroup
subtype
revised_subgroup
revised_subtype
tp53

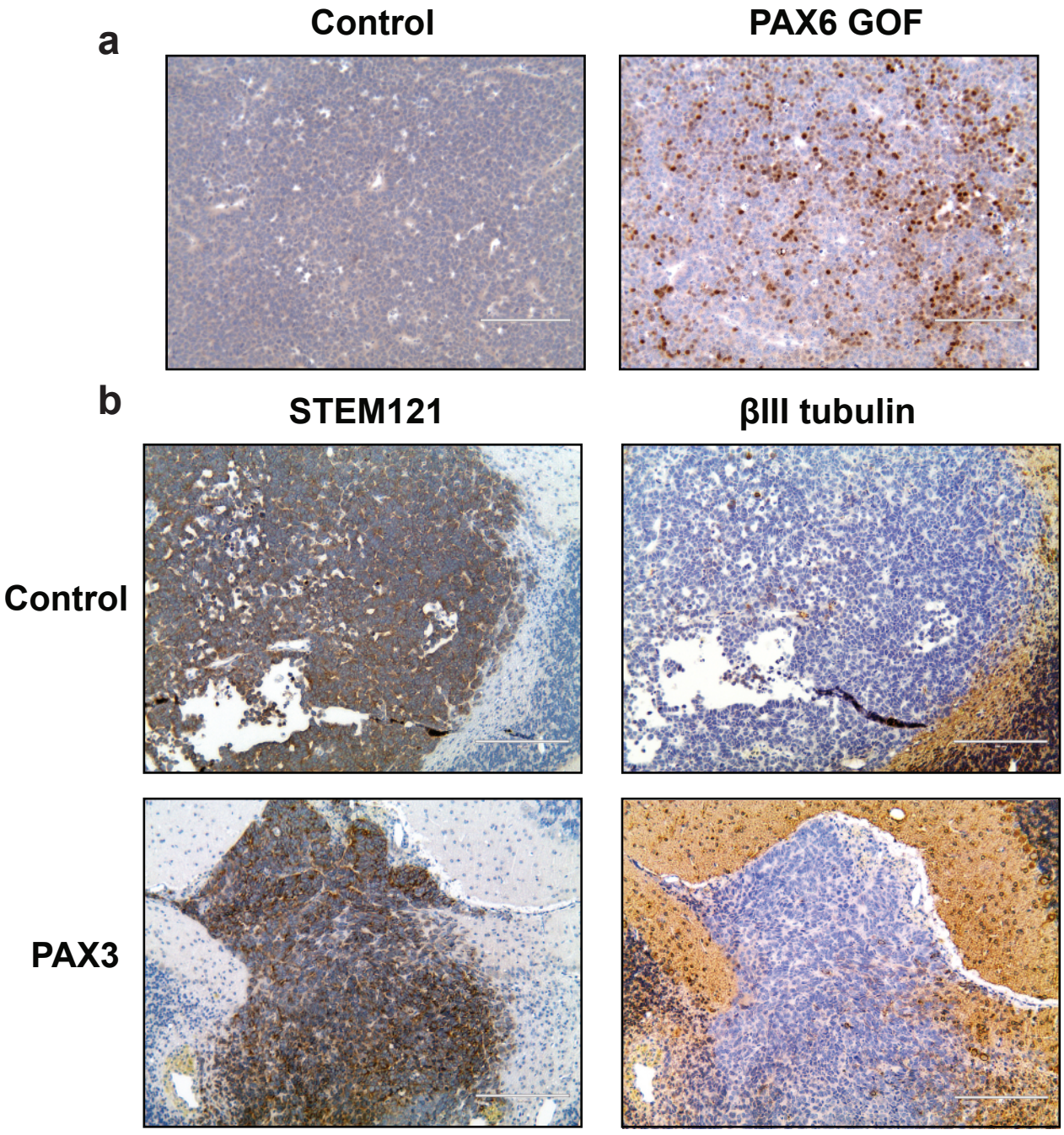
b


MYC vs. PAX6
transform_2log R=-0.118, p=0.16
2log of MYC
2log of PAX6
Samples ordered by MYC
agegroup
dead
histology
met_status_(1_met,_0_m0)
subgroup
subtype
revised_subgroup
revised_subtype
tp53

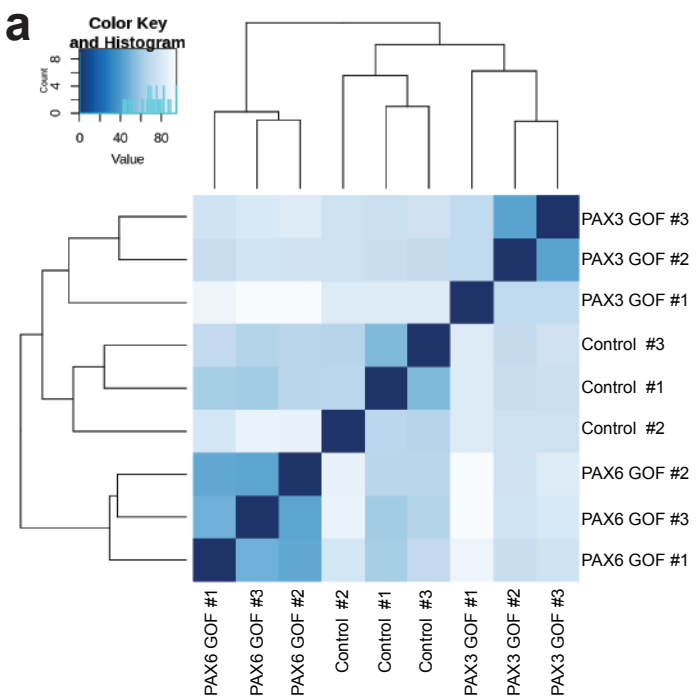
Supplementary Figure 2: *PAX*3 and *PAX6* expression are not correlated with *MYC* expression in Group 3 medulloblastoma patient samples. YY scatterplots evaluating the correlation between *MYC* and *PAX3* (a) and *MYC* and *PAX6* (b) expression in Group 3 medulloblastoma patient samples using a Pearson correlation test. Data are presented as log2-transformed signal intensity.

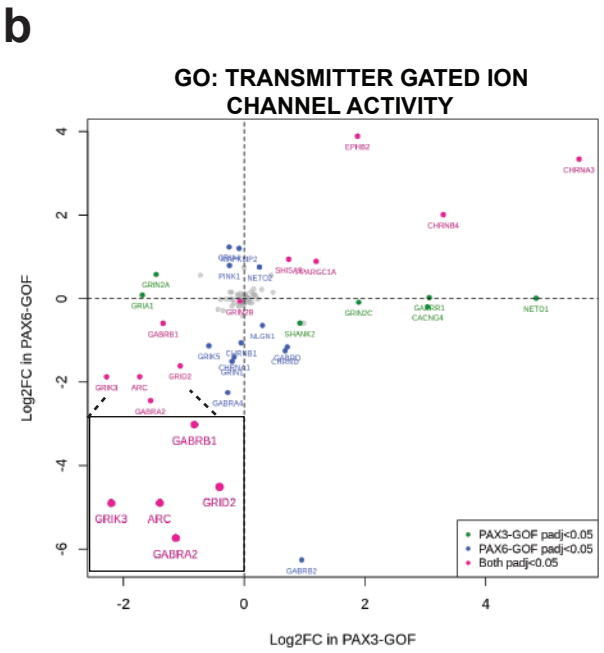

a
OTX2 protein levels
Log2 OTX2
5
0
-5
-10
WNT (3)
SHH (15)
Group 3 (14)
Group 4 (13)
SHH vs. all other subgroups:
***p<0.001
Group 3 vs. Group 4:
***p<0.001
All other pairs: NS
b
WNT MB Tumor #1
WNT MB Tumor #2
PAX3
c
SHH MB Tumor #1
SHH MB Tumor #2
PAX6

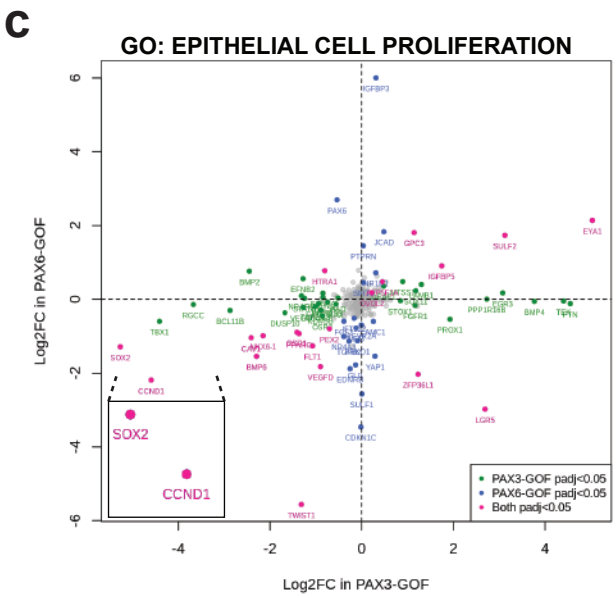
Supplementary Figure 3: OTX2, PAX3 and PAX6 levels in representative medulloblastoma patient samples. a. Proteomic analysis (Boston cohort) shows high OTX2 levels in WNT, Group 3 and Group 4 medulloblastoma patient samples (45 total). SHH vs. all others, ***$p<0.001$, Group 3 vs. Group 4, ***$p<0.001$, all other pairs NS. Significance was determined by one-way ANOVA. **b.** Images of PAX3 IHC staining in representative formalin-fixed, paraffin embedded sections from 2 primary WNT medulloblastoma samples. Scale bar: 100 μm. **c.** Images of PAX6 IHC staining in representative formalin-fixed, paraffin embedded sections from 2 primary SHH medulloblastoma samples. Scale bar: 100 μm.

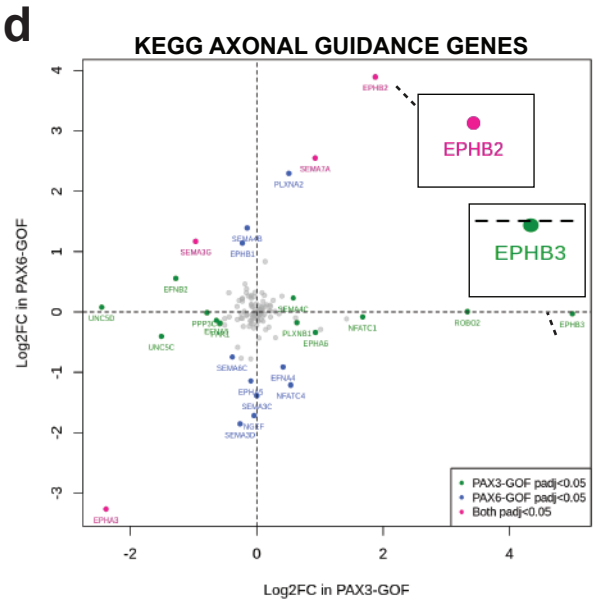

a
Control
PAX6 GOF
b
STEM121
βIII tubulin
Control
PAX3

Supplementary Figure 4: Immunohistochemical analysis following transplantation of control, PAX3 and PAX6 GOF HDMB03 medulloblastoma cells into NOD SCID mice. a. PAX6 levels in control (left) relative to PAX6 GOF (right) HDMB03 medulloblastoma tumor-bearing NOD SCID mice Scale bar: 200 μm. IHC was performed on 4 independent tumor samples for both control and PAX6 GOF. **b.** Representative sister sections depicting STEM121(left) and βIII tubulin levels (right) in control (upper) and PAX3 GOF (lower) HDMB03 medulloblastoma tumor-bearing NOD SCID mouse. Scale bar: 200 μm. IHC was performed on 4 independent tumor samples for both control and PAX3 GOF.

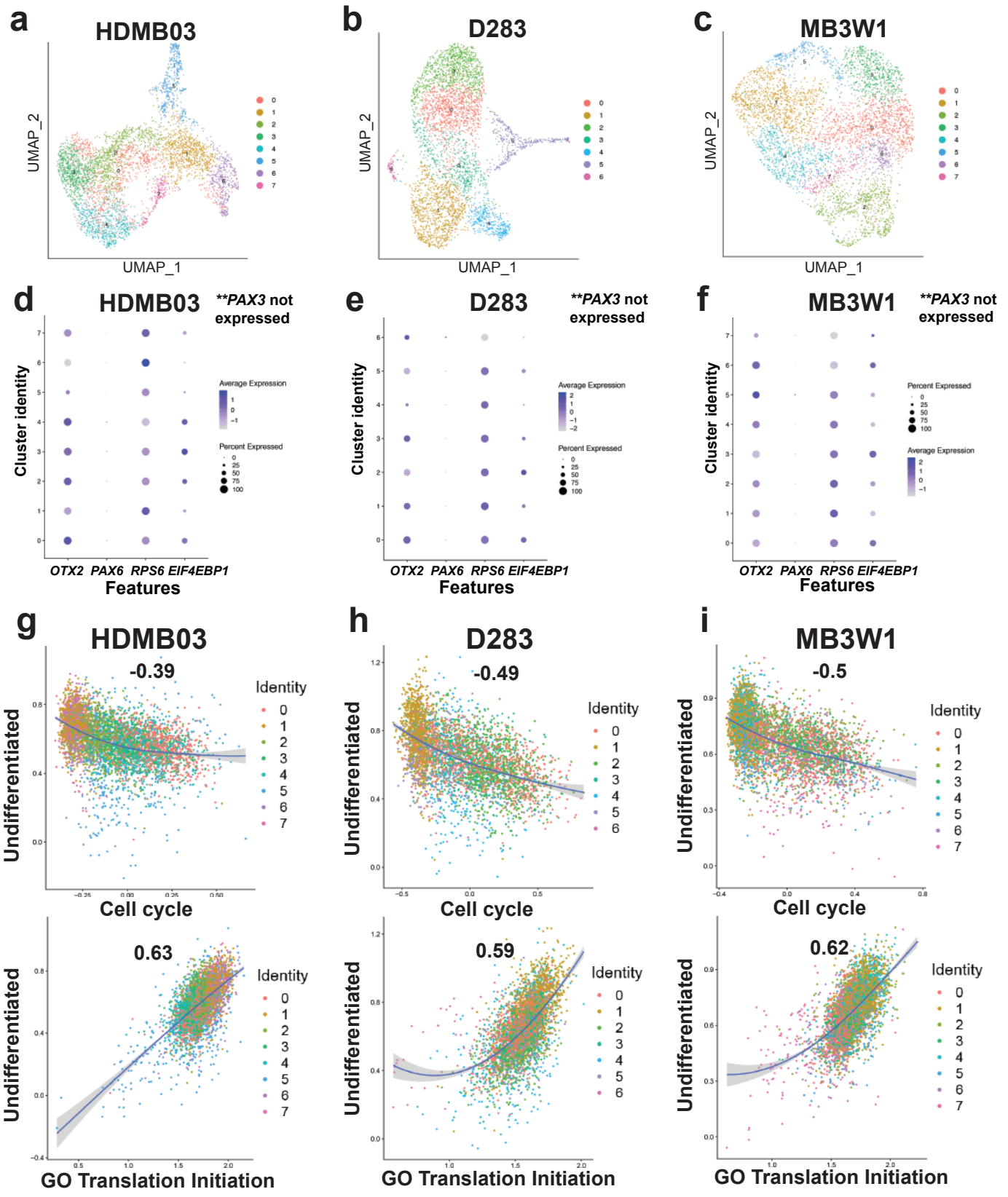

a
Color Key and Histogram
Count
Value
PAX3 GOF #3
PAX3 GOF #2
PAX3 GOF #1
Control #3
Control #1
Control #2
PAX6 GOF #2
PAX6 GOF #3
PAX6 GOF #1


b
GO: TRANSMITTER GATED ION CHANNEL ACTIVITY
Log2FC in PAX6-GOF
Log2FC in PAX3-GOF
GABRB1
GRID2
GRIK3
ARC
GABRA2
PAX3-GOF padj<0.05
PAX6-GOF padj<0.05
Both padj<0.05

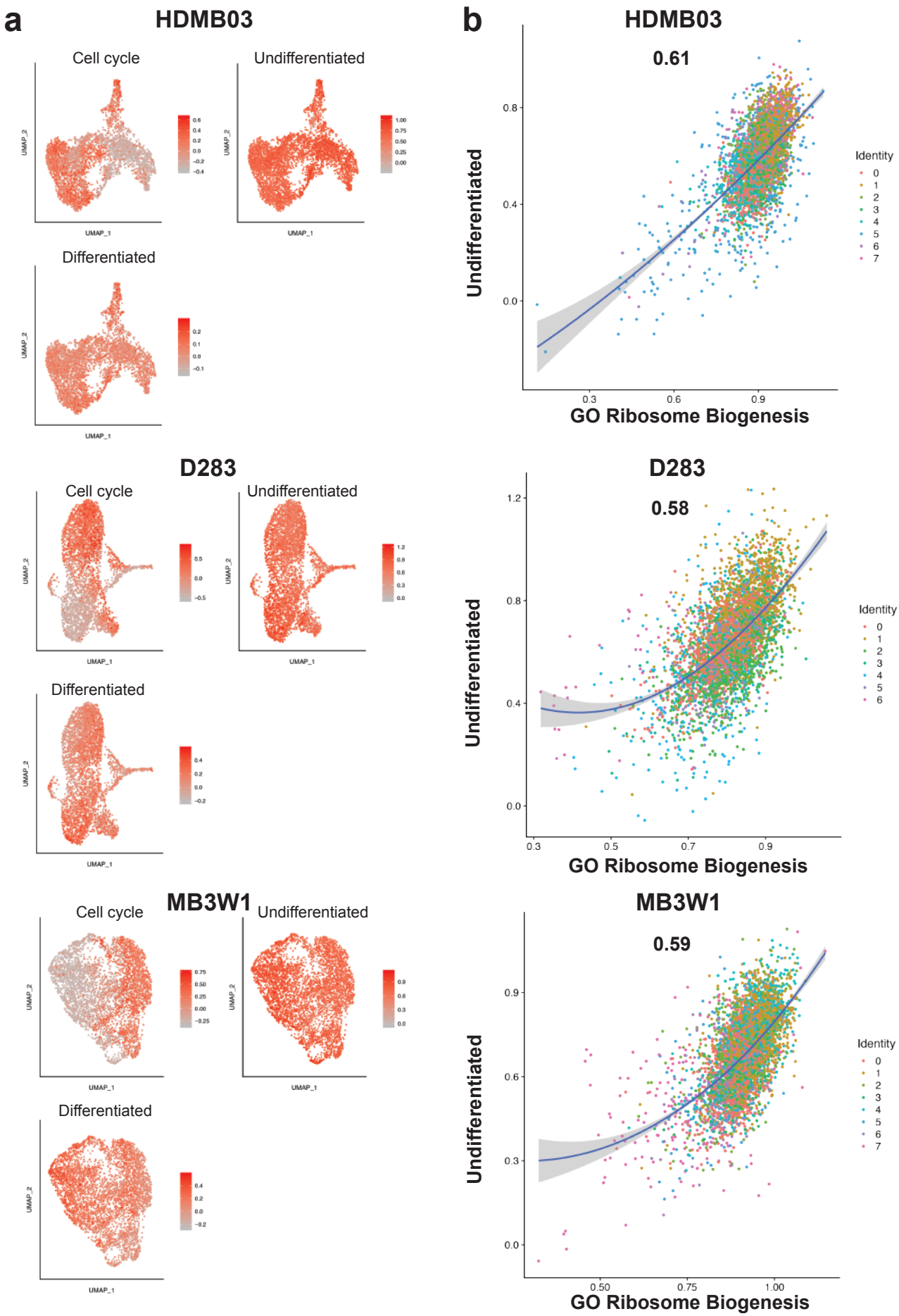

c
GO: EPITHELIAL CELL PROLIFERATION
Log2FC in PAX6-GOF
Log2FC in PAX3-GOF
SOX2
CCND1
PAX3-GOF padj<0.05
PAX6-GOF padj<0.05
Both padj<0.05


d
KEGG AXONAL GUIDANCE GENES
Log2FC in PAX6-GOF
Log2FC in PAX3-GOF
EPHB2
EPHB3
PAX3-GOF padj<0.05
PAX6-GOF padj<0.05
Both padj<0.05

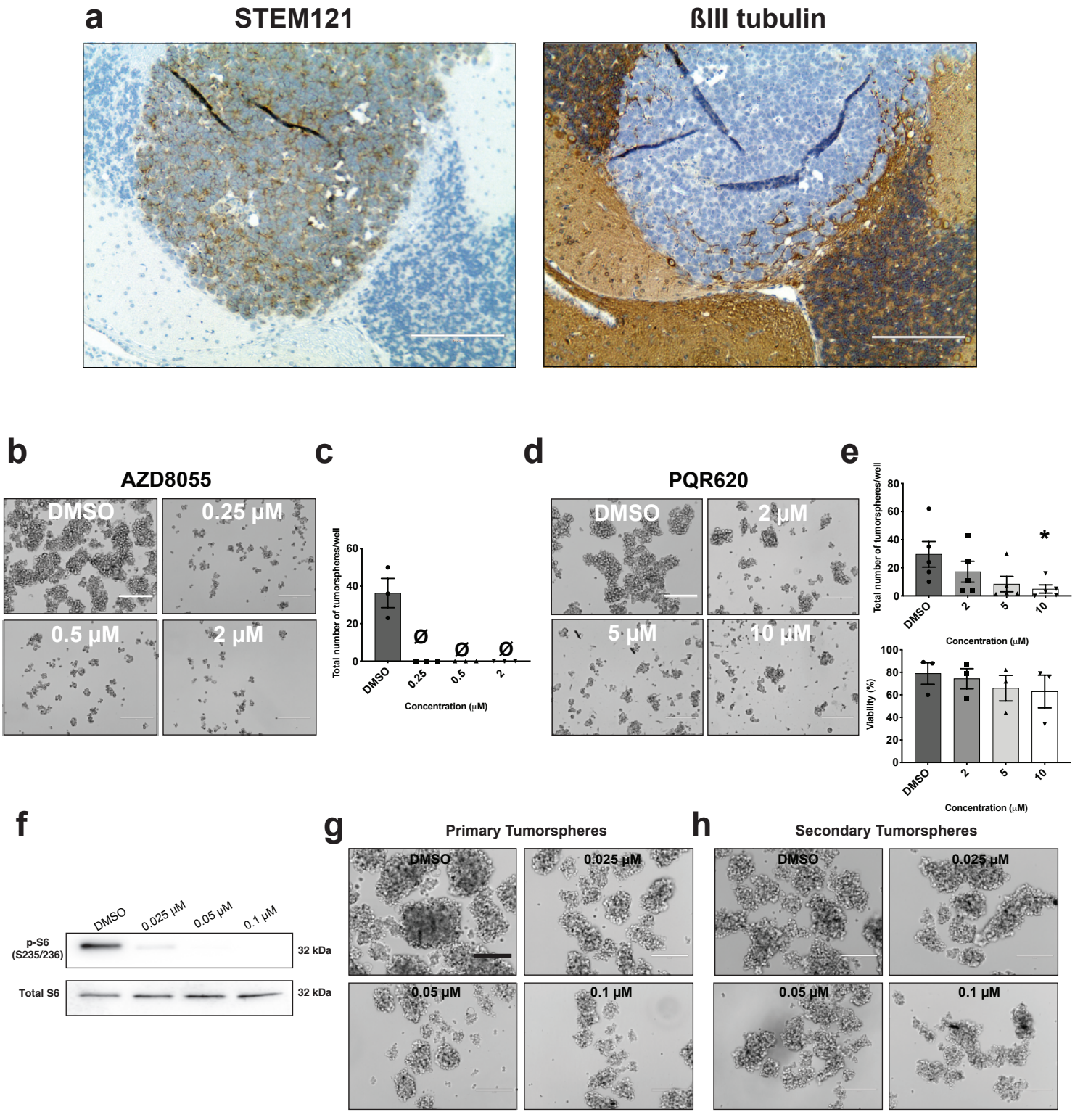
Supplementary Figure 5: PAX3 and PAX6 expression perturb Group 3 transcriptional signatures as well as genes associated with self-renewal and differentiation. a. Hierarchical clustering calculated using Euclidian distance between rlog-transformed normalized count values for all transcripts. **b-d.** Enrichment of GO molecular function/biological process terms and KEGG pathways in up- and down-regulated genes associated with PAX3, PAX6 or both PAX3 and PAX6 GOF tumorspheres. For transmitted gated ion channel activity (b), epithelial cell proliferation (c) and axonal guidance genes (d), Green= genes differentially expressed in PAX3 GOF cells, Blue= genes differentially expressed in PAX6 GOF cells, and Pink= genes differentially expressed in both PAX3 and PAX6 GOF cells. Significantly different genes were identified using a q-value (Benjamini-Hochberg corrected p-value) cut-off of 0.05. For all genes * $p<0.05$.


a
HDMB03
UMAP_2
UMAP_1
b
D283
UMAP_2
UMAP_1
c
MB3W1
UMAP_2
UMAP_1
d
HDMB03
**PAX3 not expressed
Cluster identity
Average Expression
Percent Expressed
OTX2 PAX6 RPS6 EIF4EBP1
Features
e
D283
**PAX3 not expressed
Cluster identity
Average Expression
Percent Expressed
OTX2 PAX6 RPS6 EIF4EBP1
Features
f
MB3W1
**PAX3 not expressed
Cluster identity
Percent Expressed
Average Expression
OTX2 PAX6 RPS6 EIF4EBP1
Features
g
HDMB03
-0.39
Undifferentiated
Identity
Cell cycle
0.63
Undifferentiated
Identity
GO Translation Initiation
h
D283
-0.49
Undifferentiated
Identity
Cell cycle
0.59
Undifferentiated
Identity
GO Translation Initiation
i
MB3W1
-0.5
Undifferentiated
Identity
Cell cycle
0.62
Undifferentiated
Identity
GO Translation Initiation

Supplementary Figure 6: scRNA-seq analyses of Group 3 MB tumorspheres reveals a correlation between the undifferentiated stem/progenitor cell population and protein synthesis pathways. a-c. UMAP visualization of transcriptionally distinct cell populations from HDMB03 (a), D283 (b) and MB3W1 (c) Group 3 MB tumorspheres. **d-f.** Dot plots demonstrating frequency and average expression of selected genes from single cell clusters of HDMB03 (d), D283 (e) and MB3W1 (f) Group 3 MB tumorspheres. **g-i.** Correlation plots displaying the relationship between the undifferentiated program and cell cycle (upper) as well as the undifferentiated program and translation initiation (lower) for HDMB03 (g), D283 (h) and MB3W1 (i) Group 3 MB tumorspheres. For the 3 biologically independent cell lines, n=6307 cells (HDMB03), n=5297 cells (D283), and n=5099 cells (MB3W1). Shaded area represents the 95% confidence level interval for predictions from a loess model.


a
HDMB03
Cell cycle
Undifferentiated
Differentiated
UMAP_1
UMAP_2
D283
Cell cycle
Undifferentiated
Differentiated
MB3W1
Cell cycle
Undifferentiated
Differentiated
b
HDMB03
0.61
Undifferentiated
GO Ribosome Biogenesis
Identity
D283
0.58
MB3W1
0.59

Supplementary Figure 7: Gene expression associated with transcriptional metaprograms defined by Hovestadt et al., Nature, 2019. a. Feature plots displaying the average expression of the top 30 genes in each metaprogram (cell cycle, undifferentiated and differentiated programs) overlain on the UMAP plots. **b.** Correlation plots between the undifferentiated metaprogram and genes involved in ribosome biogenesis (GO_Ribosome_Biogenesis) for HDMB03, D283, and MB3W1 Group 3 medulloblastoma tumorspheres. For the 3 biologically independent cell lines, n=6307 cells (HDMB03), n=5297 cells (D283), and n=5099 cells (MB3W1). Shaded area represents the 95% confidence level interval for predictions from a loess model.


a
STEM121
ßIII tubulin
b
AZD8055
DMSO
0.25 µM
0.5 µM
2 µM
c
Total number of tumorspheres/well
Concentration (µM)
DMSO
0.25
0.5
2
d
PQR620
DMSO
2 µM
5 µM
10 µM
e
Total number of tumorspheres/well
Viability (%)
Concentration (µM)
DMSO
2
5
10
f
DMSO
0.025 µM
0.05 µM
0.1 µM
p-S6 (S235/236)
Total S6
32 kDa
32 kDa
g
Primary Tumorspheres
DMSO
0.025 µM
0.05 µM
0.1 µM
h
Secondary Tumorspheres
DMSO
0.025 µM
0.05 µM
0.1 µM

Supplementary Figure 8: mTOR inhibitor treatment decreases Group 3 medulloblastoma tumorigenic properties *in vitro.* **a.** Representative sister sections depicting STEM121 and βIII tubulin levels in a 100 mg/kg PQR620-treated HDMB03 medulloblastoma tumor-bearing NOD SCID mouse. Scale bar: 200 μm. **b.** Representative images depicting decrease in tumorsphere number following AZD8055 treatment of MB3W1 Group 3 medulloblastoma cells. Scale = 200 μm. **c**. Quantification of total sphere number following MB3W1 Group 3 tumorsphere treatment with AZD8055. Data are presented as mean values ± SEM. N=3 biological replicates. Note that viability was not measured as there were no tumorspheres detected following treatment. **d.** Representative images depicting decrease in tumorsphere number following PQR620 treatment of MB3W1 Group 3 medulloblastoma cells. Scale = 200 μm. **e.** Quantification of total sphere number (upper) and viability (lower) following MB3W1 Group 3 tumorsphere treatment with PQR620. Data are presented as mean values ± SEM. P=0.047. N=5 biological replicates (sphere number) and N=3 biological replicates (viability). Results were analyzed using one-way ANOVA and a Dunnett test for multiple comparisons. **f.** Reduction in pS6 following 3-hour AZD8055 treatment (25-100 nM) of HDMB03 Group 3 tumorspheres. Total S6 serves as the loading control. **g.** Primary HDMB03 tumorspheres following AZD8055 treatment for 5 days at 25-100 nM. Scale = 200 μm. **h.** Secondary HDMB03 tumorspheres following AZD8055 treatment for 5 days at 25-100 nM. Scale = 200 μm.

Supplementary Table 1: List of antibodies used in the study

ChIP antibodies		
Name	Company/Catalog number	Concentration/dilution
H3K27me3	Millipore (07-449)	10 µg
H3K4me3	Abcam (ab8580)	5 µg
OTX2	Abcam (ab21990)	10 µg
Co-IP antibodies		
OTX2	Abcam (ab21990)	2 µg
Western blot antibodies		
p-4E-BP1	Cell Signaling Technology (9451)	1/1000
Total 4E-BP1	Cell Signaling Technology (9644)	1/2000
Actin	Sigma-Aldrich (A2228)	1/1000
p-AKT	Cell Signaling Technology (9611)	1/500
Total AKT	Cell Signaling Technology (9272)	1/500
Cleaved Caspase-3	Cell Signaling Technology (9664)	1/500
Doublecortin	Cell Signaling Technology (4604)	1/500
EZH2	Cell Signaling Technology (5246)	1/1000
GAPDH	Santa Cruz Biotechnology (sc-47724)	1/3000
PAX3	Abcam (ab180754)	1/500

PAX6	ThermoFisher Scientific (42-6600)	1/500
p-Raptor	Cell Signaling Technology (2083)	1/1000
Raptor	Cell Signaling Technology (2280)	1/1000
Rheb	Cell Signaling Technology (13879)	1/500
p-S6	Cell Signaling Technology (2211)	1/1000
Total S6	Cell Signaling Technology (2317)	1/1000
SOX2	Cell Signaling Technology (3579)	1/500
SUZ12	Cell Signaling Technology (3737)	1/1000
TUJ1	R&D systems (MAB1195)	1/1000
Western blot Secondary antibodies		
Goat anti-mouse HRP	Abcam (ab6789)	1/3000
Donkey anti-rabbit HRP	Jackson ImmunoResearch (711-035-152)	1/5000
IHC antibodies		
Ki67	Cell Signaling Technology (9449S)	1/800
p-4E-BP1	Cell Signaling Technology (2855)	1/1000
p-S6	Cell Signaling Technology (2211)	1/400
PAX3	DSHB (AB528426)	1/400

PAX6	ThermoFisher Scientific (42-6600)	1/150
SOX2	Cell Signaling Technology (3579)	1/100
STEM121	Clontech (Y40410)	1/500
βIII Tubulin	R&D Systems (MAB1195)	1/250
IHC secondary antibodies		
Biotin-SP sheep anti-mouse IgG	Jackson ImmunoResearch (515-065-003)	1/500
Biotin-SP goat anti-rabbit IgG	Jackson ImmunoResearch (111-065-144)	1/500
Flow cytometry antibodies		
Annexin V	BD (561012)	As per manufacturer's guidelines
BrdU	BD (552598)	As per manufacturer's guidelines

Supplementary Table 2: List of primers used in the study

qRT-PCR primers		
	Forward (5'-3')	Reverse (5'-3')
GAPDH	CCATGAGAAGTATGACAACAGCC	GGGTGCTAAGCAGTTGGTG
PAX3	CAGTATGGACAAAGTGCCTTTCATT	TGCGAAGACCAGAAACAGGG
PAX6	CCGGCAGAAGATTGTAGAGC	CGTTGGACACGTTTTGATTG
ChIP-qRT-PCR primers		
PAX3	TCTCCACTCTCTTCCTACTTCA	CCAAGAATATTCCTAGGAGCTGA
PAX6	AAGGGAGGGAGGAAAAGGGA	AATGTTAATGGCTGCGGTCG