Cooperation of Striatin 3 and MAP4K4 promotes growth and tissue invasion

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Supplementary figures

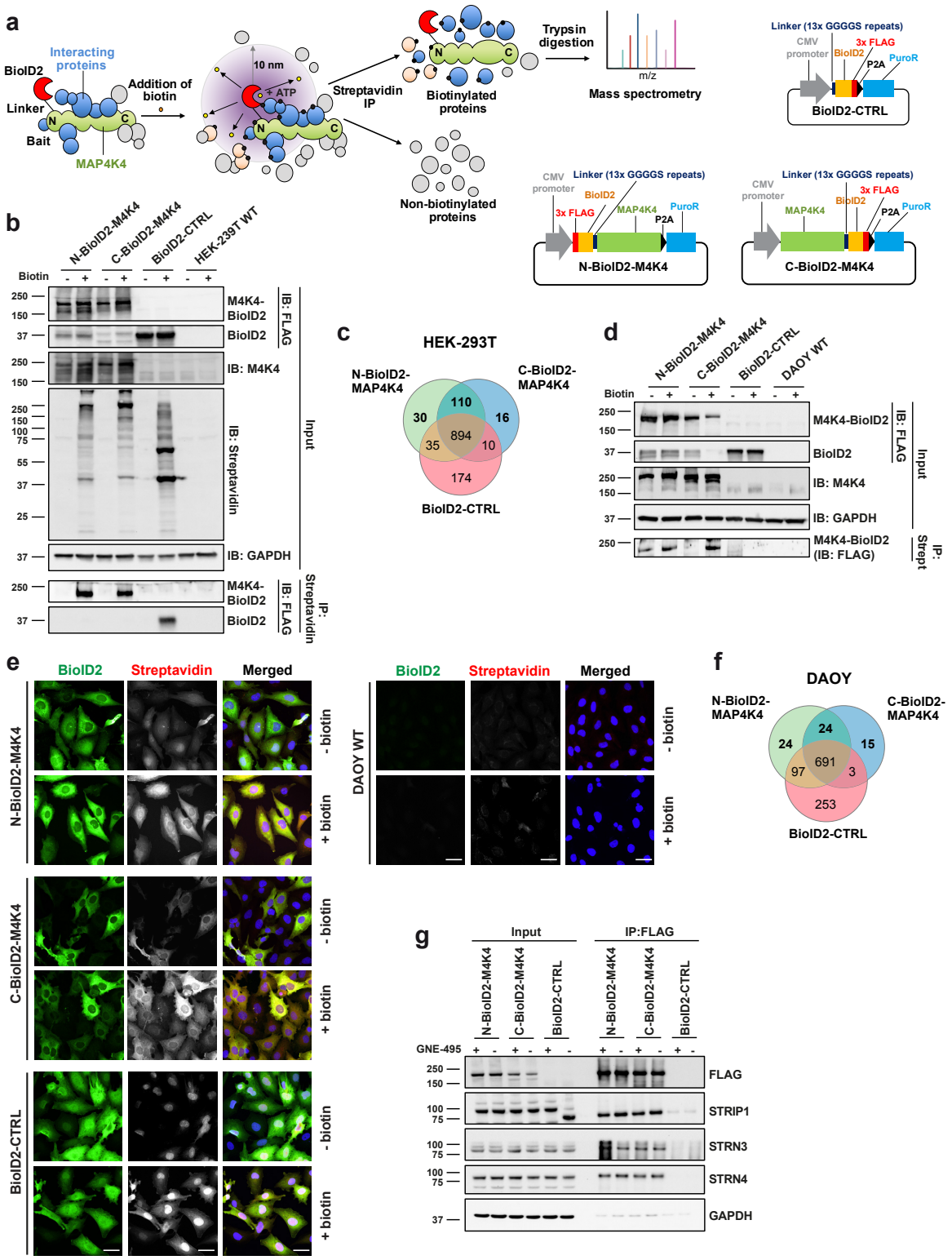


Figure S1: BioID identified MAP4K4 interactome. (a) Schematic diagram of BioID technology and schematic representation of the lentiviral vectors used to generate 3xFLAG-tagged MAP4K4-BioID2 cell lines. Biotin ligase (BioID2) was fused to either the N-terminus (FLAG-BioID2-MAP4K4) or C-terminus (MAP4K4-BioID2-FLAG) of MAP4K4. An extended flexible linker consisting of 13 repeats of GGGGS was inserted between the coding regions of MAP4K4 and BioID2. As negative control, BioID2-FLAG was used. **(b)** Immunoblot analysis (IB) of HEK-293T total cell lysate or after immunoprecipitation (IP)

with streptavidin-conjugated beads and detection with HRP-streptavidin. **(c)** Venn diagram of affinity captured proteins from HEK-293T cells. Comparison of BioID2-MAP4K4 with BioID2-CTRL is shown. Values of enriched proteins ($p < 0.05$, one-way ANOVA) are shown. *n*=3. **(d)** IB of FLAG-tagged BioID2 alone or fused to MAP4K4 in DAOY cell lysate or after pull-down with streptavidin-conjugated beads. **(e)** Confocal microscopy images of DAOY cells expressing BioID2-MAP4K4 fusion protein or BioID2 alone. Biotinylated proteins were labeled with streptavidin (red) and predominantly co-localized with BioID2 (green). Considerable biotinylation is observed in biotin (50 µM) supplemented cells expressing BioID2. DNA is labeled with Hoechst (blue). Scale bar: 30 µm. **(f)** Venn diagram of affinity-captured proteins from DAOY BioID2-MAP4K4 cells compared to BioID2-CTRL. *n*=2. **(g)** DAOY cells expressing BioID2-MAP4K4 fusion protein were serum-starved overnight and treated with 1 µM GNE-495 for 16 h where indicated. FLAG-immunoprecipitated MAP4K4 was subjected to immunoblot analysis for STRIPAK components. GAPDH was used as loading control.

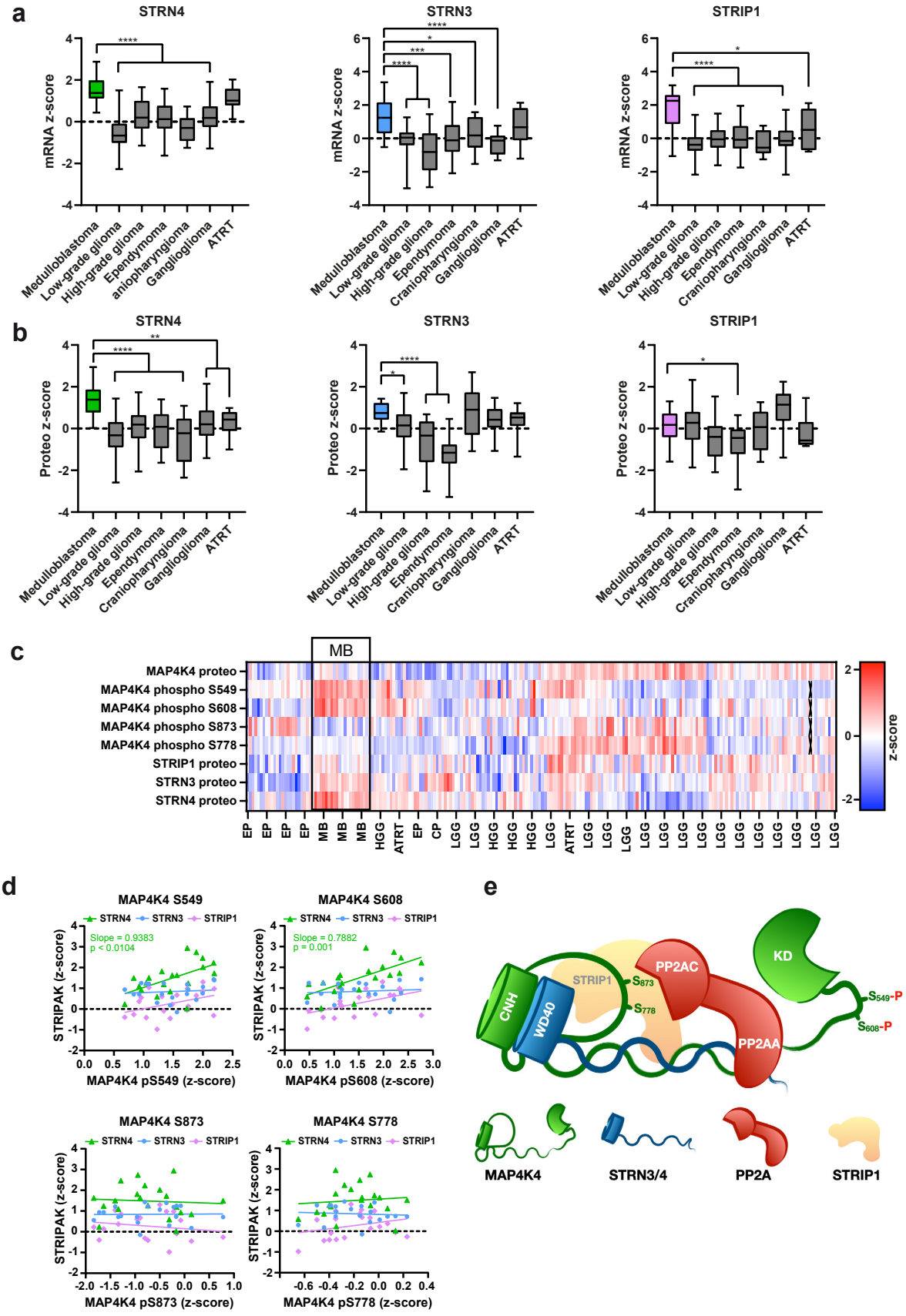


Figure S2: STRIPAK complex members are highly expressed in MB patients. (a, b) mRNA (a) and protein (b) levels of STRN4, STRN3, and STRIP1 in a cohort of 218 pediatric brain tumor samples representing seven histological types, including medulloblastoma (MB, *n*=22), low-grade glioma (LGG,

n=93), high-grade glioma (HGG, *n*=25), ependymoma (EP, *n*=32), craniopharyngioma (CP, *n*=18), ganglioglioma (GG, *n*=18), and atypical teratoid rhabdoid tumor (ATRT, *n*=12). The graphs represent the Z-score values for protein and mRNA in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (one-way ANOVA). **(c)** Heatmap for protein or phosphoprotein levels of the indicated proteins in the same cohort of patients as in a. **(d)** Scatterplot showing the phospho-abundance of the indicated residues of MAP4K4 versus the protein expression levels of STRN4, STRN3, or STRIP1 in MB samples. The data were obtained from the CPTAC data portal (http://pbt.cptac-data-view.org/). **(e)** Schematic prediction of MAP4K4-STRN3/4 interaction via the citron homology (CNH) domain of MAP4K4 and the WD40 domain of the STRNs (according to 53, 72). Source data for quantifications shown in (a) and (b) are available in supplementary data 11.

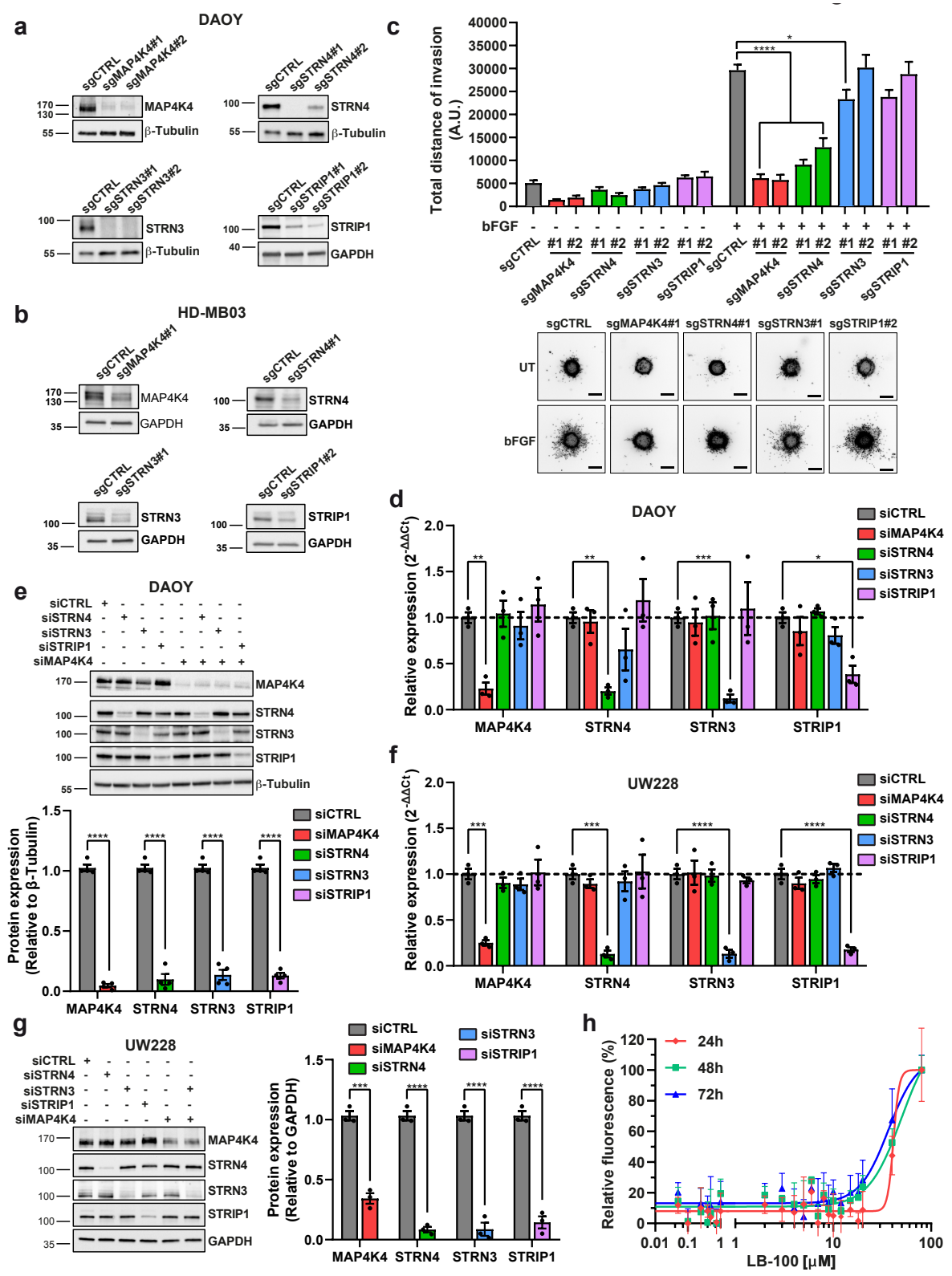


Figure S3: CRISPR/Cas9 and siRNA-mediated downregulation of MAP4K4 and STRIPAK complex members. (a, b) Immunoblots showing CRISPR/Cas9-mediated depletion of MAP4K4, STRN4, STRN3, or STRIP1 in DAOY (A) or HD-MBO3 (B) cells. The numbers indicate the sgRNA used for each gene. **(c)** Invasion and representative images of 3D spheroid invasion assay (SIA) of DAOY cells KO for MAP4K4, STRN4, STRN3, or STRIP1 ± bFGF (100 ng/ml) (*n*=3, means ± SEM). Scale bar: 300 µm. **(d)** qRT-PCR analysis of *MAP4K4*, *STRN4*, *STRN3*, and *STRIP1* in DAOY cells 72 h after transfection

with the indicated siRNA (*n*=3, means ± SEM). **(e)** IB analysis and quantification of MAP4K4, STRN4, STRN3, or STRIP1 protein expression level in DAOY cells 72 h after transfection with the indicated siRNA (*n*=4, means ± SEM). **(f)** qRT-PCR analysis of *MAP4K4*, *STRN4*, *STRN3*, and *STRIP1* in UW228 cells 72 h after transfection with the indicated siRNA (*n*=3, means ± SEM). **(g)** Immunoblot analysis and quantification of MAP4K4, STRN4, STRN3, or STRIP1 protein expression level in UW228 cells 72 h after transfection with the indicated siRNA (*n*=3, means ± SEM). **(h)** CellTox Green viability analysis of DAOY spheroids treated for 24, 48, and 72 h with increasing concentrations of LB-100. (*n*=4, means ± SD). Statistical analyses in c, d and f were performed by one-way ANOVA, in e and g by unpaired Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Source data for quantifications shown in (c-h) are available in supplementary data 12.

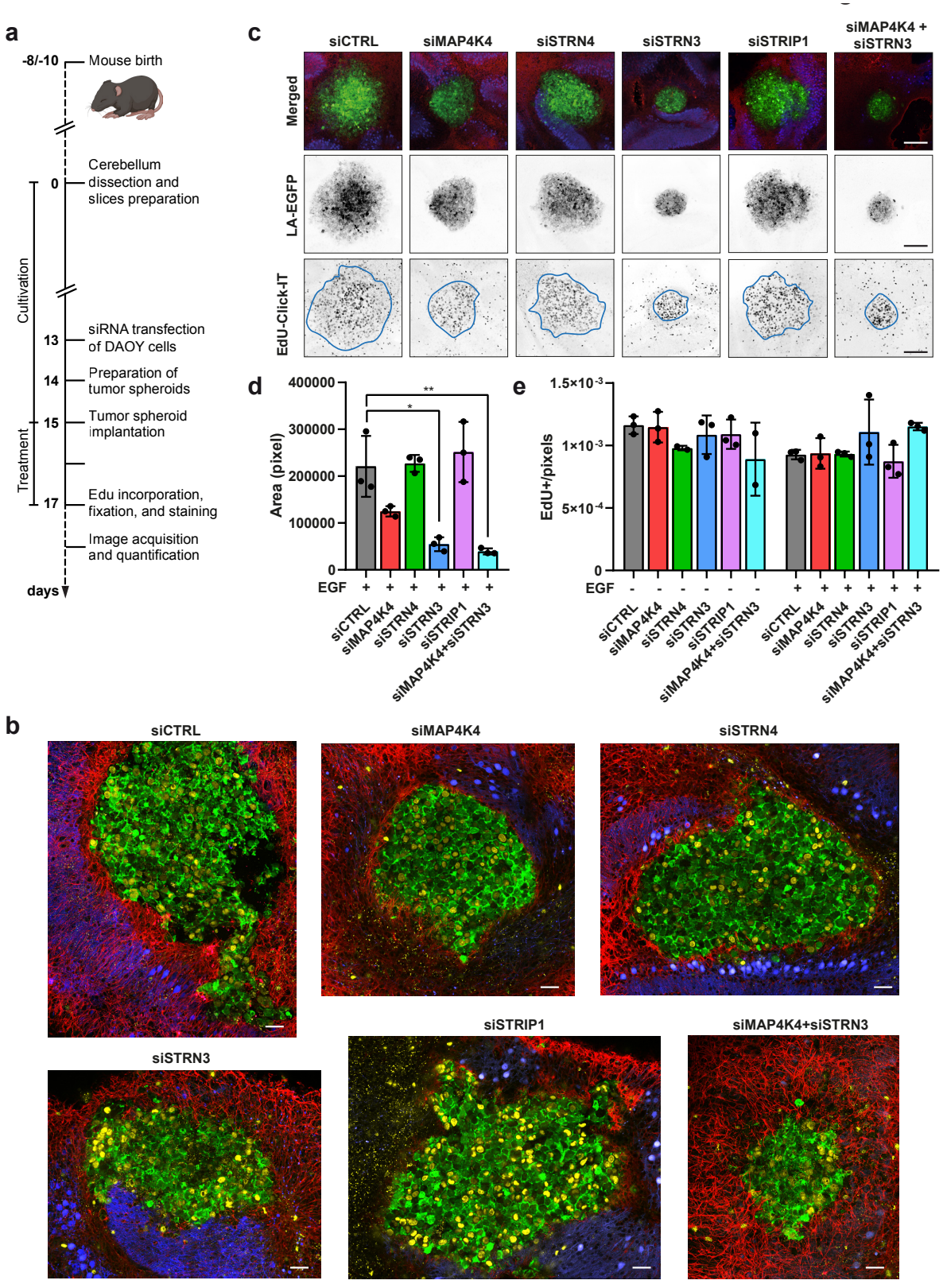


Figure S4: Depletion of MAP4K4 and STRN3 blocks tissue invasion in SHH MB cell model. (a) Timeline of organotypic cerebellum slice culture (OCSC) and tumor spheroid implantation. **(b)** High-resolution (63x) images of OCSCs co-cultured with siRNA-transfected DAOY tumor cell spheroids 72 h after transfection without GF treatment. Green: Lifeact-EGFP; blue: calbindin (Purkinje cells); red: GFAP; yellow: Edu-Click-IT. Scale bar: 50 µm. **(c)** Maximum intensity projections (MIP) of representative

confocal sections of OCSCs implanted with DAOY tumor cell spheroids and treated with 30 ng/ml EGF for 48h. Green: Lifeact-EGFP; blue: calbindin (Purkinje cells); red: GFAP. Middle: Inverted greyscale images of MIP of LA-EGFP channel, lower: Inverted greyscale images of MIP of EdU-Click-IT staining. Scale bar: 200 µm. **(d)** Quantification of LA-EGFP area of spheroids shown in c (*n*=3, means ± SD). **(e)** Number of EdU-positive nuclei normalized by the area of the tumor spheroids shown in c (*n*=3, means ± SD). * $p < 0.05$, ** $p < 0.01$ (unpaired Student's *t*-test). Source data for quantifications shown in (d) and (e) are available in supplementary data 13.

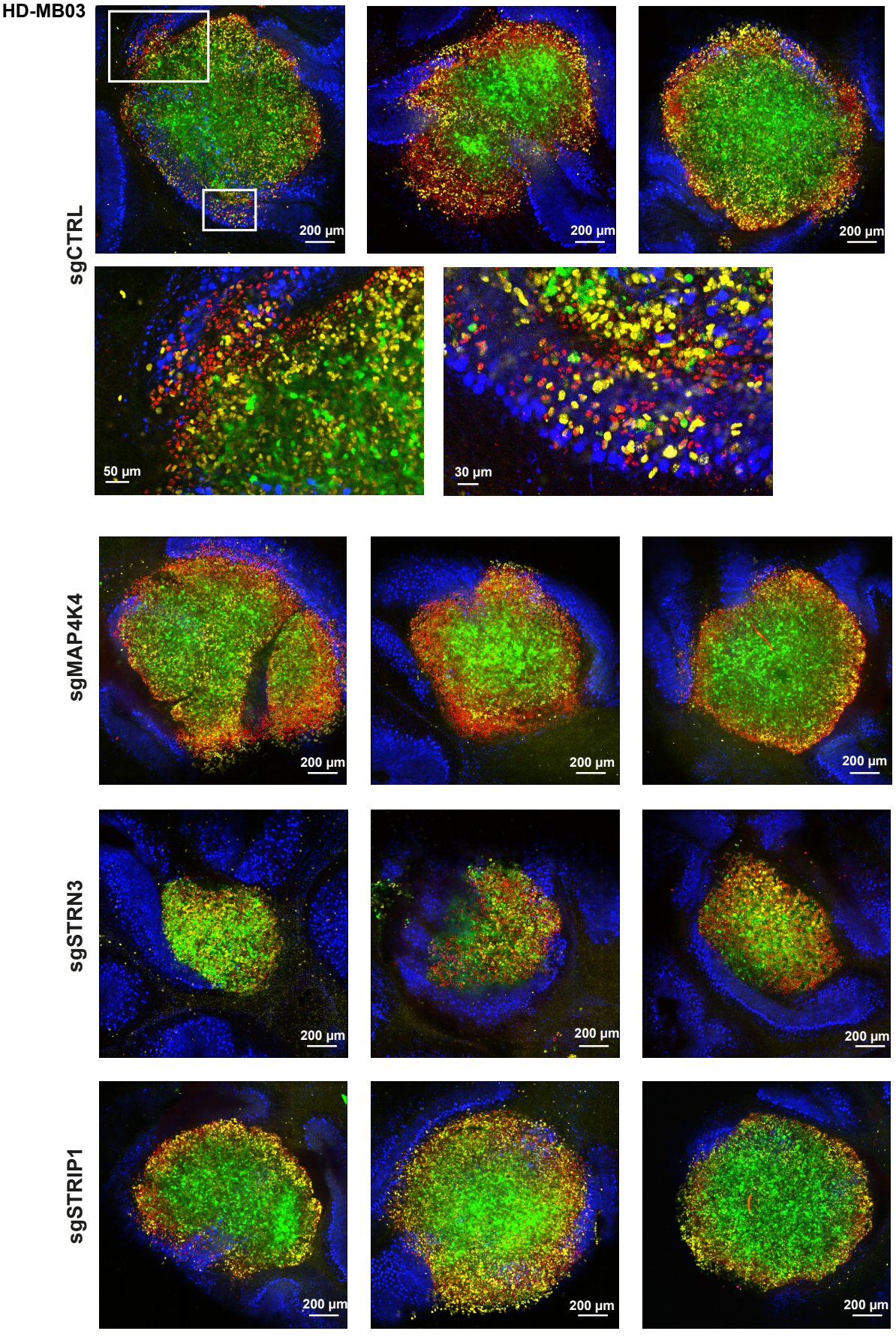


Figure S5: High-resolution images of organotypic cerebellum slice culture implanted with HD-

MBO3 spheroids. Single confocal microscopy sections of OCSCs implanted with tumor spheroids derived from HD-MBO3 cells with CRISPR/Cas9-mediated knockout of MAP4K4, STRN3, or STRIP1. 63x image acquisition of slices five days after implantation and treatment with 12.5 ng/ml bFGF. 4x magnifications of boxed areas are shown. Green: Lifeact-EGFP; blue: calbindin (Purkinje cells); red: human nuclei; yellow: Edu-Click-IT. Scale bar: 200 µm.

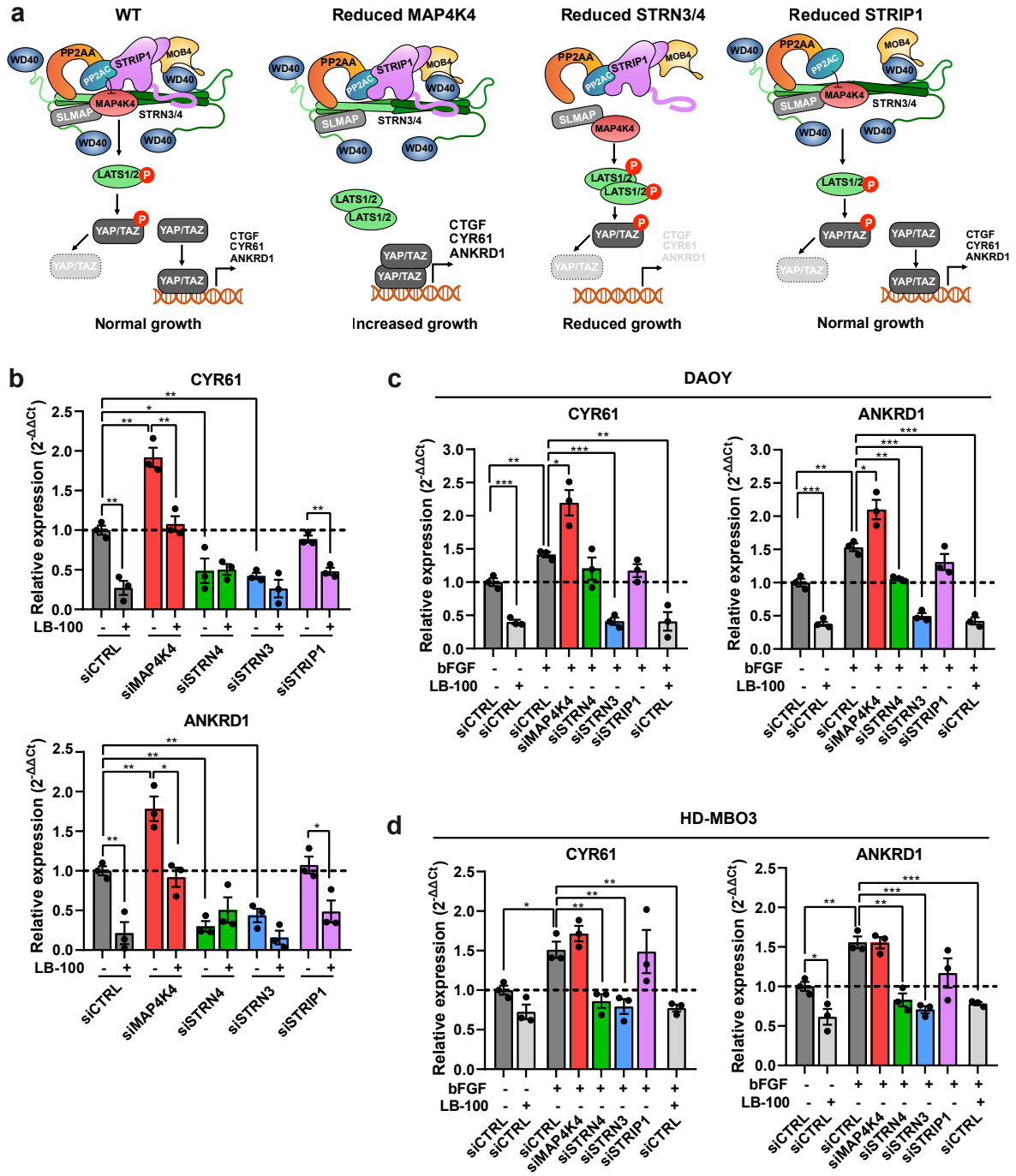


Figure S6: STRIPAK enables FGFR-PP2A-mediated activation of YAP/TAZ transcriptional program. (a) Models for MAP4K4 and STRIPAK regulation of Hippo pathway and YAP/TAZ target gene expression. **(b)** qRT-PCR analysis of *CYR61* (upper) and *ANKRD1* (lower) expression in DAOY cells 48 h after transfection with the indicated siRNA ± treatment for 4 h with 5 µM LB-100 (*n*=3, means ± SEM). **(c, d)** qRT-PCR analysis of *CYR61* and *ANKRD1* expression in DAOY (c) or HD-MBO3 cells (d) transfected with siRNA for 48 h, serum-starved overnight, and treated with 100 ng/ml bFGF for 1 h and/or 5 µM LB-100 for 4 h where indicated (*n*=3, means ± SEM). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (unpaired Student's *t*-test). Source data for quantifications shown in (b-d) are available in supplementary data 14.

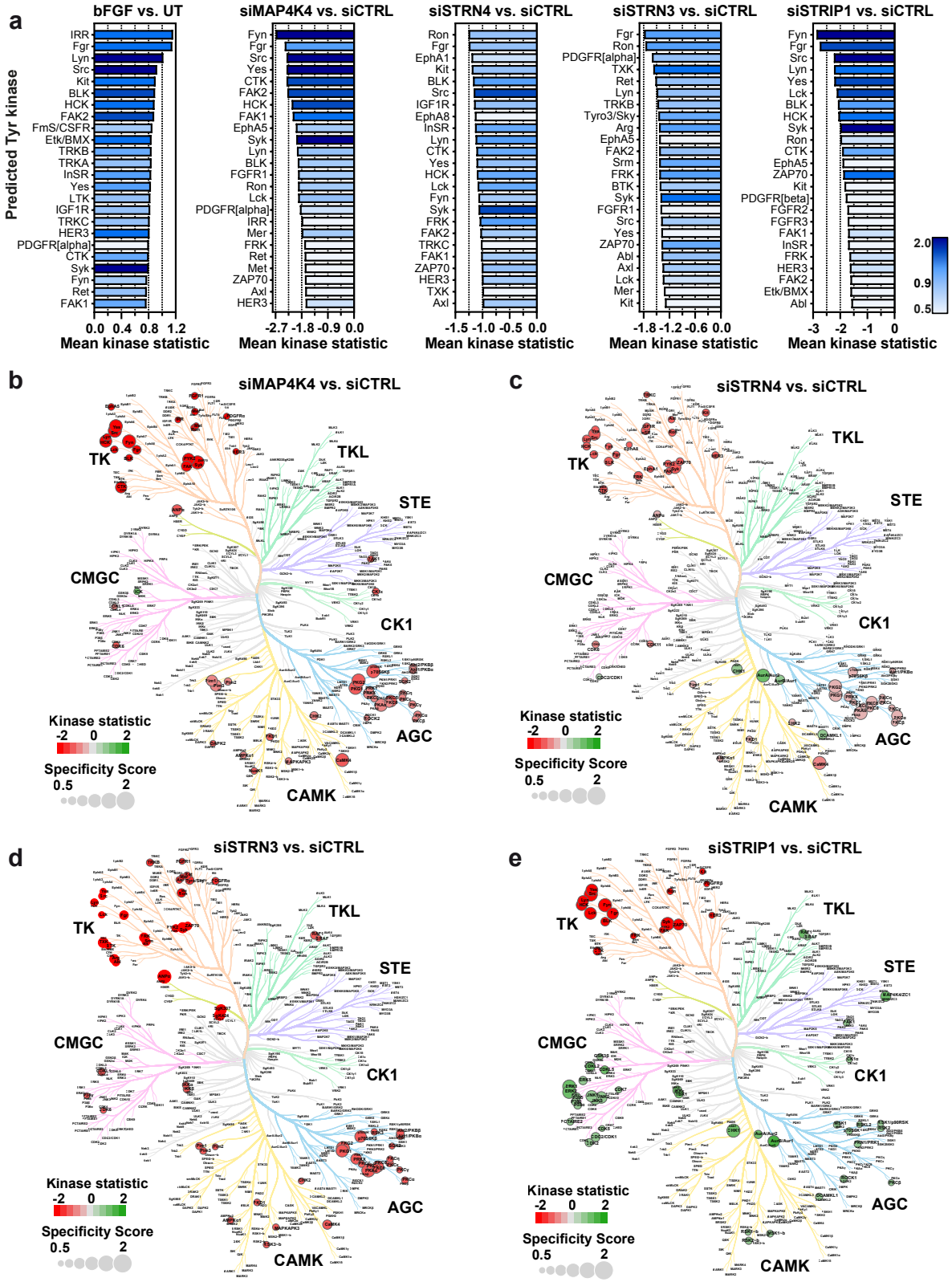


Figure S7: Phylogenetic distribution of the kinases predicted the peptide chip array. **(a)** Upstream kinase prediction analysis of DAOY cells transfected with the indicated siRNA ± treatment with 100 ng/ml bFGF. The plots show the top differentially activated putative upstream tyrosine kinases (PTK) predicted to phosphorylate the phosphosites on the PamChip®. The x-axis indicates the values for the mean

kinase statistic, which represents the difference in the activity of the predicted protein kinase between the two compared groups, with effect size (values) and direction (>0=activation; <0=inhibition). The color of the bars represents the specificity score (darker the color, higher the specificity). Values of the specificity score >0.9 were considered statistically relevant. First graph from left: comparison of bFGF stimulated siCTRL cells vs. untreated (UT); all other graphs: comparison of the indicated siTarget vs. siCTRL in the presence of bFGF. *n*=3. **(b-e)** Phylogenetic kinome trees illustrating the family distribution of the upstream kinases predicted to phosphorylate the serine/threonine (STK) and protein tyrosine (PTK) consensus peptides of the PamChip®. Colored dots highlight the kinases where predicted activity is significantly different in the indicated siTarget (b: siMAP4K4; c: siSTRN4; d: siSTRN3, e: siSTRIP1) compared to siCTRL in bFGF stimulated conditions in DAOY cells. The coloring scale is based on the mean kinase statistic and ranges from −2 (strong decrease of kinase activity in siTarget vs. siCTRL, red color) to +2 (strong increase of kinase activity in siTarget vs. siCTRL, green color). The size of the circle represents the specificity score. Source data for quantifications shown in (a) are available in supplementary data 15.

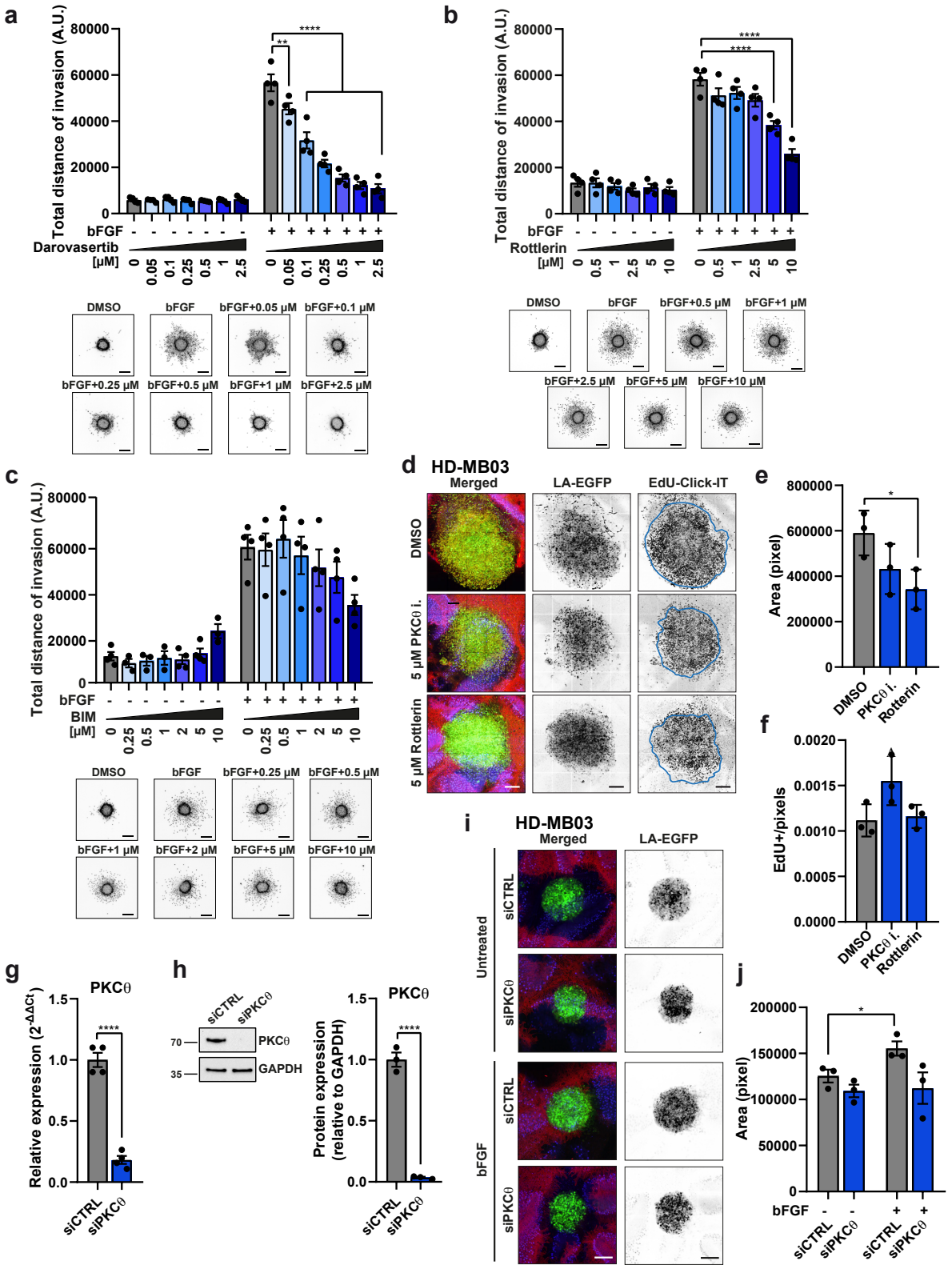


Figure S8: Novel protein kinases C are pro-migratory effectors of MAP4K4 and STRN3. (a-c) Quantification and representative images of SIA with DAOY cells stimulated with 100 ng/ml bFGF and treated with increasing concentrations of Darovasertib (PKCθ inhibitor, a), Rottlerin (PKCδ inhibitor, b) or Bisindolylmaleimide I (BIM, PKCα/β/γ inhibitor, c). Invasion was quantified as the sum of invasion distances from the center of the spheroids. **(***n*=4, means ± SEM). Scale bar: 300 µm. **(d)** Maximum

intensity projections (MIP) of representative confocal sections of OCSCs implanted with HD-MBO3 tumor cell spheroids and treated with 5 µM PKCθ inhibitor, 5 µM Rottlerin, or DMSO for five days. Green: Lifeact-EGFP; blue: calbindin (Purkinje cells); red: GFAP; yellow: Edu-Click-IT. Scale bar: 200 µm. **(e)** Quantification of LA-EGFP area of HD-MBO3 spheroids shown in d (*n*=3, means ± SD). **(f)** Quantification of the number of EdU-positive nuclei normalized by the area of HD-MBO3 spheroids shown in d (*n*=3, means ± SD). **(g)** qRT-PCR analysis of *PRKCQ* (PKCθ) mRNA expression in DAOY cells 72 h after siRNA transfection (*n*=4, means ± SEM). **(h)** IB analysis and quantification of siRNA-mediated PKCθ depletion in DAOY cells 72 h after transfection (*n*=3, means ± SEM). **(i)** MIP of representative confocal sections of OCSC implanted with transfected HD-MBO3 tumor cell spheroids and treated with 12.5 ng/ml bFGF for 48h. Green: Lifeact-EGFP; blue: calbindin (Purkinje cells); red: GFAP. Scale bar: 200 µm. **(j)** Quantification of LA-EGFP area of HD-MBO3 spheroids shown in i (*n*=3, means ± SD). Statistical analyses in a-c were performed by one-way ANOVA, in e-h and j by unpaired Student's t-test. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. Source data for quantifications shown in (a-c), (e-h) and (j) are available in supplementary data 16.

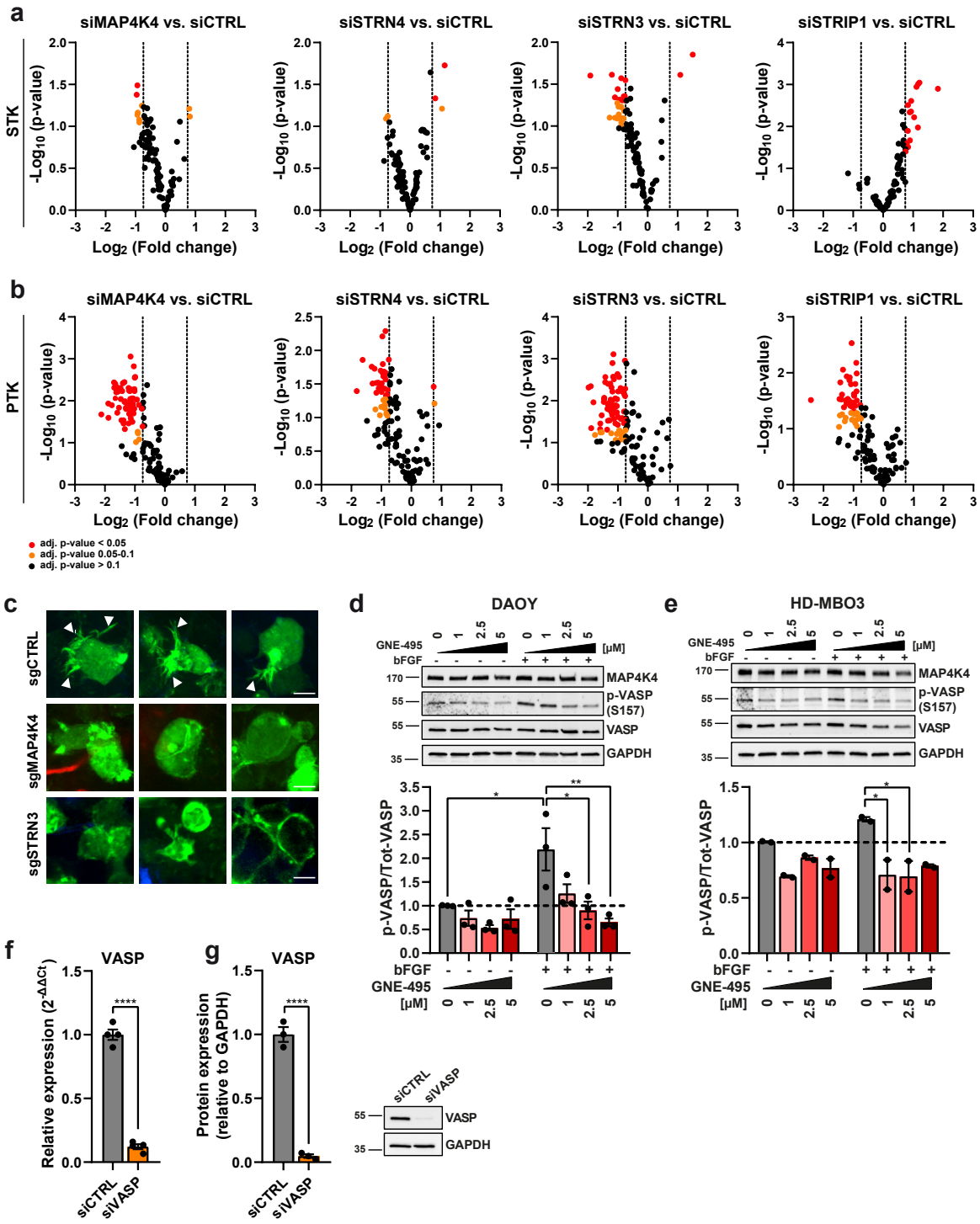


Figure S9: MAP4K4 mediates VASP$_{S157}$ phosphorylation. (a, b) Individual volcano plots representing the changes in phosphorylation of phospho-serine/threonine (STK, a) and phospho-tyrosine (PTK, b) peptides in the indicated siTarget versus siCTRL in bFGF-stimulated DAOY cells. The p-values were calculated versus siCTRL by ANOVA and post-hoc Dunnett's test in the BioNavigator software. *n*=3. The peptides that showed significant changes in phosphorylation are highlighted in red ($p < 0.05$) or orange ($p < 0.1$). **(c)** Maximum intensity projection of LA-EGFP fluorescence of tissue-invading HD-MBO3 CTRL or KO MAP4K4 or STRN3 cells. Scale bar: 10 µm. Arrowheads indicate filopodia-like protrusions. **(d-e)** IB and quantification of VASP$_{S157}$ phosphorylation in DAOY (d) and HD-MBO3 cells

(e) after treatment for 4 h with increasing concentration of GNE-495 ± 100 ng/ml bFGF for 15 min. Quantification represents the ratio of p-VASP$_{S157}$ over total VASP relative to untreated control (d: *n*=3, means ± SEM. e: *n*=2, means ± SEM). **(f)** qRT-PCR analysis of *VASP* mRNA expression in DAOY cells 72 h after siRNA transfection (*n*=4, means ± SEM). **(g)** IB analysis and quantification of siRNA-mediated VASP depletion in DAOY cells 72 h after transfection (*n*=3, means ± SEM). Statistical analysis in d and e was performed by one-way ANOVA, in f and g by unpaired Student's t-test. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. Source data for quantifications shown in (d-g) are available in supplementary data 17.

Supplementary information antibodies and sequences used, uncropped immunoblots.

Supplementary table 1:

Antibodies			
Ab name	**Company**	**Identifier**	**Dilution**
Anti-β-tubulin	Sigma Aldrich	T5201	1:1000 (IB)
Anti-GAPDH (14C10)	Cell signaling technologies	2118s	1:2000 (IB)
Anti-FLAG® M2	Sigma Aldrich	F1804	1:1500 (IB) 3 µg/mg lysate (IP)
Streptavidin-HRP	Jackson Immunoresearch	16-030-084	1:500 (IB)
Anti-MAP4K4	Abcam	ab80418	1:2000 (IB)
Anti-STRN4	Abcam	ab194948	1:2000 (IB)
Anti-STRN3 (S68)	Thermo Fisher Scientific	MA1-46461	1:2000 (IB)
Anti-STRIP1	Abcam	ab199851	1:2000 (IB)
Anti-VASP (9A2)	Cell signaling technologies	3132	1:1000 (IB) 1: 400 (IFA)
Anti-phospho-VASP (Ser157)	Abcam	ab47268	1:750 (IB)
Anti-mouse HRP linked	Cell signaling technologies	7076	1:5000 (IB)
Anti-rabbit HRP linked	Cell signaling technologies	7074	1:5000 (IB)
easyBlot anti-mouse HRP	GeneTex	GTX221667-01	1:1000 (IB)
Anti-BioID2	Novus	NBP2-59940	1:200 (IFA)
Streptavidin Alexa Fluor 594 conjugate	Invitrogen	S11227	1:500 (IFA)
Anti-Calbindin	Abcam	ab108404	1:1000 (IFA)
Anti-GFAP	Abcam	ab53554	1:250 (IFA)
Anti-human nuclei (3E1.3)	Millipore	MAB4383	1:250 (IFA)

Donkey anti-Mouse IgG Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	A32766	1:400 (IFA)
Cy3-conjugated Donkey anti-Rabbit IgG	Jackson Immunoresearch	711-165-152	1:250 (IFA)
Brilliant Violet 421 Donkey Anti-Goat IgG	Jackson Immunoresearch	705-675-147	1:100 (IFA)
Goat anti-Mouse IgG Secondary Antibody, Alexa Fluor 405	Thermo Fisher Scientific	A31553	1:200 (IFA)
Hoechst	Sigma Aldrich	B2883	1:2000

Supplementary table 2:

sgRNA Oligonucleotides		
Name	**Exon**	**Target sequence**
sgCTRL	-	GTAGCGAACGTGTCCGGCC
sgSTRN4#1	Exon 1	GCTCAGGTGGCCTTCCTTCA
sgSTRN4#2	Exon 2	TTCCTTCAGGGAGAGAGGAA
sgSTRN3#1	Exon 2	AGGTCAAGAGAACCTGAAGA
sgSTRN3#2	Exon 2	AGTATGCATTAAAACAAGAA
sgSTRIP1#1	Exon 4	TGCCAGGGAGAAGAGACTCA
sgSTRIP1#2	Exon 4	GGATGGCTTGGAAGTCACTG
sgMAP4K4#1	Exon 7	GGGCGGAGAAATACGTTCAT
sgMAP4K4#2	Exon 4	CAGGACATGATGACCAACTC

Supplementary table 3:

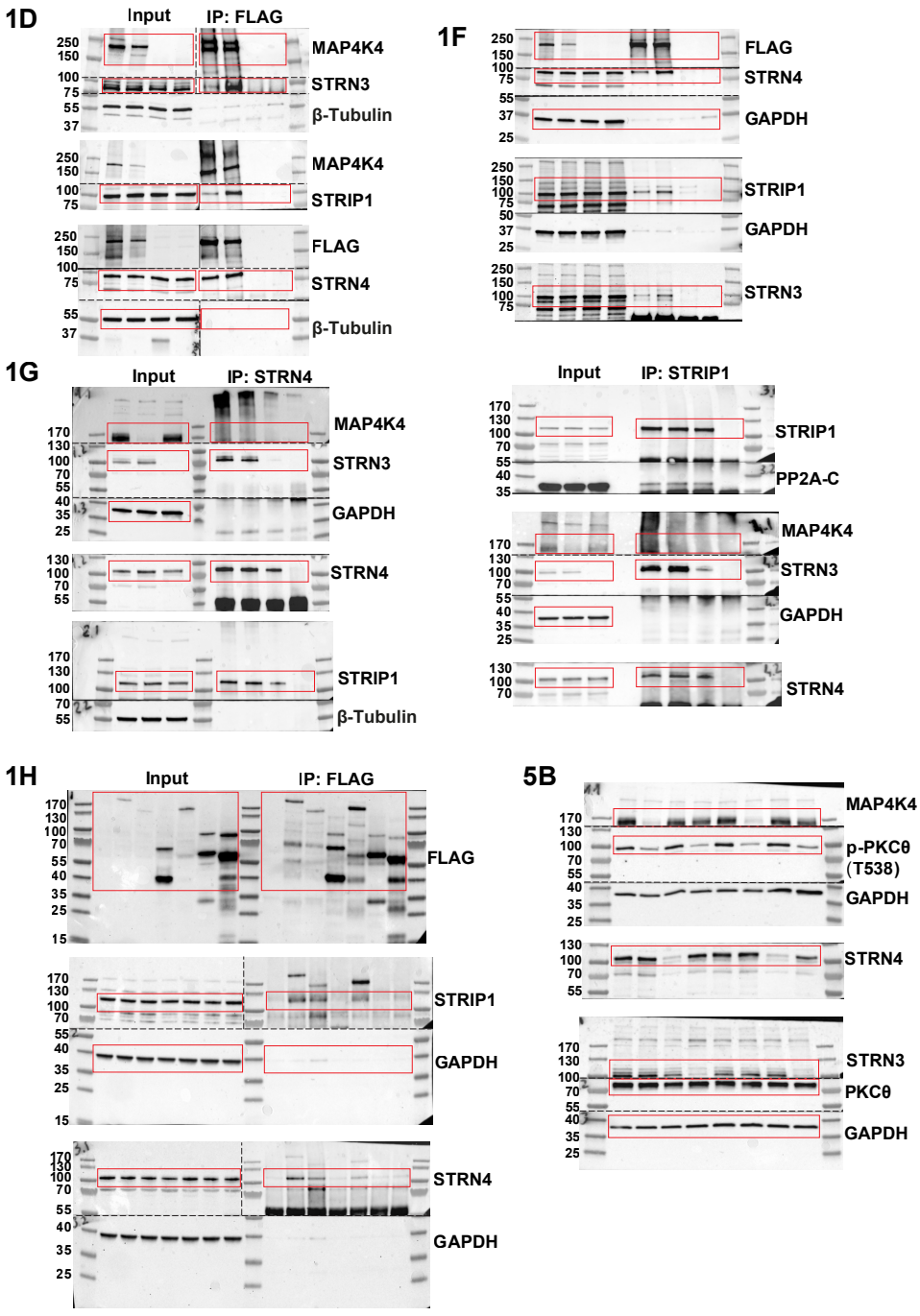
siRNA			
Name	**Target sequence**	**Company**	**Identifier**
siCTRL	UAAGGCUAUGAAGAGAUAC	Dharmacon	D-001210-02-05
siSTRN4	GGAUCAAGAUGCUAGAGUA	Dharmacon	D-020389-01-0002

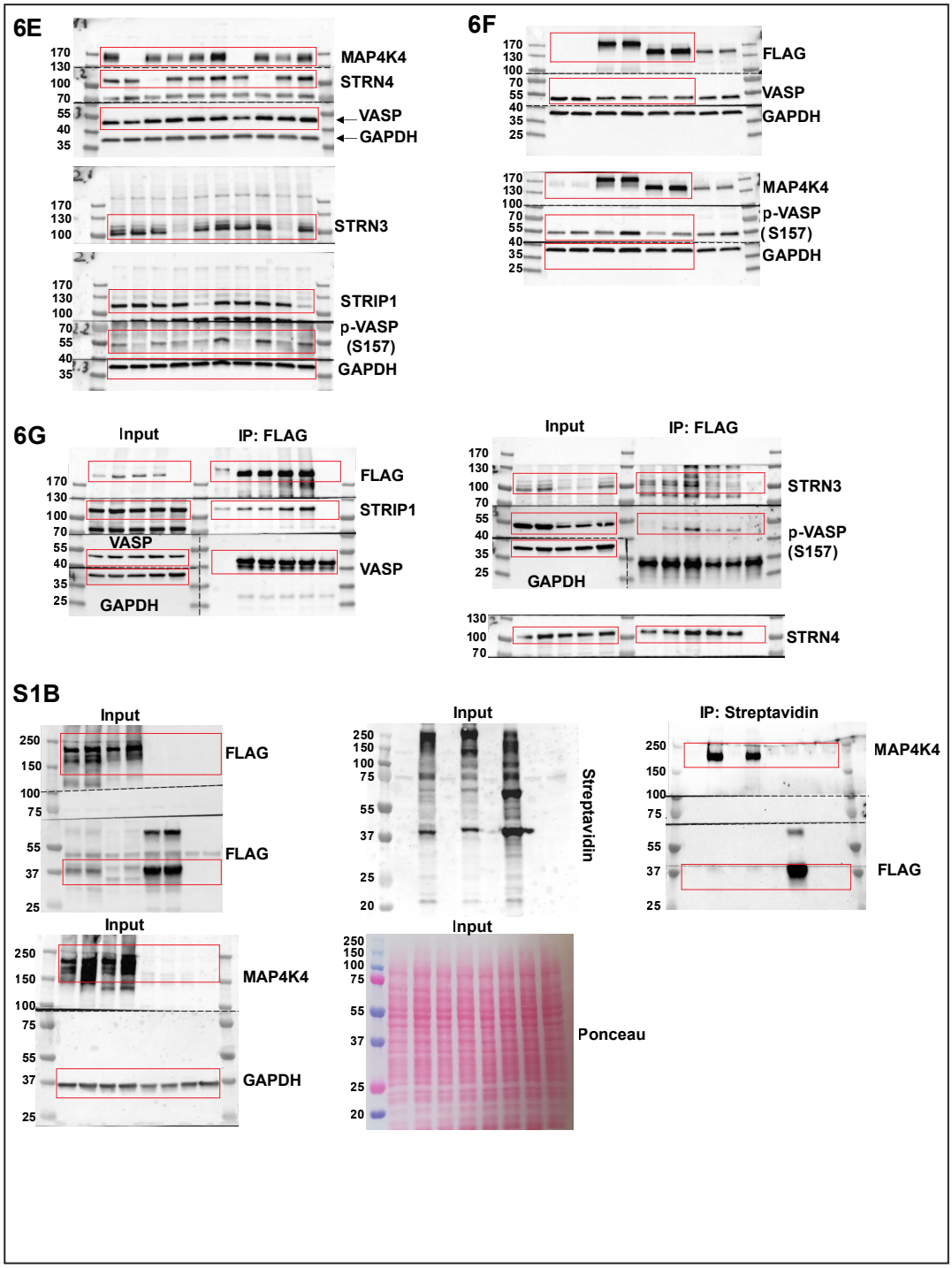
siSTRN3	GGAGGAGGCAAGUCAUUUA	Dharmacon	D-019145-01-0002
siSTRIP1	GCAGCAAAUUUAUAGGUUA	Dharmacon	D-021516-01-0002
siMAP4K4	UAAGUUACGUGUCUACUAU	Dharmacon	D-003971-05-0002

Supplementary table 4:

qPCR primers			
Target genes		**Sequence**	**Product size**
18s	F	GGATGTAAAGGATGGAAAATACA	23 bp
	R	TCCAGGTCTTCACGGAGCTTGTT	
MAP4K4	F	GTTACACTAATGCGCACCAC	193 bp
	R	GTACTTGCCACCAGTCTGCT	
STRN4	F	CTCAGGTGGCCTTCCTTCAG	20 bp
	R	TTTGGCCCTTTCCTGCTTCA	
STRN3	F	TGGCACAGAATGGGCTGAAC	101 bp
	R	CTCCAAGGCCCAGTACACTT	
STRIP1	F	CGCAAAGACTCAGAGGGCTA	109 bp
	R	GCCCTTCCGTGTAGCTGTAA	
CTGF	F	CACCCGGGTTACCAATGACA	119 bp
	R	GGATGCACTTTTTGCCCTTCTTA	
CYR61	F	ACAGCAGCCTGAAAAAGGGC	104 bp
	R	GGGCCGGTATTTCTTCACACT	
ANKRD1	F	TAGCGCCCGAGATAAGTTGC	97 bp
	R	GTCTGCCTCACAGGCGATAA	

Original uncropped immunoblot images

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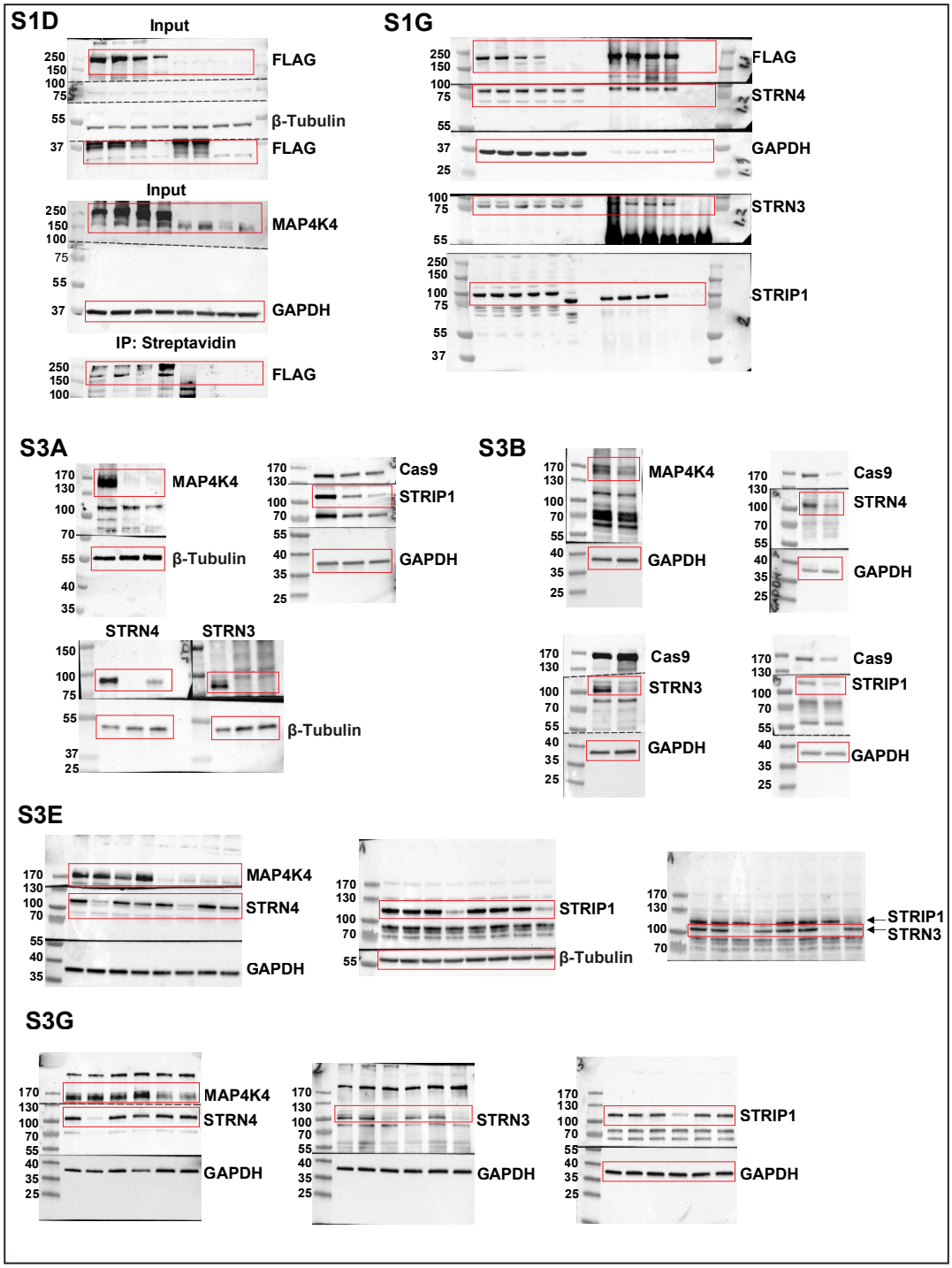
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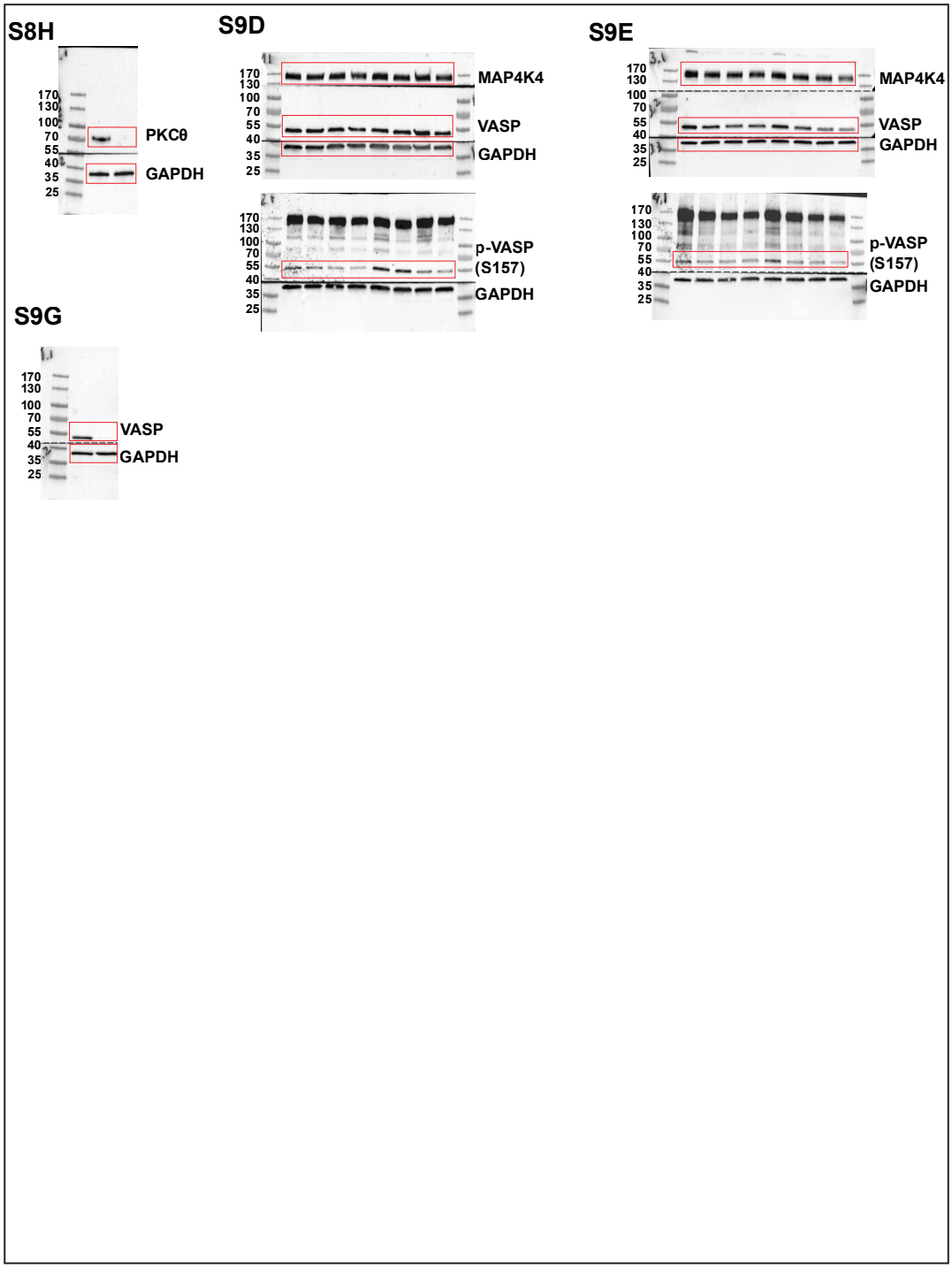
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